Identification and Assessment of Molecular Biomarkers in Haematopoietic Stem Cell Transplantation

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Thesis

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Abstract

Haematopoietic stem cell transplantation (HSCT) has become a central treatment modality in the management of various hematologic malignancies, but it is not without treatment sequelae. The major complication of HSCT is acute or chronic graft-versus host disease (GvHD). GvHD is an immunologically mediated disease that contributes substantially to transplant-related morbidity and mortality. One reason for the lack of progress in the treatment of acute GvHD (aGvHD) is the lack of reliable biomarkers. There is a need to develop diagnostic tools that can identify patients who are at higher risk of aGvHD progression following allogeneic HSCT and predict GvHD occurrence before clinical symptoms manifest. During the past decade, many reports have identified genetic variants such as single nucleotide polymorphisms (SNPs) that influence the risk of aGvHD after allogeneic HCT. In addition, since miRNAs are key regulators of gene expression, miRNA-related SNPs including SNPs in miRNA genes and target sites may function as regulatory SNPs through modifying miRNA regulation to affect the phenotypes and disease susceptibility.

Firstly, this study investigated the impact of rs2910164 and rs2431697 in miR-146a, rs3027898 in IRAK1 and rs10511792 in MICA for their association with HSCT outcome and showed that there was a significant association between carrying the C variant in rs2910164 in miR-146a and an increased non relapse mortality (NRM) post-HSCT. For rs2431697 in miR-146a, the presence of the T allele was associated with a trend towards an increased NRM in patients post-HSCT. In the case of rs3027898 in IRAK1, the C allele was associated with a decreased risk of relapse in patients which was more apparent when patients were homozygous for the C allele. For rs1051792 in MICA, this study showed that the MICA-129 Met variant was significantly associated with low overall survival (OVS) post-HSCT, which was more apparent in the group of patients receiving non-TCD treatment. This study also revealed that the presence of the MICA-129 Met allele in patients was significantly associated with an increased risk of relapse and the presence of the MICA-129 Val variant in patients was significantly associated with an increased risk of developing aGVHD post-HSCT. Investigation of gene expression and the protein levels of *MICA* in the GI tract showed that there was a significant association between decreased expression of MICA and aGvHD which was observed again in the case of MICA protein levels, where high levels of MICA protein were observed in patients with no active GIGvHD. Assessment of the levels of

soluble MICA in sera of patients post HSCT showed a significant association between high levels of soluble MICA and aGvHD post-HSCT. Alongside MICA, this study investigated the mRNA and protein levels of a panel of genes (*C1QTNF7, LGALS7, ANP32A, HTRA1, PIK3AP1, PSTPIPI, MSR1* and *CXCL9)* in RNA from blood samples and patient sera at different time points pre and post-HSCT. This study showed that there was a significant downregulation in the expression levels of *MSR1* and *ANP32A* in aGvHD patients post-HSCT while a significant upregulation in the expression levels of *CXCL9* was observed in aGvHD patients. Investigation of the association between the levels of proteins and the incidence of aGvHD showed that there was a significant association between upregulated protein levels of LGALS7 and aGvHD. Finally, a microRNA profiling in GI samples taken from aGvHD patients was performed aiming for the identification of miRNAs associated with the incidence of aGvHD in the GI tract after HSCT. This study identified 4 miRs that were dysregulated in patients in association with aGvHD, and a validation study was carried out for hsa-miR-34a-5p which expression was shown to be significantly decreased in patients with aGvHD (1-4) compared those patients with no aGvHD.

Author’s Declaration

I declare that, except where explicit reference is made to the contribution of others, this dissertation is the result of my own work and has not been submitted for any other degree at Newcastle University or any other institution.

Rihab Gam M.Sc.

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Dedication

I dedicate this thesis to my parents Mondheur and Jamila, to my sisters Ryhem and Siwar, to my brothers Gaith and Achraf, to my grandparents, aunts and uncle.

“Our deepest fear is not that we are inadequate. Our deepest fear is that we are powerful beyond measure.”

— Marianne Williamson

Acknowledgement

A PhD is an exciting and emotional journey. You are forced to confront all of your fears and doubts and somehow overcome them. Anything is one of a million paths, following this PhD path for me was following my passion for science. This PhD project helped me find where my heart is, and for that I would like express my deepest appreciation to Prof Anne Dickinson for giving me the honour to carry on this project as part of her team at the Haematological sciences department. I am forever grateful to her, to Newcastle University and to the Marie Curie Initial Training Network 'CellEurope'.

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List of Abbreviations

ADAM17	A disintegrin and metalloproteinase domain 17
Ago2	Argonaute
aGvHD	Acute graft versus host disease
AIF1	Allograft Inflammatory Factor 1
ALL	Acute lymphoblastic leukaemia
Allo-HSCT	Autologous heamatopoietic stem cell transplant
AML	Acute myeloid leukaemia
ANOVA	Analysis of variance
ANP32A	Acidic (Leucine-Rich) nuclear phosphoprotein 32
APC	Antigen presenting cell
ATG	Anti-thymocyte globulin
Auto-HSCT	Allogeneic haematopoietic stem cell transplant
BAFF	B cell activating factor
BioGRID	Biological general repository for interaction datasets
BM	Bone marrow
BMT	Bone marrow transplantation
BSA	Body surface are
C. elegans	Caenorhabditis elegans
C1QTNF7	C1q And tumour necrosis factor related protein 7
C2	Complement component 2
CARD11	Caspase recruitment domain family, member 11
CCL3L1	Chemokine (C-C Motif) ligand 3-like 1
CD	Cluster of differentiation
CDR	Complementary determining region
CDR3	Complementarity determining region
CEACAM4	Carcinoembryonic antigen-related cell adhesion molecule 4
cGvHD	Chronic graft versus host disease
CML	Chronic myelogenous leukaemia
CMP	Common myeloid progenitor
CMV	Cytomegalovirus
CNV	Copy number variation
Cq	Quantitation cycle
CsA	Cyclosporine
Ct	Cycle threshold
CTGF	Connective tissue growth factor
CTL	Cytotoxic T lymphocyte
Cy	Cyclophosphamide
DAMP	Damage-associated molecular pattern molecule

DC	Dendritic cell
DLI	Donor lymphocyte infusion
DMSO	Dimethyl sulphoxide
dNTP	Deoxyribonucleotide triphosphate
ds	Double-stranded
DTT	Dichloro-diphenyl- trichloroethane
ECP	Extracorporeal photochemotherapy
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FOXP3	Forkhead box p3
FRET	Fluorescence resonance energy transfer
GCSF	Granulocyte-colony stimulating factor
GEO	Gene expression omnibus
GI	Gastrointestinal tract
GMP	Granulocyte-monocyte progenitors
GvH	Graft versus host
GvHD	Graft versus host disease
GvHR	Graft-versus-host reaction
GvL	Graft-versus-leukaemia
GvT	Graft versus tumour
HCLS1	Haematopoietic cell-specific lyn substrate 1
HDMEC	Human dermal microvascular endothelial cell
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPC	Haematopoietic progenitor cells
hsa-	Homo sapiens
HSC	Haematopoietic stem cell
HSCT	Haematopoietic stem cell transplantation
hsTCRB	Human T-cell receptor beta
HTP	High-throughput sequencing
HTRA1	High-temperature requirement a serine peptidase 1
I2D	Interologous Interaction database
IGFBP5	Insulin-Like growth factor binding protein 5
IGFBP5	Insulin-Like growth factor binding protein 5
IL-1b	Interleukin 1, beta
INF-γ	Interferon gamma
IRAK1	Interleukin-1 receptor-associated kinase 1
IRAK2	Interleukin-1 receptor-associated kinase 2
IRAKM	Interleukin-1 receptor-associated Kinase M

IRF5	Interferon regulatory factor 5
IκBα	Inhibitor of kappa B
JAK	Janus Kinase
KIR	Killer cell immunoglobulin-like receptor
LGALS7	Lectin, galactoside-binding, soluble, 7
LPS	Lipopolysaccharide
LST1	Leukocyte specific transcript 1
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A
miHa	Minor histocompatibility antigen
miR	MicroRNA
MME	Membrane Metallo-Endopeptidase
MMP	Multiple myeloid progenitors
MSC	Mesenchymal stem cell
MSR1	Macrophage scavenger receptor 1
MUD	Matched unrelated donor
NF-κB	Nuclear factor kappa B
NHL	Non-Hodgkin’s lymphoma
NIH	National institute of health
NOD2	Nucleotide-binding oligomerization domains containing 2
NRM	Non-relapse mortality
OVS	Overall survival
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBSC	Peripheral blood stem cell
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PIK3AP1	Phosphoinositide-3-Kinase Adaptor Protein 1
Pre-miRNA	Precursor microRNA
Pri-miRNA	Primary microRNA
PSTPIP1	Proline-serine-threonine phosphatase interacting protein 1
PTGER2	Prostaglandin E receptor 2

PTPN7	Protein tyrosine phosphatase, non-receptor type 7
RCC	Resource compiler
RFU	Relative fluorescence units
RIN	RNA integrity number
RISC	RNA-induced silencing complex
RPM	Revolutions per minute
RT	Reverse transcription
RT-qPCR	Reverse transcription- quantitative polymerase chain reaction
SAHA	Suberonylanilide hydroxamic acid
SCID	Severe combined immunodeficiency
SCT	Stem cell transplantation
SD	Standard deviation
SEM	Standard error of the mean
SIB	Sibling donor
siRNA	Small interfering ribonucleic acid
sMICA	Soluble MHC class I polypeptide-related sequence A
SNP	Single nucleotide polymorphism
STAT1-a	Signal transducers and activators of transcription 1- alpha
TAP1	Transporter 1, ATP-binding cassette, sub-Family B
TCD	T cell depletion
TGFβ	Transforming growth factor beta
TGM2	Transglutaminase 2
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
TRAF6	TNF receptor-associated factor 6, E3 ubiquitin protein ligase
Treg	Regulatory T cell
TREM2	Triggering receptor expressed on myeloid cells 2
Tx	Transplant
UBD	Ubiquitin D
UTR	Untranslated region
NKG2D	Natural killer group 2D

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R. Gam, J. Norden, K. Pearce, Clare Lendrem, M. Gionni, S.Hafner, R. Crossland, E. Holler, R. Dressel, A. M. Dickinson. MICA-129 dimorphism, MICA mRNA and sMICA levels association with the outcome of HSCT. European Cells and Materials Vol. 29. Suppl. 3, (2015), page 53.

Rihab Gam, Pranali Shah, Rachel E. Crossland, Jean Norden, Anne M. Dickinson, Ralf Dressel. Genetic Association of Haematopoietic Stem Cell Transplantation Outcome beyond Histocompatibility Genes. Manuscript submitted to Frontiers in Immunology.

Rihab Gam, Jean Norden, Rachel Emily Crossland, Kim Pearce, Clare Lendrem, Massimiliano Caioni, Silvia Haffner, Ernst Holler, Ralf Dressel, Anne. Mary. Dickinson. Impact of MICA-129 dimorphism, MICA gene expression and soluble MICA levels on haematopoietic stem cell transplantation outcome. Manuscript in preparation for submission.

R. Gam, J. Norden1, R. Crossland, K. Pearce, A. M. Dickinson. IRAK1 and miR-146a for predicting the outcome of allogeneic haematopoietic stem cell transplantation. 41st Annual Meeting of the European Society for Blood and Marrow Transplantation. (http://www.fondazioneime.it/newime/documenti/bmt2015abstract.pdf)

R. Gam, J. Norden, R. Crossland, K. Pearce, E. Holler, R. Dressel, A. M. Dickinson. MICA genotype, serum and expression level effects on the outcome of HSCT. The 41st Annual Meeting of the European Society for Blood and Marrow Transplantation. (http://www.fondazioneime.it/newime/documenti/bmt2015abstract.pdf)

Oral Presentations

R. Gam, J. Norden, K. Pearce, Clare Lendrem, R. Crossland, E. Holler, R. Dressel, A. M. Dickinson. MICA-129 dimorphism, MICA mRNA and sMICA levels association with the outcome of HSCT. GvH/GvL. The 13th GvH GvL meeting, Regensburg, Germany, March 2016

R. Gam, J. Norden, R. Crossland, K. Pearce, E. Holler, R. Dressel, A. M. Dickinson. MICA genotype, serum and expression level effects on the outcome of HSCT. The 41st Annual Meeting of the European Society for Blood and Marrow Transplantation, Istanbul, Turkey, March 2015.

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41st Annual Meeting of the European Society for Blood and Marrow Transplantation, Istanbul, Turley, March 2015.
R. Gam, J. Norden, R. Crossland, K. Pearce, E. Holler, A. M. Dickinson. Aspect of MICA and IRAK1 in graft versus host disease. CellEurope, Project The Marie Cure ITN midterm review for CellEurope, Number315963, July 2014.

R. Gam. Knowledge Transfer: Project Showcase presentation. Fred Hutchinson Cancer Research Center, Seattle Washington, USA September 2015.

R. Gam. Impact of MICA on HSCT outcome. ICM Seminar, Institute of Celular Medicine, Faculty of Medical Sciences, Newcastle University, March 2015

R. Gam. Novel SNPs in HSCT. ICM Seminar, Institute of Celular Medicine, Faculty of Medical Sciences, Newcastle University, March 2014

Poster Presentations

R. Gam, J. Norden, K. Pearce, Clare Lendrem, R. Crossland, E. Holler, R. Dressel, A. M. Dickinson. MICA-129 dimorphism, MICA mRNA and sMICA levels association with the outcome of HSCT. GvH/GvL. The 13th GvH GvL meeting, Regensburg, Germany, March 2016.

R. Gam, J. Norden1, R. Crossland, K. Pearce, A. M. Dickinson. IRAK1 and miR-146a for predicting the outcome of allogeneic haematopoietic stem cell transplantation. 41st Annual Meeting of the European Society for Blood and Marrow Transplantation, 24th March 2015, Istanbul Turkey.

R Gam, J Norden, R Crossland, K Pearce, E Holler, R Dressel, AM Dickinson MICA genotype, serum and expression level effects on the outcome of HSCT. Annual meeting of the Tissue and Cell Engineering Society 19-20 July 2015, Southampton, Great Briton.

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Chapter 1. Introduction and study aims

1.1 Haematopoietic stem cells and generation of blood and immune cells

The bone marrow (BM) represents the most regenerative tissue, with relatively one trillion (10^{12}) cells being produced on a daily basis (Doulatov *et al.*, 2012). Establishment and maintenance of the blood system depends on self-renewing haematopoietic stem cells (HSCs) residing as rare cells in specific niches (Taganov *et al.*, 2007; Ferrara *et al.*, 2009; Doulatov *et al.*, 2012). In humans, haematopoiesis −the process by which blood cells are formed− begins in the yolk sac and transitions into the liver temporarily before finally establishing definitive haematopoiesis in the bone marrow and thymus (Ferrara *et al.*, 2009; Tavian *et al.*, 2010; Rusca and Monticelli, 2011a; Shaw and Madrigal, 2012).

The hierarchy model of haematopoiesis, shown in Figure 1.1, depicts that haematopoietic stem cells are at the top of a hierarchy of progenitors that become progressively restricted to several or single lineages including red blood cells, megakaryocytes, myeloid cells (monocyte/macrophage and neutrophil) and lymphocytes (Orkin and Zon, 2008). Macrophages and neutrophils play a major role in the innate immune system and provide a first line of defence against many common pathogens. These cells also play a crucial part in the initiation and subsequent direction of adaptive immune responses, as well as participating in the removal of pathogens that have been targeted by adaptive immune responses (Sant'Angelo and Janeway, 2002).

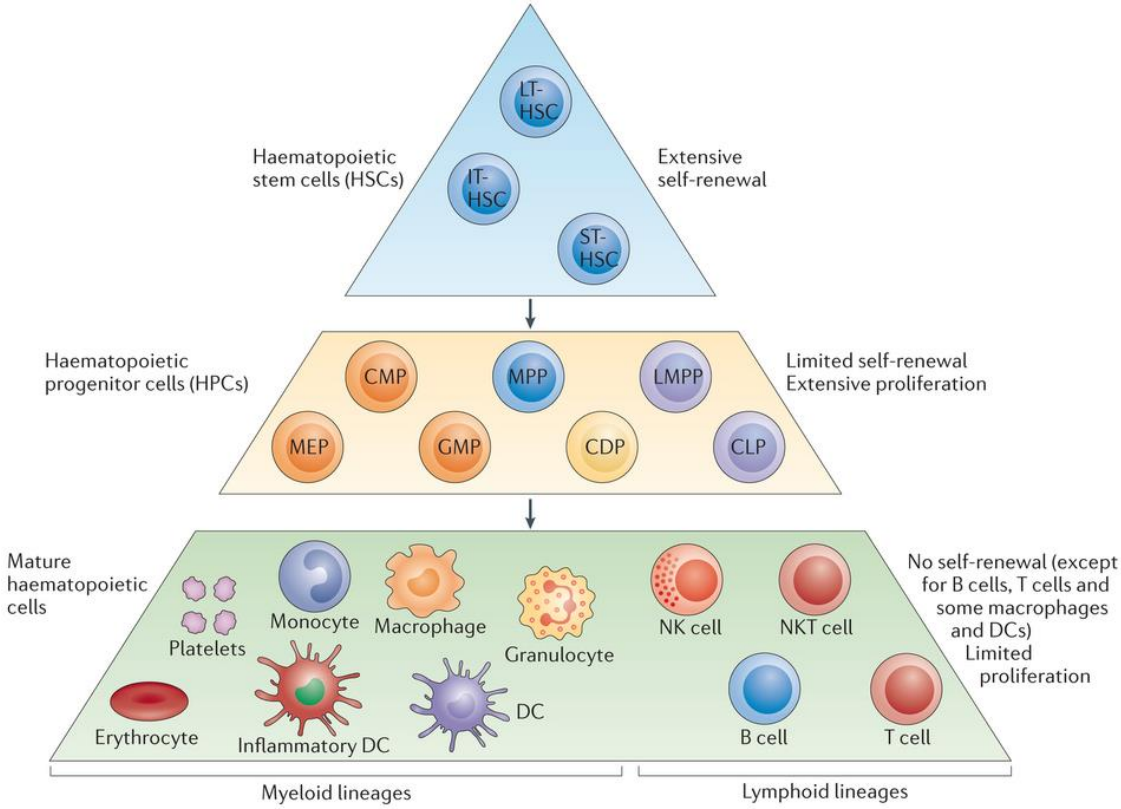


Figure 1.1 Model of the haematopoietic hierarchy (adopted from Manz and Boettcher, 2014). HSCs are at the top of the hierarchy and are characterized by their self-renewal capacity and the potential to give rise to all haematopoietic cell types (multi-potency). HSCs generate multiple types of haematopoietic progenitor cells (HPC), which are characterized by an extensive proliferative potential but only very limited (if any) self-renewal capability and thus, these cells need to be continuously replenished from the HSC pool. Throughout differentiation, an HSC first loses self-renewal capacity, then loses lineage potential step-by-step as it commits to become a mature functional cell of a specific lineage. Multipotent progenitors give rise to oligo-potent progenitors including the common lymphoid progenitors (CLP), which gives rise to mature B lymphocytes, T lymphocytes, and natural killer (NK) cells. The common myeloid progenitor (CMP) gives rise to granulocyte-macrophage progenitors, which differentiate into monocytes/macrophages and granulocytes, and megakaryocyte/ erythrocyte progenitors, which differentiate into megakaryocytes/platelets and erythrocytes. Both CMPs and CLPs have been proposed to give rise to dendritic cells (Bryder *et al.*, 2006; Manz and Boettcher, 2014).

1.1.1 Innate and adaptive immune cell specificities and pathogen recognition

Penetration of the epithelial surface by microorganisms such as bacteria, immediately activates cells and molecules that can mount an innate immune response (Elphick and Mahida, 2005). By means of surface receptors, phagocytic macrophages become activated and engulf the pathogen, followed by secretion of chemokines and cytokines which attract neutrophils and monocytes from the blood stream (Albert *et al*, 2002). Local inflammation and phagocytosis of the bacteria may also be initiated as a result of activation of the complement cascade on the bacterial cell surface. The main cell types contributing to the inflammatory response in its initial phases are neutrophils. This influx of neutrophils is followed by monocytes, which differentiate into macrophages. Macrophages and neutrophils are thus also known as inflammatory cells (Janeway *et al*, 2001).

This innate immune response increases the flow of lymph containing antigen and antigen-bearing cells in the lymphoid tissue and induces an adaptive immune response, beginning with the ingestion of the pathogen by immature dendritic cells (DCs) in the infected tissue (Forster *et al.*, 2008). Eventually, all tissue-resident DCs migrate through the lymph node to the regional lymph node where they interact and recruit naïve lymphocytes (Janeway *et al*, 2001).

The recognition mechanism used by lymphocytes of the adaptive immune system is more sophisticated than the innate immune system, in order to enable recognition of an almost infinite diversity of antigens so that each different pathogen can be targeted specifically.

1.2 Haematopoietic stem cell transplantation

The field of HSCT has undergone several advancements since it was introduced as a therapy for otherwise incurable haematological disorders.

The marrow transplantation story began in 1949 with Jacobson et al. (Jacobson *et al.*, 1949). During their studies, they demonstrated that shielding the spleen of a mouse during irradiation allowed the survival of this mouse. Further studies by Lorenz et al. (Lorenz *et al.*, 1951) showed that the infusion of spleen or marrow cells could protect the mice during irradiation. The "protection against irradiation" phenomenon was thought to be due to humoral factors, however in 1954, Barnes and Loutit reviewed their work along with other studies and stated, "the chemical hypothesis has not been proved by the complete exclusion of the cellular hypothesis" (Barnes and Loutit, 1954). A subsequent study performed by Main and Prehn in 1955 showed great support for the cellular hypothesis, when they demonstrated that the infusion of allogeneic marrow protected the mice from irradiation and resulted in tolerance to a donor skin graft (Main and Prehn, 1955).

Later, Ford et al. showed that the protection of the mice was due to the infusion of cytogenetic characteristics of the donor along with the marrow infusion (Ford and Hamerton, 1956). The principles of HSCT came from the first experiment performed by Nowell and Ford (Ford and Hamerton, 1956; Nowell *et al.*, 1956), where they transfused bone marrow cells from one mouse into a lethally irradiated mouse and observed restoration of the entire repertoire of haematopoietic cells.

In 1957, Thomas performed the first unsuccessful allogeneic transplant, followed by a successful syngeneic transplant in 1959, using the bone marrow of an identical twin (Thomas *et al.*, 1957). In 1968, Dr Robert Good and his team performed the first matched sibling donor transplant in an infant with immunodeficiency (Gatti and Good, 1971) and in 1969, it was repeated for a patient with leukaemia (Thomas and Storb, 1970). In 1969, the marrow transplant team in Seattle began a series of marrow transplantations using HLA matched sibling donors for patients in the end stages of leukaemia or aplastic anaemia (Main and Prehn, 1955; Thomas et al., 1972).

In the 1970s it was difficult to evaluate the role of stem cell transplantation (SCT) in the treatment of leukaemia, due to the fact that almost all patients had been transplanted for advanced diseases after failure of normal therapy. However, in 1972, a review

article presented the current state of BMT knowledge at that time. This study included 73 patients with leukaemia and 37 with aplastic anaemia. All underwent transplantation after failure of conventional therapy and it was observed that the engraftment was successful in some patients with aplastic anaemia and survival with grafts in remission was observed in few patients with leukaemia (Thomas *et al.*, 1972). In 1977, an American study reported on 100 patients with advanced acute leukaemia who were conditioned with cyclophosphamide (Cy) and total body irradiation (TBI) and given marrow from HLA matching siblings (Thomas *et al.*, 1977). At the time of the report, 17 out of 100 survived 1 to 3 years later and 8 of those 17 remain alive and well (Thomas *et al.*, 1972). The analyses of disease-free survival demonstrated that some patients with advance leukaemia might be cured (Thomas *et al.*, 1972). Since then the HSCT field has come a long way from this pioneering research. Current estimates of annual numbers of HSCT are between 55,000 and 60,000 worldwide (Lad et al., 2012).

Different types of HSCT are best suited for different diseases (Figure 1.2). Indeed, autologous HSCT (auto-HCST) (Figure 1.3), a process by which removal, storage and reinfusion of patient's own HSC is performed to re-establish the patient's depleted bone marrow after a high dose of myeloablative therapy, can be indicated for conditions such as: multiple myeloma, non-Hodgkin's lymphoma, Hodgkin's disease, acute myeloid leukaemia, neuroblastoma, germ cell tumours, autoimmune disorders (systemic lupus erythematosus (SLE), systemic sclerosis) and Amyloidosis (Daelken *et al.*, 2008).

Allogeneic HSCT (allo-HSCT), consists of infusing the mature and immature blood cells from the bone marrow, umbilical cord or peripheral blood of a sibling, relative or unrelated donor (Figure 1.3) as a possible procedure to restore the patient's bone marrow with a new immune system after a conditioning regimen (myeloablative or non myeloablative chemotherapy). Allo-HSCT can be indicated for: acute leukaemia, chronic leukaemia, myeloproliferative disorders, myelodysplastic syndromes, multiple myeloma, lymphoma, aplastic anaemia, Fanconi anaemia, thalassemia major, severe combined immunodeficiency (SCID), and many other conditions (Hołowiecki, 2008).

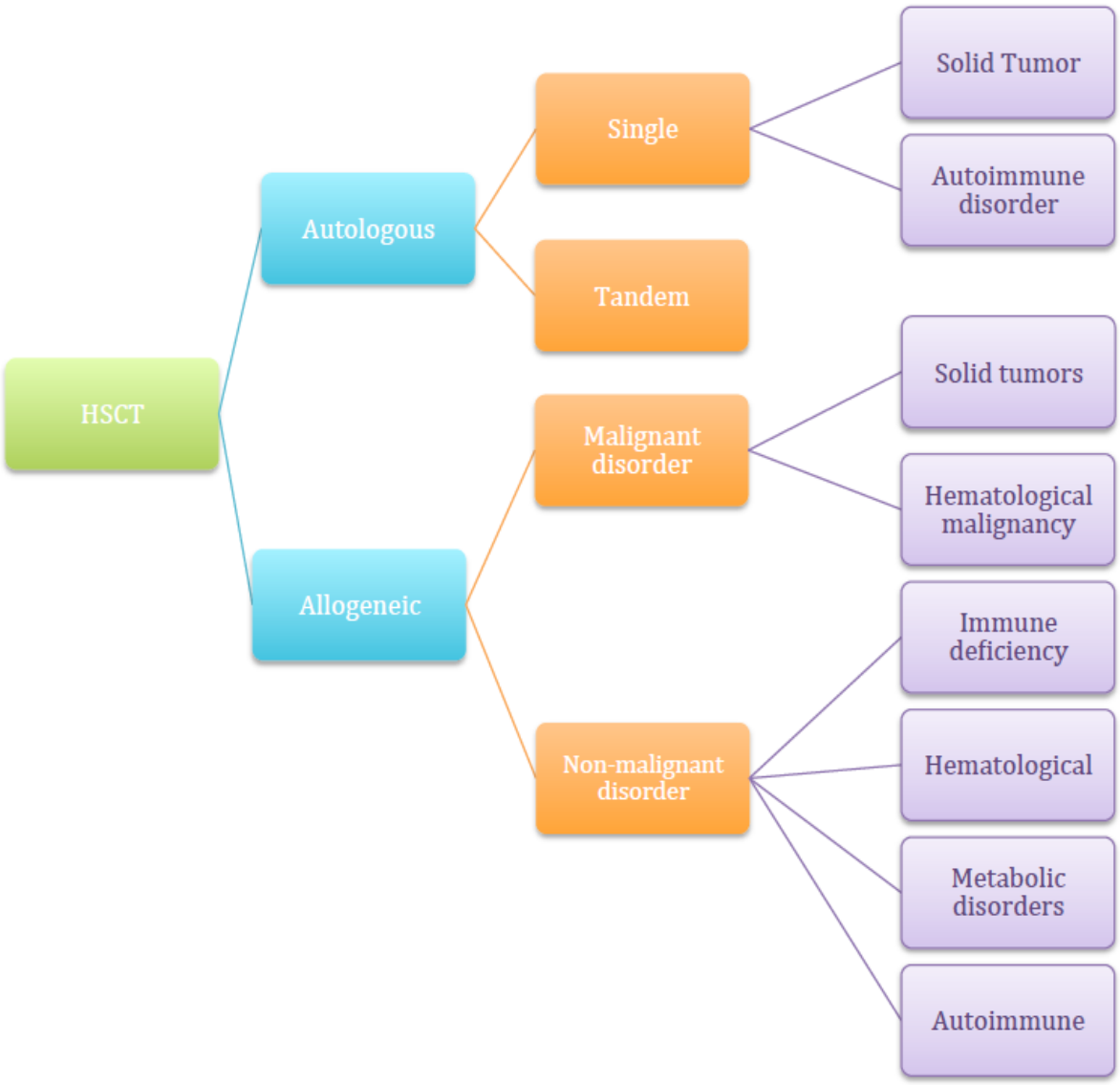


Figure 1.2 Examples of indication for HSCT according to the underlying condition (adapted from Hołowieck et al., 2008). Autologous HSCT is indicated for conditions such as multiple myeloma, non-Hodgkin's lymphoma, Hodgkin's disease, acute myeloid leukaemia, neuroblastoma, germ cell tumours, autoimmune disorders (systemic lupus erythematosus (SLE), systemic sclerosis) and Amyloidosis. Allogeneic HSCT can be indicated for: acute leukaemia, chronic leukaemia, myeloproliferative disorders, myelodysplastic syndromes, multiple myeloma, lymphoma, aplastic anaemia, Fanconi anaemia, thalassemia major, severe combined immunodeficiency (SCID), and many other conditions (Hołowiecki, 2008).

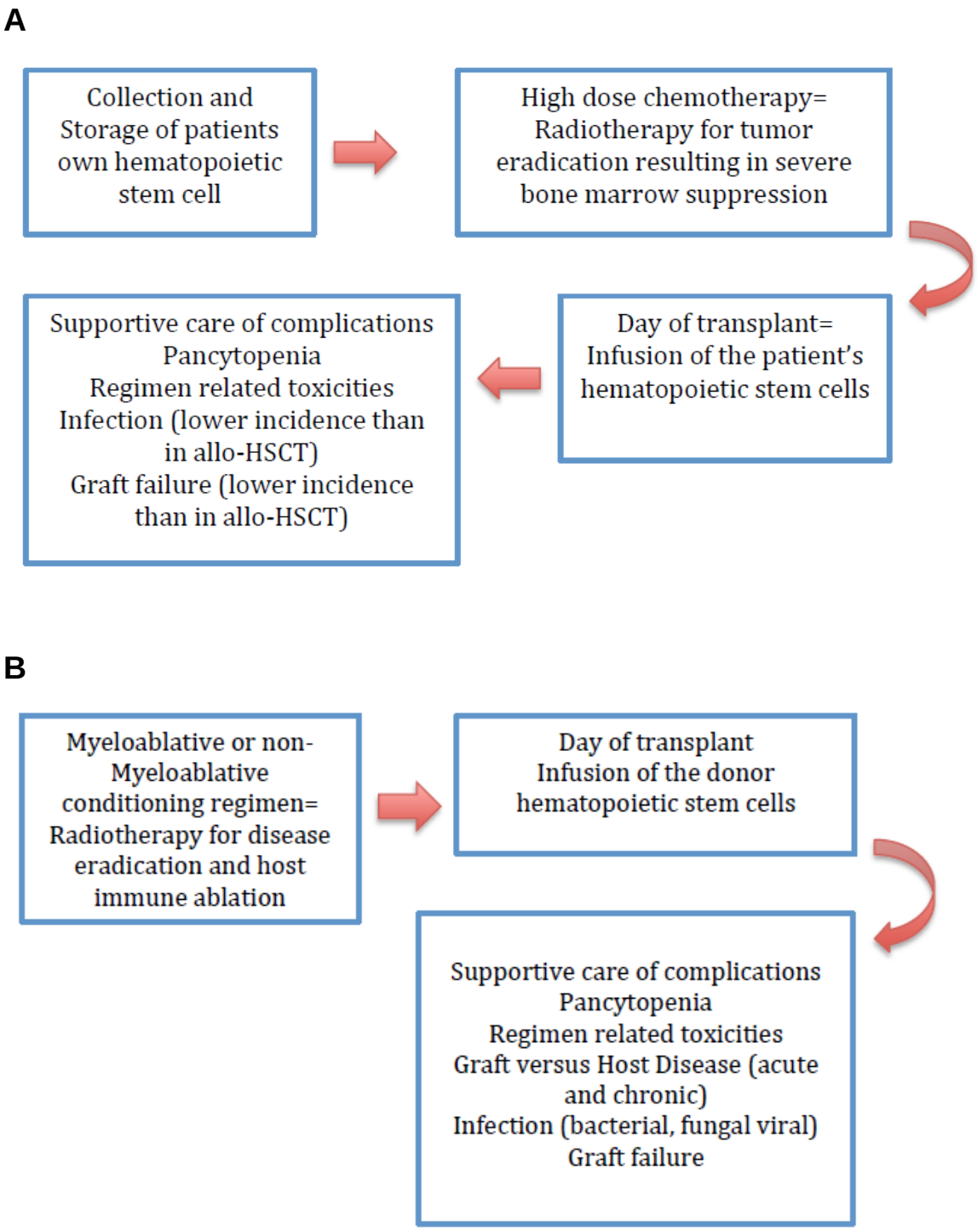


Figure 1.3. Haematopoietic Stem Cell Transplantation procedure (adapted from Hołowieck et *al.*, 2008). (A) Autologous HSCT procedure. (B) Allogeneic HSCT procedure (Hołowiecki, 2008).

1.3 Donor selection

The donor selection is a critical element contributing to the success HSCT. Certain aspects should be taken into consideration when selecting a donor for allo-HSCT. The donor should have satisfactory cardiac, pulmonary, hepatic and renal functions in order to tolerate general or local anaesthesia. Donors with active cancer or history of cancer are generally excluded (Lee *et al.*, 2003). The state of positivity for hepatitis B, hepatitis C, CMV and HIV should be assessed in allogeneic donors (Choi *et al.*, 2005; Parody *et al.*, 2006; Schmidt-Hieber *et al.*, 2013). In addition, it is of primary importance in HSCT to have a sufficient donor-recipient human leucocyte antigen (HLA) match to ensure engraftment and acceptable rates of complications such as graft versus host disease (GvHD) (Park and Seo, 2012). Therefore, the selection of HSCT donors includes a rigorous assessment of the availability and human leukocyte antigens (HLA) match status. HLA plays critical roles in HSCT, and its involvement is constantly evolving due to the change of technologies and variation in clinical transplantation results (Park and Seo, 2012). The increased availability of donors through the use of HLA-mismatched related and unrelated donors is feasible, with a more complete understanding of permissible HLA mismatches in HSCT (Lee *et al.*, 2007a).

1.3.1 The major histocompatibility complex and HLA matching

Tissue compatibility is determined by genes of the major histocompatibility complex (MHC), known as the HLA system in humans (Hedrick, 1994). These genes are clustered on the short arm of chromosome 6. The HLA region of the genome is a multigenic system that encodes structurally homologous cell surface glycoproteins characterized by a high degree of allelic polymorphism in the human population (Le Bouteiller, 1994). The function of HLA molecules is to present peptide antigens to T cells, where they play a major role in T cell mediated adaptive immune responses. A major barrier in HSCT is caused by immune responses directed against incompatible HLA antigens and thus, the accuracy of histocompatibility testing and matching criteria have important consequences on HSCT outcome (Hedrick, 1994).

The homologous HLA Class I (HLA-A,-B,-C) and Class II (HLA-DR,-DQ,-DP) antigens are co-dominantly expressed and have different structures, tissue distribution and characteristics in peptide presentation to T cell (Weyand *et al.*, 1992).

HLA class I molecules are expressed on most nucleated cells and are composed of an α chain (encoded in the MHC), non-covalently associated with β2-microglobulin (encoded on chromosome 15) and two outermost α1 and α2 domains of the heavy chain which form the peptide binding site (Figure 1.4). Peptides presented by class I HLA molecules are usually 8-10 amino acids and are commonly recognized by CD8+ cytotoxic T cells (CTL) (Koziel *et al.*, 1995). HLA class II antigens are expressed on a subset of T cells referred to as antigen presenting cells (APCs) such as B cells, activated T cells, macrophages and dendritic cells (Brown *et al.*, 1993). HLA class II molecules are comprised of two membrane bound alpha and beta chains encoded by two genes co-localized in the MHC, and the peptide pocket is formed by the most distal domains of the two chains (Figure 1.4) (Leddon and Sant, 2010). Peptides presented by HLA Class II molecules are recognized by CD4+ T helper cells (Koziel *et al.*, 1995).

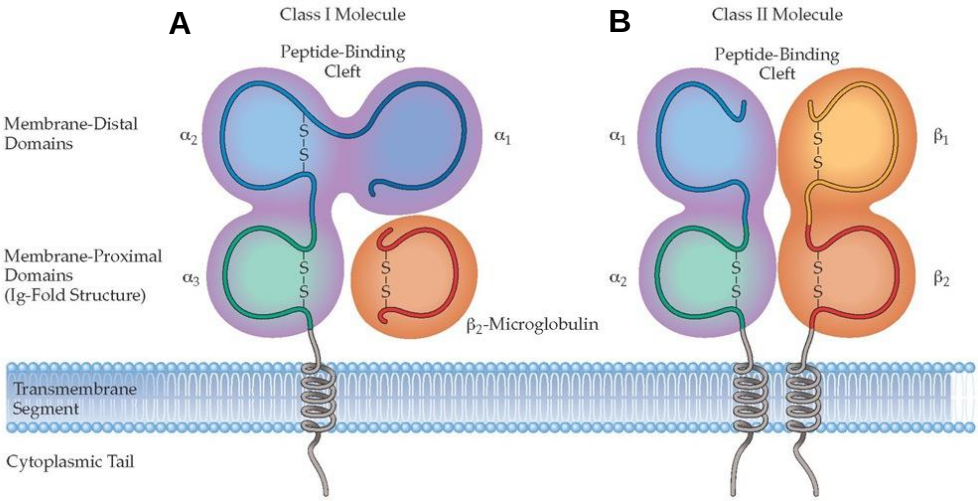


Figure 1.4 Structural composition of the MHC molecules (adapted from Milford and Carpenter, 2004). (A) MHC class I molecules consist of heavy chains made up of three polypeptide domains (α1, α 2, α 3) and a non-covalently associated light chain, β2-microglobulin. (B) MHC class II molecules are heterodimers of α and β chains with a very similar overall structure and peptide-binding surface (Milford and Carpenter, 2004).

These peptide-HLA complexes are the ligands of clonally distributed TCRs, which are capable of recognizing HLA molecules. 1 to 10% of the peripheral blood lymphocytes of a donor can respond to a given allo-MHC (Benichou *et al.*, 2011). Immune responses against incompatible HLA antigens may be extreme such as the case of GvHD and thus, represent a major barrier in HSCT (Benichou *et al.*, 2011).

During the process of HLA matching, the optimum donor is a HLA genotypically matched sibling as determined by family typing. Family typing is also used to verify the patient's genotype (Choo, 2007). Low resolution typing for HLA-A, -B, -DR (serology or 2-digit DNA typing (ABDR typing) is sufficient, in most cases, to determine the maternal and paternal haplotypes present in the patient and their potential donor. Thus, ABDR typing can confirm genotypic identity for the whole set of HLA genes (example 12/12 match) (Figure 1.5) (Petersdorf *et al*., 2015).

Due to the weak linkage disequilibrium between DP and the DR/DQ loci, a low level of a DP mismatch sibling donors (1-2%) can be identified because of the recombination (Huang *et al.*, 2006). An HLA-A/B or B/DRB1 recombination event is detected by routine HLA-A/B/DR typing in about 2% of families. The chance of a sibling match (genotypically identical sibling) is 25% and thus, approximately 70% of patients do not have a sibling match (Huang *et al.*, 2006). In contrast, the possibility of identifying a haploidentical donor is 50% (Huang *et al.*, 2006).

In cases of mismatched related HSCT, the risk of GvHD and graft failure increase with the number of HLA disparities. In some cases a differential effect of Class I and Class II has been described, in which GvHD risk was associated with Class II disparities (Petersdorf, 2007).

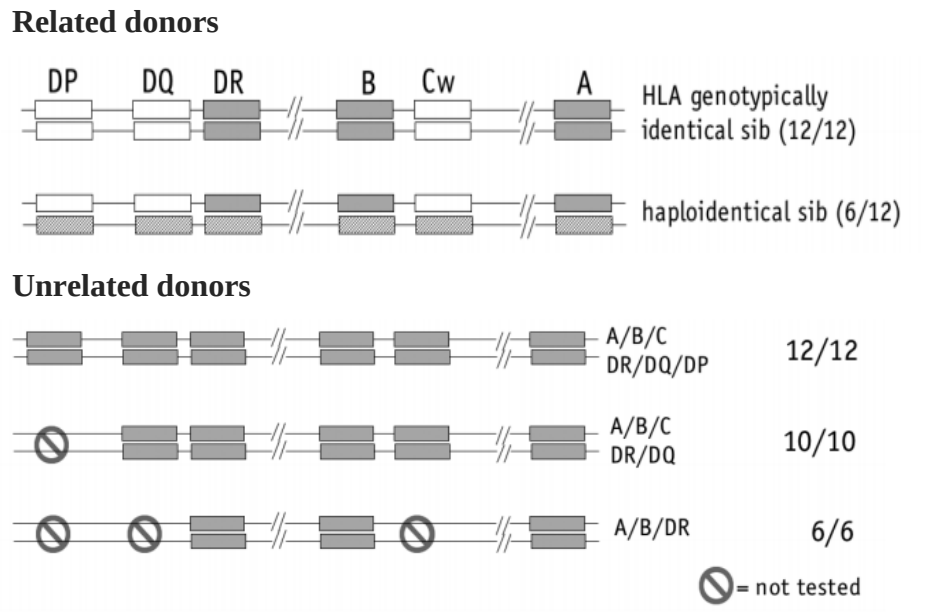


Figure 1.5. Matching criteria for related and unrelated HSCT (adapted from Shaw and Madrigal, 2012). An HLA-genotypically identical sibling donor is compatible at the allele level at all the loci on both chromosomes (12/12 match). In unrelated HSCT, matching for *A/B/C/DRB1/DQB1* loci is usually searched for (10/10 match). In addition to DRB1 compatibility, some centres also consider DRB3/DRB5 polymorphism. DRB3 mismatches occur frequently in DR13 haplotypes. Because of strong linkage disequilibrium with DRB1, the DRB5 locus is usually not tested. In DR15/16 haplotypes, DRB5 mismatches usually co-occur with DRB1 disparities. Searching for a 12/12 match implies DPB1 typing. Donors with an 8/8 match (not shown) or a 6/6 match apply, respectively, when HLAC/DP, or HLA-C/DQ/DP are not tested (Marsh *et al.*, 2010; Shaw and Madrigal, 2012).

1.3.2 The minor histocompatibility antigens

HLA matching remains the major factor influencing donor selection and the outcome following transplantation. However, extensive research of the human genome has shown that certain polymorphisms of nucleotides in genes that are non-HLA related also play a major role in generating alloimmune responses (Hansen *et al.*, 2010). Minor histocompatibility antigens (miHAs) are considered as immunogenetic non-HLA related factors encoded by polymorphic genes, which may differ between the recipient and the donor and thus, may influence transplant outcomes.

MiHAs are polymorphic peptides comprising 9 to 12 amino acids (Granados *et al.*, 2014). After binding to the antigen recognition site of either Class I or Class II HLA molecules present on the cell surface, miHAs can be recognized by T cells. Thus, the occurrence of miHAs depends on the presence of specific HLA antigens, which is referred to as MHC restriction (Simpson *et al.*, 1993). MiHAs can either be encoded by autosomal chromosomes or by the Y-chromosome (Simpson *et al.*, 1993). There are two patterns of miHAs tissue distribution: restricted and broad. Autosomal HA-3, HA-8 and the majority of miHAs encoded by the Y chromosome are predominant in various tissues including GvHD target tissue: skin, intestine, and liver (Falkenburg *et al.*, 2003). Most autosomal and 2 miHAs encoded by the Y chromosome (B8/HY and B53/HY) are only present in HSC, including leukaemic cells DCs, NK and multiple myeloma cells (Dzierzak-Mietla *et al.*, 2012). MiHAs are key molecules driving allo-immune responses in both GvHD and graft versus leukaemia (GvL) reactivity in HLA-matched HSCT. The genetic basis of miHA immunogenic T-cell epitopes is caused by polymorphic genes. The most common form of genetic polymorphisms leading to miHAs are non-synonymous SNPs, but disparities of miHAs may also result from gene deletion (Dzierzak-Mietla *et al.*, 2012). Although the characterization of miHAs has contributed to our basic knowledge of genetic polymorphism, immunobiology, and immungenetics, their key role is related to their clinical applicability. The role of miHAs in HSCT has been extensively explored and miHAs mismatching has been clinically associated with an increased risk of GvHD (Goulmy *et al.*, 1996; Tseng *et al.*, 1999; Falkenburg *et al.*, 2003; Marijt *et al.*, 2003; Cavanagh *et al.*, 2005).

Despite the improved matching of donor-recipient pairs via the implementation of high resolution technology for molecular HLA typing, improved outcomes following transplantation are still limited by a high number of complications: GvHD, engraftment problems (lack or loss of engraftment) and relapse (Vogelsang *et al.*, 2003). The long-term survival after allo-HSCT is currently estimated in the range of 40-70% (Dzierzak-Mietla *et al.*, 2012). Failures are mainly due to infectious complications and GvHD (30-40% each), organ toxicity following chemotherapy (20%) and relapse (20-30%) (Dzierzak-Mietla *et al.*, 2012).

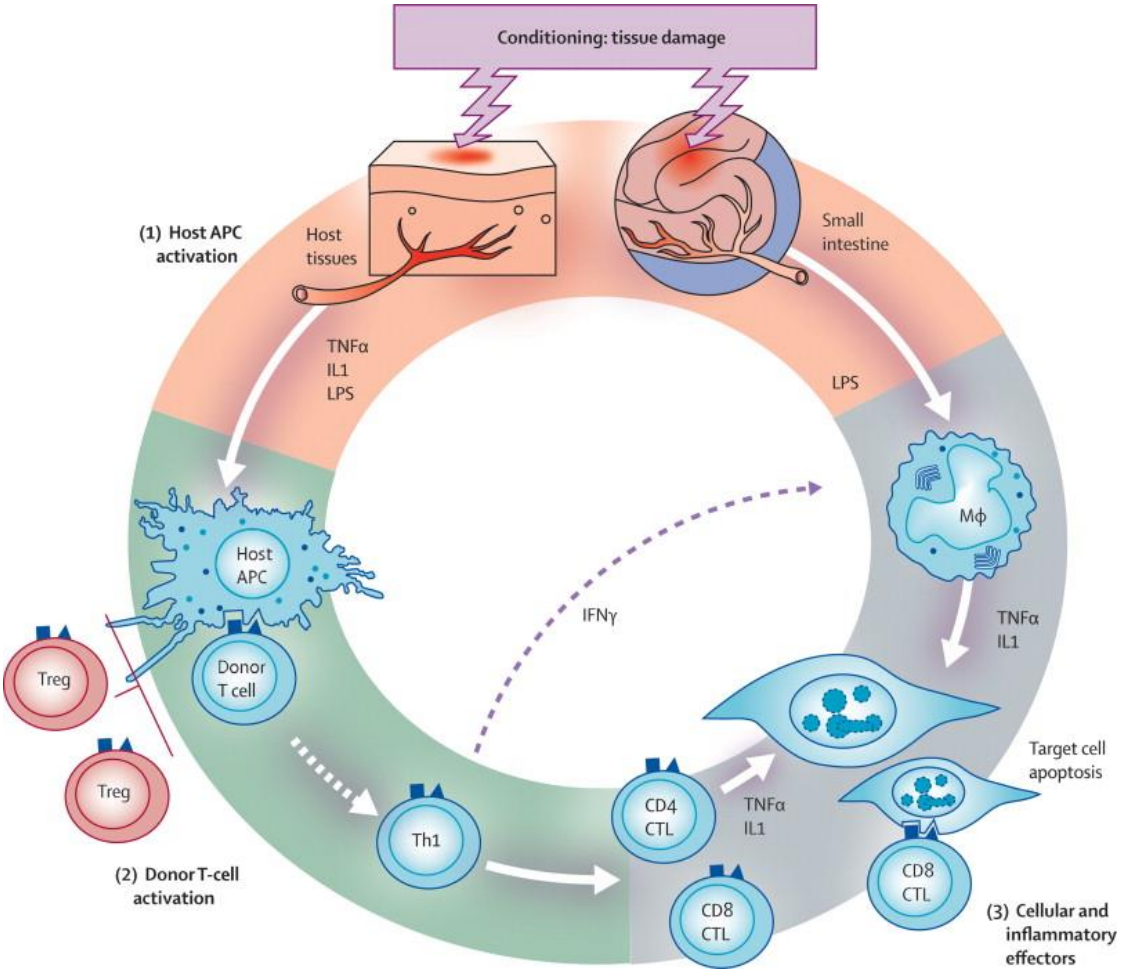
1.4 Complications after haematopoietic stem cell transplantation

1.4.1 Graft versus host disease

Graft versus host disease may occur after allogeneic BMT. GvHD occurs when the donated bone marrow or stem cells (graft) view the recipient's body as non-self, which causes these cells to attack the body of the recipient (Welniak *et al.*, 2007). The risk of GvHD is very low when a patient receives bone marrow or cells from an identical twin, but increases to 30-40% when the donor and recipient are related and it rises further to 60-80% when the donor and recipient are not related (Sykes *et al* 2011). GvHD may be lethal and thus, limits the effect of many measures that have been developed to improve HSCT outcome, such as infection prophylaxis, immunosuppressive medications, supportive care and DNA-based tissue typing. As allo-HSCT is increasingly becoming an attractive therapeutic option, the need for novel approaches to predict GvHD has accelerated. This is particularly true as the number of patients receiving transplants from unrelated donors is expected to double, significantly increasing the population of patients with GvHD (Ferrara *et al.*, 2009).

1.4.2 Pathophysiology of graft versus host disease

GvHD has a complex pathophysiology and should therefore be studied as a pathway that has its own triggers, sensors, mediators and effectors (Figure 1.6).



IL 1=interleukin 1. IFN γ=interferon γ. LPS=lipopolysaccharide. Treg=regulatory T cell. Th1=T-helper 1 cell. CTL=cytotoxic T lymphocyte

Figure 1.6 GvHD pathophysiology (adapted from Ferrara *et al.,* 2009). In phase I, the recipient conditioning regimen damages host tissues and causes release of inflammatory cytokines such as TNFα IL-1 and IL-6. Increased levels of these cytokines leads to activation of host antigen presenting cells (APCs). In phase II, host APCs activate mature donor cells. The subsequent proliferation and differentiation of the activated T cells produces additional effectors that mediate the tissue damage, including cytotoxic T lymphocytes, NK cells, TNFα and IL-1. Lipopolysaccharide (LPS) that has leaked through the damaged intestinal mucosa triggers additional TNFα production. TNFα can damage tissue directly by inducing necrosis and apoptosis in the skin and GI tract through either TNFα receptors or Fas pathway. TNFα plays direct role in intestinal GvHD damage, which further amplifies damage in the skin, liver and lung in “cytokine storm”. Phase III, is a complex cascade of cellular mediators (such as cytotoxic T cell and NK cells) and soluble inflammatory agents (eg, TNFα, interferon γ, interleukin 1 and nitric oxide). These molecules work synergetically to amplify local tissue injury and further promote inflammation and target issue destruction (Welniak *et al.*, 2007; Shaw and Madrigal, 2012) (Ferrara *et al.*, 2009)**.**

1.4.2.1 Triggers for induction of GvHD

As with all immune responses, specific factors are important in initiating a graft versus host reaction. Disparities between histocompatibility antigens is the first trigger for GvHD, this can be at the level of MHC, referred to as MHC mismatch, or it can be at the level of miHA complex known as MHC matched but miHA mismatched. The severity of aGvHD may be directly related to the degree of MHC mismatch (Tang et al., 2004). In the case of bone marrow transplant where the MHC is matched but the miHA is disparate, the donor T cells will still be able to recognize MHC peptides expressed with the polymorphic miHAs of the recipient (Den Haan *et al.*, 1995; Murata *et al.*, 2003).

The damage caused by different conditioning regimens and the underlying disease (Ishida *et al.*, 2012) is the second trigger of GvHD, by initiating an innate immune response. The innate immune system can be triggered by different exogenous and endogenous stimuli. These include specific receptors of innate immunity, such as Toll like receptor (TLRs) or nucleotide-binding oligomerization domains containing 2 (NOD2) present on APCs. APCs recognize conserved damage associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) and initiate a “cytokine storm”. DAMPs and PAMPs are usually released during the chemotherapeutic and radio-therapeutic-conditioning regimens performed before HSCT donor cell infusion, and play a critical role in GvHD (Hill and Ferrara, 2000; Holler *et al.*, 2004; Holler *et al.*, 2006).

1.4.2.2 Sensors of GvHD

Antigen presenting cells might be considered as the sensors for aGvHD. As previously mentioned, the APCs have pattern recognition receptors for DAMPs and initiate aGvHD by presenting protein ligated to MHC or miHA and thus, avoid critical secondary and tertiary signals for activation of alloreactive T cells (Reddy, 2013). Allodisparity between peptide complexes and MHC is usually sensed by APCs. One of the most important APCs are DCs, as they are the primary sensors of allodisparity (Banchereau and Steinman, 1998).

At the time of transplant, recipient DCs process and present MHC and peptide complexes to donor T cells (Shlomchik *et al.*, 1999). Donor DCs take over this role at a later point (Matte *et al.*, 2004). Initially the recipient DCs present endogenous antigens to the donor CD4+ T cells and the exogenous antigens to the donor CD8+ T cells. This indicates that the DCs represent one of the most important mediators for GvHD, as they contribute to its initiation. In addition to DCs and APCs, the DAMPs along with the inflammatory cytokines represent the third signal to enhance interactions between APCs of the recipient and T cells of the donor. Different modulations of the APCs also have an effect on GvHD. Recent studies have shown that exposure to granulocyte colony-stimulating factor (GCSF) shortly after HSCT, combined with a total body irradiation (TBI)-conditioning regimen, significantly enhanced GvHD in mice (Morris *et al.*, 2009). In contrast, modulating the host DCs function via inhibiting histone deacetylase using suberonylanilide hydroxamic acid (SAHA) can reduce GvHD in murine models (Reddy *et al.*, 2004; Reddy and Beal, 2008).

1.4.2.3 Mediators of GvHD

Mediators of GvHD include donor T-cell subsets and donor NK cells. It has been shown that alloreactive donor T cells have several subsets with different characteristics, including stimuli responsiveness, activation thresholds and effector functions (Wu and Ritz, 2006). The alloantigen composition of the donor determines which subset of the T cells will differentiate and proliferate. Either CD4+ or CD8+ subsets, or both, can induce aGvHD in HLA-matched HCT in response to miHAs (Wu and Ritz, 2006).

Moreover, the repertoire and immunodominance of the GvHD associated peptides presented by MHC class I or II has not been defined until now (Spierings *et al.*, 2006). Some studies have shown that it is possible to modulate the alloreactivity of the naïve donor T cells by mixed chimerism, deletion of cytokine modulation or co-stimulation blockage (Anderson, *et al*. 2003). Donor T cells that are not alloreactive cannot induce GvHD, but can mediate GvL (Zheng *et al.*, 2008). Another type of T cell that plays a key role in GvHD are regulatory T cells (Tregs), these cells have a negative effect on GvHD. Tregs also have different subsets, such as the naturally occurring CD4+CD25+ that express the Forkhead Box Protein P3 (FOXP3), CD4+CD25- IL10+ Treg cells, gamma delta T cells, double negative (DN) T cells and NKT cells (Zeng *et al.*, 1999; Roncarolo *et al.*, 2001; Young *et al.*, 2003; Maeda *et al.*, 2005; Cohen and Boyer, 2006).

Studies performed in murine BMT models showed that these naturally occurring donor-derived Tregs have the ability to prevent GvHD and preserve GvL, depending on the ratio of effector T cells to Tregs (Cohen *et al.*, 2002; Edinger *et al.*, 2003; Coghill *et al.*, 2008). Thus, finding ways to increase the number of Tregs and enhance their function is of high importance in allo-HSCT.

T cells are usually divided into subsets based on the dominant cytokines produced after their activation. Based on this, subsets such as Th1, Th2 and Th17 cells can be defined. Cytokines produced by Th1 cells (INF-γ, IL-2 and TNFα) are implicated in the pathophysiology of aGvHD (Reddy, *et al* 2009). IL-2 and its receptor have been the target for many therapeutic and prophylactic procedures, in an attempt to control aGvHD (Ratanatharathorn *et al.*, 1998; Liu *et al.*, 2004). On the other hand, emerging data shows that another important role of IL-2 is the generation and the maintenance of CD4+CD25+Foxp3+ Tregs, so inhibiting IL-2 may have a negative effect on the development of long-term tolerance after allogeneic HCT (Zeiser *et al.*, 2006; Liston and Rudensky, 2007).

Recently, donor NK cells have been identified as emerging key effectors in the GvH process. It has been shown that they specifically down regulate the activation of alloreactive donor T cells and this could be by directly killing the host APCs that activate donor T cells (Asai *et al.*, 1998; Baker *et al.*, 2001).

1.4.2.4 Effectors of GvHD

This final phase leads to the damage of the target organ after a cascade that involves cytolytic cellular effectors such as CD8 CTLs, CD4 T cells, NK cells and inflammatory molecules such as IL-1b, INF-γ, TNFα as well as reactive oxygen species (Ferrara and Deeg, 1991). Cell-to-cell contact is required to cause damage to the target tissue and this is mediated via the activation of perforin granzyme, Fas-FasL (CD95-CD95L), or TNFR-TRAIL pathways (Brown *et al.*, 2005). Other damage pathways including the TNF related weak inducer of apoptosis (TWAEK), which is a small pleiotropic cytokine of the TNF super family involved the stimulation of cell growth and angiogenesis, have also been reported to be implicated in GvHD (Kägi *et al.*, 1994; Schmaltz *et al.*, 2002; van den Brink and Burakoff, 2002; Zimmerman *et al.*, 2005). The cell-mediated

pathways require cell-to-cell contact and are implicated in both GvHD and GvL (Matte-Martone et al., 2008).

The inflammatory pathways, however, do not require cell-to-cell contact to kill target cells and are thought not to be critical for GvL (Paczesny *et al.*, 2010). Both the cellular pathways and the inflammatory pathways can cause GvHD damage (Figure 1.10).
The pathophysiology of GvHD may be summarized in the 'cyclical three step model: (step 1) damage related to the conditioning regimen leads to the release of DAMPs such as LPS, (step 2) proliferation of donor T cells and (step 3) target organ damage by effectors (Sung, *et al* 2011). However, it is important to consider that the biology of GvHD is a very complicated systemic process with many unknowns and it is therefore not a simple linear or cyclical process. Nonetheless, based on the current research and knowledge, some agents that reduce inflammatory cytokines such as TNF and IL-1, but at the same time spare T cell CTL functions and enhance donor NK cell and Treg functions, may be of high importance to reduce GvHD without compromising GvL.

1.4.3 Acute graft versus host disease

1.4.3.1 Classification

Acute GvHD can occur when the donor's bone marrow or stem cells engraft in the transplant recipient. According to the Seattle classification of 1991, aGvHD occurs in the first 100 days after bone marrow or stem cell transfusion (Ferrara and Deeg, 1991). However, more recently due to changes in conditioning regimens, the National Institute of Health (NIH) have updated the classification to include late-onset aGvHD (after 100 days) and an overlap syndrome, which shows symptoms of both acute and chronic GvHD and might develop in the skin, liver or gastrointestinal tract (Kreisel *et al.*, 1994). The NIH consensus conference held in 2005, proposed the term "overlap" GvHD to describe the situation when both acute and chronic GvHD are present. According to the proposed NIH criteria, aGvHD manifestations occurring more than 100 days after HSCT are classified as "persistent", "recurring" or "late onset" aGvHD depending on the antecedent history of aGvHD and absence of other cGvHD manifestations (Vigorito *et al.*, 2009).
Classic chronic GvHD is defined by diagnostic manifestations of chronic GvHD without characteristic features of acute GvHD, and an "overlap" subtype of chronic GvHD is

defined by simultaneous features of both chronic and acute GvHD (Jagasia *et al.*, 2007; Arora *et al.*, 2009; Cho *et al.*, 2009; Pidala *et al.*, 2011).

1.4.3.2 Target organs, grades and symptoms

Onset of aGvHD symptoms typically occurs 2-3 weeks after transplant (Gilleece, 2011). The primary organs affected by aGvHD are the skin, liver, and gastrointestinal (GI) tract (Gilleece, 2011).

In the skin, symptoms classically manifest as an erythematous, macropopular rash with or without pruritus involving the pinnae, palms and soles. This rash often spreads to involve the neck and trunk with later involvement of the extremities. Severity in determined by the percentage of body surface area involved and may range from a mild, nonpuritic rash to bullous formation and desquamation reminiscent of toxic epidermal necrolysis (Gilleece, 2011).

In the liver, symptoms include an elevated serum bilirubin as a typical manifestation, although elevated alkaline phosphate may also be an indicator of impending disease. A variant of liver aGvHD has also been described that manifests as hepatitis with transaminitis and elevated alkaline phosphatase. However, these are not classic findings and are not specific (Gilleece, 2011). Liver biopsy post transplantation is a rare and dangerous procedure because thrombocytopenia early after transplant can greatly increase its risk and thus, the diagnosis of liver involvement in aGvHD is one of exclusion. The liver is a difficult organ to study as hepatic disease caused by GvHD may be difficult to distinguish from other causes of liver dysfunction following BMT such as drug toxicity or viral infection. However, if related to GvHD, the histological features of hepatic malfunctions are endothelialitis, lymphocytic infiltration of the portal areas, pericholangitis and bile duct destruction (Snover *et al.*, 1984).

The GI tract presents symptoms such as diarrhea, vomiting, anorexia and abdominal pain in severe cases (Ferrara and Deeg, 1991). Histological features include patchy ulcerations, apoptotic bodies in the base of the crypts, crypt abscesses, and loss as well as flattening of the surface epithelium (Snover *et al.*, 1985). Depending on the involvement of these three organs, the degree of severity of GvHD can be determined (Wiesner *et al.*, 2003).

The overall grades of GvHD are classified as I (mild), II (moderate), III (Severe) and IV (very severe). Severe GvHD has a poor prognosis, with 25% long-term survival for grade III and 5% for grade IV (Cahn, 2005). In the skin, the clinical grading system is based on the percentage of skin affected by rash and the severity of the rash, in the liver it is based on elevations of bilirubin level and finally in the GI tract the grade depends on the volume of diarrhea (Kreisel *et al.*, 1994). For a patient, the overall aGVHD score takes into account the grading for each organ. Overall grades are from 0 to IV (0: None, I: Mild, II: Moderate, III: Severe, IV: Life threatening (Spitzer T. Children's National Medical Center, USA) (Table 1.1).

Table 1.1: **Grading and staging of aGvHD based on organ involvement (Przepiorka et al.1995).**

State	Skin	Liver (bilirubin)	Gut (stool output/day)
0	No GvHD rash	< 2 mg/dl	< 500 ml/day or persistent nausea.
1	Maculopapular rash< 25% BSA	2–3 mg/dl	500–999 ml/day
2	Maculopapular rash 25 – 50% BSA	3.1–6 mg/dl	1000–1500 ml/day
3	Maculopapular rash > 50% BSA	6.1–15 mg/dl	Adult: >1500 ml/day
4	Generalized erythroderma plus bullous formation	>15 mg/dl	Severe abdominal pain with or without ileus
Grade			
I	Stage 1–2	None	None
II	Stage 3 or	Stage 1 or	Stage 1

Abbreviations: BSA=body surface area; GI=gastrointestinal; GVHD=graft-versus-host disease.

1.4.4 Chronic graft versus host disease

Chronic GvHD is the major cause of late non-relapse mortality after HSCT. It may present in a progressive way (aGvHD merging into cGvHD), quiescent (aGvHD that resolves completely but is later followed by cGvHD) or it may occur *de novo*. The advanced age of a recipient and a history of aGvHD are the greatest risk factors for cGvHD (Carlens *et al.*, 2002) and thus, the same strategies to prevent aGvHD may help prevent the chronic form. Usually, the manifestations of cGvHD are of an autoimmune nature with resemblance to scleroderma, wasting syndrome and chronic immunodeficiency (Shulman *et al*. 2004). Symptoms usually appear within 3 years after allogeneic HCT and they may be restricted to a single organ or tissue or may be widespread. cGvHD can lead to severe complications such as contractures, loss of sight, end-stage lung disease and may even cause death due to profound chronic immune suppression leading to recurrent or life threatening infections (Shulman *et al*. 2004). The incidence rates of cGvHD range from 6% to 80% according to recipient age, donor type, HCT source, graft manipulation (T-cell depletion) and use of post transplantation donor lymphocyte infusion (DLI) (Sullivan *et al.*, 1991; Rocha *et al.*, 2002).

1.5 Graft versus host disease prophylaxis and treatment

1.5.1 Treatment of acute GvHD

Acute GvHD generally develops during the phase of continued treatment (MacMillan *et al.*, 2002a; Ferrara *et al.*, 2009). Steroids are the standard of treatment for aGvHD with efficient anti-lymphocyte and anti-inflammatory activity. In many centres, mild GvHD of the skin (grade I) is treated with topical steroid only, but in the case of a more severe disease and any degree of visceral GvHD involvement, high-dose systemic steroids are usually employed (Ferrara and Deeg, 1991).

For less than half of patients, administration of steroids results in complete remission however more severe GvHD is less likely to respond to treatment (Cragg *et al.*, 2000). In a prospective study, the addition of anti-lymphocyte globulin to steroids as primary treatment was not beneficial (Cragg *et al.*, 2000), although in a retrospective study, the

use of anti-thymocyte globulin in patients who showed early signs of steroid resistance increased the response rates and was beneficial (MacMillan *et al.*, 2002b). However, not all studies have confirmed such a benefit and for this reason, this antibody preparation is not used as standard because of raised infection risks (Cutler *et al.*, 2005).

A promising approach for the treatment of aGvHD is the infusion of mesenchymal stromal cells (MSCs), after being expanded in culture either from the original donor or from a third party. This approach produced 55% complete response in a phase II study of patients with steroid-resistant GvHD (Le Blanc *et al.*, 2008).

Another strategy for the treatment of aGvHD is extracorporeal photopheresis. During this procedure, the patient's white blood cells are gathered by apheresis, incubated with the DNA-intercalating agent 8-methoroxypsolen, exposed to ultraviolet light, and returned to the patient. This procedure is known to induce cellular apoptosis, which has anti-inflammatory effects in several systems, including prevention of rejection of solid organ grafts (Sanchez-Jimenez *et al.*, 2013). However, randomized multicentre studies of this approach are needed to establish its place in the clinical management of aGvHD (Sanchez-Jimenez *et al.*, 2013).

A different strategy to treat GvHD is blockade of the inflammatory cytokine TNFα. Two anti-TNFα monoclonal antibodies have been used: infliximab, a chimeric monoclonal antibody that binds to TNFα and that lyses cells producing TNFα; and etanercept, a recombinant DNA protein composed of TNF receptor II linked to the Fc portion of human IgG1. Infliximab resulted in a 19% complete response rate in patients treated for grade II-IV steroid-refractory acute GvHD in a multicentre, retrospective analysis (Patriarca *et al.*, 2004).

1.5.2 Treatment of chronic GvHD

In contrast to aGvHD, the pathophysiology of cGvHD remains poorly understood and the disease is treated with a wide range of immunosuppressive agents. The response to cGvHD treatment is unpredictable, and mixed responses in different organs can occur in the same patient. Variables such as infection and comorbidities also make responses hard to measure.

The use of corticosteroids with or without calcineurin inhibitor is the standard of care, but findings of a randomized trial of over 300 patients with cGvHD noted differences between cyclosporine plus prednisone versus prednisone alone (Koc *et al.*, 2002).

Chronic immunosuppressants, especially those containing steroids, are highly toxic and can result in infectious deaths. Many second line therapies have been studied, but none have achieved widespread acceptance. ECP showed promise with significant response rates in high-risk patients. The best responses were observed in skin, liver, oral mucusa, eye and lung (Couriel *et al.*, 2006). This observation is particularly relevant because lung GVHD has the potential to be a particularly devastating complication necessitating lung transplant as the only therapeutic option (Rabitsch *et al.*, 2001; Sano *et al.*, 2005).

1.5.3 Prevention of GvHD

Recently, tissue-typing laboratories have developed and are using more precise and sophisticated DNA tests to select the best HLA matched donor for transplant patients. In order to lower the risk of developing GvHD, prophylactic immunosuppressive medicines and intravenous immunoglobulins (Anti-thymocyte globulin (ATG) and Campath, alemtuzumab) can be given to patients after HSCT, to prevent the donor T cells from initiating an immune response against the recipient tissue (http://www.cancer.org). However, using this prophylactic regimen comes with the risk of developing fungal, bacterial and viral infections due to the immune system being suppressed with a decreased ability to fight infection (http://www.cancer.org). New technologies as well as new and improved methods to prevent GvHD are being studied in clinical trials. These include novel immunosuppressive drugs and new monoclonal antibodies administrated to patients after transplantation, removing donor T cells before transplant and the use of photopheresis (Martin *et al*., 1990; Chao *et al*., 1993; Zic *et al*., 1999; Greinix *et al*., 2000).

1.5.4 Novel therapeutic approaches

Traditional therapies have targeted T cells, yet immune stimulatory DCs also are critical in the pathogenesis of GvHD. Other cellular therapies, notably Tregs and MSCs mediate important effects through DC and show promise for the prevention and treatment of GvHD in early human studies (Stenger, *et al*. 2012). Therapies are likely

to be more effective if they have synergistic effects or target both DC and T cells *in vivo*, such as tol DC or Treg (Stenger, *et al*. 2012).

1.6 Other allogeneic allo-HSCT outcomes

As early as 1956, it was found that transplanted allogeneic immunocompetent cells could eliminate leukaemic cells in mice independent of chemoradiotherapy (Barnes *et al.*, 1956). This was then termed a graft versus tumour (GVT) effect (Weiden *et al.*, 1979; Weiden *et al.*, 1981). Initial evidence for GVT effects in humans came from studies reporting reduced leukaemic relapse rates in allo-grafted patients who developed acute and/or chronic GvHD compared to patients who did not (Weiden *et al.*, 1979; Weiden *et al.*, 1981). The GVT effect was confirmed by other investigators who observed increased risks of relapse in patients receiving T-cell-depleted (Maraninchi *et al.*, 1987) and syngeneic transplantation (Horowitz *et al.*, 1990). Direct support for antitumor effects of allogeneic cells came from observations that DLI could induce complete remission in some patients with haematological malignancies who had relapsed after allo-HSCT (Kolb *et al.*, 1995; Collins *et al*., 1997; Kolb *et al.*, 2004). The use of reduced intensity conditioning (RIC) and non-myeloablative conditioning regimens has shifted some or all of the burden of tumour-cell kill from the conditioning regimen to the GVT effect (Slavin *et al.*, 1998; pitzer TR *et al.*, 2000; Sorror *et al.*, 2004). Every patient undergoing transplantation has some degree of GVT reaction, otherwise the underlying disease for which the patient had been transplanted for would not be eliminated. Thus, it is crucial in the clinic to maintain the balance between GvH and GVT as this can ensure patient recovery from cancer without disease relapse.

Alongside GvHD and GVT, several additional outcomes are assessed post allo-HSCT such as disease relapse, overall survival (OVS) and non-relapse mortality (NRM). All HSCT outcomes are affected by three main types of clinical risk factors that affect transplantation: pre-transplantation, peri-transplantation and post-post transplantation (Gratwohl, 2012a). These risk factors were introduced as part of the European Group for Blood and Marrow Transplantation (EBMT) risk score (Gratwohl, 2012a).

Pre-transplant factors including patient's age, disease stage, time interval, diagnosis, donor HLA type and gender combination, are assessed to calculate risk scores for patients to predict transplantation outcome. This can help to adapt specific allo-HST

procedure such as patient care, conditioning regimen, GvHD prophylaxis and also the source of stem cells (Gratwohl, 2012a). Peri-transplant factors are those involved during the HSCT procedure and include transplantation technique, conditioning regimen, GvHD prophylaxis and source of stem cells.

Post- transplant risk factors are usually the most complex factors, as they are relatively unpredictable at the time of allo-HSCT such as GvHD incidence and severity, relapse and infections which are a major post-HSCT complication as the patient's immune system is compromised to receive the donor cells (Gratwohl, 2012b).

1.7 Non-HLA immunogenetics in graft versus host disease

A considerable proportion of the risk of adverse outcome after HSCT is genetic and can be attributed to various factors including HLA matching, KIR matching, miHAg and non-HLA gene polymorphisms (Harkensee *et al.*, 2012).

Outcomes such as aGvHD and cGvHD, relapse and survival have been shown to be modified by functionally relevant polymorphisms in non-HLA genes that are involved in immune responses (Porter *et al.*, 2010). Such regulatory polymorphisms are complicated to pinpoint among other polymorphisms localized near these genes which have no direct effects on gene function. Reliable identification of polymorphisms that result in differences in gene expression or protein function and affect the outcome of HSCT is challenging. However, these polymorphisms are expected to have a critical role in the molecular characterization of complex traits manifesting post HSCT (Sachidanandam *et al.*, 2001).

1.7.1 Genes involved in the immune response

The MHC complex is the most important genomic region that could contribute to the risk of GvHD after HSCT. Matching of MHC class I and class II genes is essential for the success of transplantation. However, the MHC contains additional genes that could also contribute to the risk of developing acute GvHD (Novota *et al.*, 2011a).

The HLA complex, is organized into segments of closely linked genetic variants that are inherited as haplotypes on the same DNA strand. HLA haplotypes can be defined by HLA class I and II alleles and they are in strong linkage disequilibrium with defined genetic variants of non-class I/non-class II genes within the haplotype blocks within this region.

Interestingly, HLA haplotype mismatching in 10/10 fully matched unrelated donors transplants was associated with an increased risk of severe acute GvHD (Petersdorf, 2007). This finding demonstrates that the HLA complex encodes, in addition to HLA-A, B, C, DRB1, and DQB1, further unidentified genes that contribute significantly to the risk of developing acute GvHD. HLA gene expression profiling may be an alternative strategy to identify HLA genes that are involved in the pathophysiology of GvHD.

There is still a need to identify genes that contribute significantly to the risk of developing acute GvHD. These genes or gene markers may be used to assess the risk of developing GvHD, for the diagnosis of GvHD, for monitoring treatment of GvHD, and for screening for immunomodulating substances which may be useful in the treatment of GvHD.

The novel use of gene markers as a method of predicting the risk for a patient developing GvHD was developed under a patent (application number PCT/EP2011/072804) by Prof Ralf Dressel, Prof Anne Dickinson, Prof Bent Rolstadt and Lutz Walter (Dressel *et al*., 2011). The invention relates to methods of monitoring the efficacy of GvHD treatment, and could also be used to screen new candidate drug/antibodies for therapies. The inventors identified rat and human MHC and NKC genes and non-MHC and non-NKC genes that are regulated during GvHR in skin explant assays and could therefore serve as biomarkers for GVHD. The method involves determining the prognostic transcript of one or more genes selected from the following genes consisting of *UBD, C2, LST1, AIF1, C1QTNF7, CEACAM4, MME*, *IGFBP5, TAP1, CTGF, ANP32A, HCLS1, HTRA1, LGALS7, PTGER2, PTPN7, TGM2, TREM2* and *CARD11, PIK3AP1, PSTPIPI, MSR1*; or their corresponding cDNAs, or their expression products.

1.7.2 Single nucleotide polymorphisms and risk associated genotypes

SNPs are the most studied and evaluated variants of the human genome. Mullally et al, combined knowledge from different studies and accumulated understanding of different structural variants (such as CNVs), and summarized that the dissimilarities between all individuals are much greater than previously thought (Mullally and Ritz, 2007). This insight into the diverse complexity of the genome was of great benefit to the field of HSCT on two levels: the generation of the transplant antigens and the individual susceptibility to transplant related toxicities. Advances in studies and techniques used for DNA sequencing made it easier to perform genome-wide analysis using high throughput standard procedures to test for genetic characteristics and details associated with patients and donors before performing the transplantation. Applying and incorporating these finding into clinically meaningful results will be the next challenge for transplant clinicians.

The extent of the human genome is apparent when studying SNPs. Indeed, the International Hapmap Project reported more than one million SNPs in the human genome in October 2005 (Altshuler *et al.*, 2005). Different types of genome variations were described in the Hapmap project, including whole gene deletions, multiple copy gene duplication, inverted gene sequences, large-scale copy number variants and segmental duplications (hapmap.ncbi.nlm.nih.gov). Regarding SNPs, 11,500 were catalogued as non-synonymous coding SNPs. According to studies on copy number variations (CNVs) carried out in the following years, the normal human genome contains at least 600 structural variants, comprising at least 100 million bases of DNA sequence. These numbers continue to increase with new structural variants being discovered (Fredman *et al.*, 2004; Sharp *et al.*, 2005; Feuk *et al.*, 2006)

Numerous studies on SNPs have shown that genes which bear genetic variation are to be enriched significantly during immune responses (Tuzun *et al.*, 2005). This means that genes stimulated in, or responsible for, the immune response (e.g. cytokines) contain more structural rearrangements that other genes. Some genes were reported to be implicated in the adaptability and fitness of an organism in response to an external stimulus. Thus, the structural variations that occur in the genome represent the process of adoptive evolution. An example of these observations is the selection of gene copy number that has been reported for CCL3L1, an immune response gene,

where lower than average copy number is associated with HIV/AIDS (Gonzalez *et al*., 2005).

Understanding the human genetic diversity will help dissect the susceptibility and response to different diseases and specifically, these studies are of great significance to the field of HSCT. Indeed, along with deletions, non-synonymous SNPs can generate potentially immunogenic transplant antigens (Mullally *et al.*, 2006). Non-HLA SNPs can influence the immune response. A study by Cho et al. suggested that significant variability in cytokine and chemokine expression after Toll-like receptor stimulation has been observed between individuals. This suggests that particular aspects of immune response, such as TLR stimulation in the case of this study, are closely associated with genetic variation (Cho *et al.*, 2013).

To analyse the SNPs in relation to cGvHD, Clark et al suggested that SNPs in target genes can lead to better understanding of the biological basis of the different subtypes of cGvHD (Clark *et al*. 2010). Genes that could be subject to copy number variation include KIR, MHC, and the gene encoding Fc and immunoglobulin receptor. Genes involved in drug detoxification, which are also subject to structural variation leading to CNV, are of potential relevance to HSCT. These include genes relevant to the glutathione s-transferase gene family, the cyclophosphamide (cytochrome p450, GST family) and calcineurin inhibitor (cytochrome p450, UGT2B family metabolism). This suggests that normal gene structural variations could have an impact on individual outcomes during HSCT (Sebat *et al.*, 2004).

The majority of the SNPs arise in non-coding regions including intronic, intergenic regions and untranslated regions (UTRs) (Engle *et al*., 2006). Those which are within genes, including genes affecting the immune response, can alter the expression of the gene or the structure of the encoded proteins (Dickinson and Norden, 2015a). Indeed, many of the genes which were associated with HSCT outcome, were also associated with autoimmune disease, however only few remained significant following genome wide association studies (GWAS) (Dickinson and Norden, 2015a).

Since the original work regarding candidate gene associations published by Middleton et al, multiple studies investigating lager cohort gene associations have been reported on SNPs located in more than 20 genes that either code for cytokines or other molecules playing a significant role in the biology of HSCT (Dickinson and Norden,

2015a; Cavet *et al.*, 1999; Socie *et al*., 2001; Mullighan *et al*., 2007; Espinoza *et al*., 2011). The relationship between SNPs in the NOD2 gene with GvHD and HSCT outcome has been extensively studied several groups. SNPs which were originally identified in the NOD2 gene for their association with Crohn's disease have been associated with HSCT outcome (Holler *et al.,* 2006; Grube *et al.,* 2015). Patients carrying one variant of rs206684 (SNP8), rs2066845 (SNP12) or rs41450053 (SNP 13) have a 2 to 4-fold increase of developing Chron's disease and this risk increases to 20-fold in patients who are homozygotes or compound heterozygotes (Economou *et al.,* 2008; Chien *et al*., 2012). NOD2 plays a major role in defense against infection as it recognises pathogen-associated patters and thus induces cytokine cytokine response and it is also regulated by pro-inflammatory cytokines (Rosenstiel *et al.*, 2003).

Another example is the FOXP3 gene region, within which more than 90 SNPs have been identified and several among these SNPs have been identified as risk factors for a number of malignant and autoimmune diseases (Eastell et al., 2007). Tregs, defined as $CD4^+CD25^+FOXP3^+$ T cells are involved in the maintenance of immunological tolerance (Beres *et al*., 2013) and have been the focus of several HSCT studies due to their ability to supress alloreactivity during GVHD (Hoffmann *et al*., 2002). A SNP, rs3761548, in the promotor region of *FOXP3*, resulting in A->C base exchange was shown to cause loss of binding to E47 and c-Myb factors and thus, leading to defective transcription of the *FOXP3* gene (Shen *et al.,* 2010). In HSCT setting, this polymorphism was shown to be associated with the development of auto or alloimmune conditions, including type I diabetes, and graft rejection in renal transplantation (Noriega *et al*., 2015). In patients transplanted from donors carrying short alleles (≤(GT)15), this polymorphism was shown to be associated with a lower incidence of severe GvHD (grade 3-4) (Noriega *et al*., 2015). This polymorphism however had no effect on relapse, event free survival or overall survival in patients with aGvHD and cGvHD (Noriega *et al*., 2015).

Specific polymorphisms in genes for IL-10, IL-6, TNF- α and IFN-γ in a pediatric cohort of 57 HLA-identical sibling myeloablative transplants were reported by Goussetis and colleagues who retrospectively studied these polymorphisms and found a significant association between the *IL10* promoter haplotype polymorphism at -1082, -819 and -592 with the incidence of severe aGVHD (Goussetis *et al.,* 2011). The authors reported that patients with the haplotype GCC showed a decreased risk of sever aGvHD in

comparison with other *IL10* haplotypes (Goussetis *et al.,* 2011). Chien et al, reported that 2 SNPs in the same gene *IL10,* rs1800872 and rs1800872, were associated with a 30% decrease of the risk of severe aGvHD (Chien *et al*., 2011). In the case of *IL6*, the donor genotype for rs1800795 in *IL6*, was associated with a 20% to 50% increase in the risk for aGvHD (II-IV) and the *IL2* polymorphism rs2069762 in the donor genotype was showed to be associated with a 1.3-fold increase in the risk of grade III-IV aGvHD (Chien *et al*., 2011). The Ogawa group identified three new loci that were shown to be significantly associated with severe aGvHD (II to IV) including the SNP rs17473423 within the *KRAS* locus (Stao *et al.,* 2015). In a study by Bair et al, two GvHD susceptibility loci (rs17114803 and rs17114808) in the suppressor of fused homolog (SUFU) gene have been found (Bari *et al.,* 2015). The Incidence of aGvHD was shown to be higher in patients who were homozygous for CC at SUFU rs17114808 (Bari *et al.,* 2015).

In microRNAs (miRNAs), SNPs can alter regulatory properties but elucidation of the functions of these SNPs is not straight forward (Hudson, 2003). Moreover, SNPs located in miRNAs can affect the miRNAs maturation, function, and target selection. To date, a number of studies have demonstrated that SNPs in target sites or miRNA genes are associated with diseases such as chronic lymphocytic leukaemia, non-small-cell lung cancer, papillary thyroid carcinoma and breast cancer (Chin et al., 2008; Jazdzewski et al., 2008; Sethupathy and Collins, 2008; Mencía et al., 2009).

1.7.3 Implication of microRNAs in the pathophysiology of graft versus host disease

MicroRNAs (miRNAs) represent a promising source of biomarkers for GvHD because they play critical roles in the development and function of the immune system (Banerjee *et al*. 2010; Rodriguez *et al.* 2007). MicroRNAs are a family of 19-24 nucleotide noncoding RNAs, which affect the regulation of gene expression in eukaryotic cells by binding to the 3´-untranslated region of target messenger RNAs (mRNAs). They play an important role in many cellular processes such as development, stem cell division, apoptosis and cancer (Ajit, 2012).

MiRNAs regulate gene expression by binding to the mRNA and the seed sequence is essential for this binding. The seed sequence (or seed region) is a conserved

heptametrical sequence which is mostly situated at positions 2-7 from the miRNA 5´-end. Even though perfect base pairing of miRNA and its target mRNA is required, the “seed sequence” has to be perfectly complementary (Felekkis *et al.*, 2010). Human miRNA biogenesis is a two-step process including both nuclear and cytoplasmic cleavage events, performed by two ribonuclease III endonucleases, Drosha and Dicer (Figure 1.11) (Denli *et al.*, 2004; Gregory *et al.*, 2004; Han *et al.*, 2004). The miRNA-processing pathway includes the production of the primary miRNA transcript (pri-miRNA) by RNA polymerase II or III and cleavage of the pri-miRNA by the microprocessor complex Drosha–DGCR8 (Pasha) in the nucleus. The resulting precursor hairpin, the pre-miRNA, is exported from the nucleus by Exportin-5–Ran-GTP (Du and Zamore, 2005). In the cytoplasm, the RNase Dicer in complex with the double-stranded RNA-binding protein TRBP cleaves the pre-miRNA hairpin to its mature form (Lee *et al.*, 2007b). The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs. This may be via cleavage, translational repression or deadenylation, and the passenger strand is usually degraded (Winter *et al.*, 2009) (Figure 1.7).

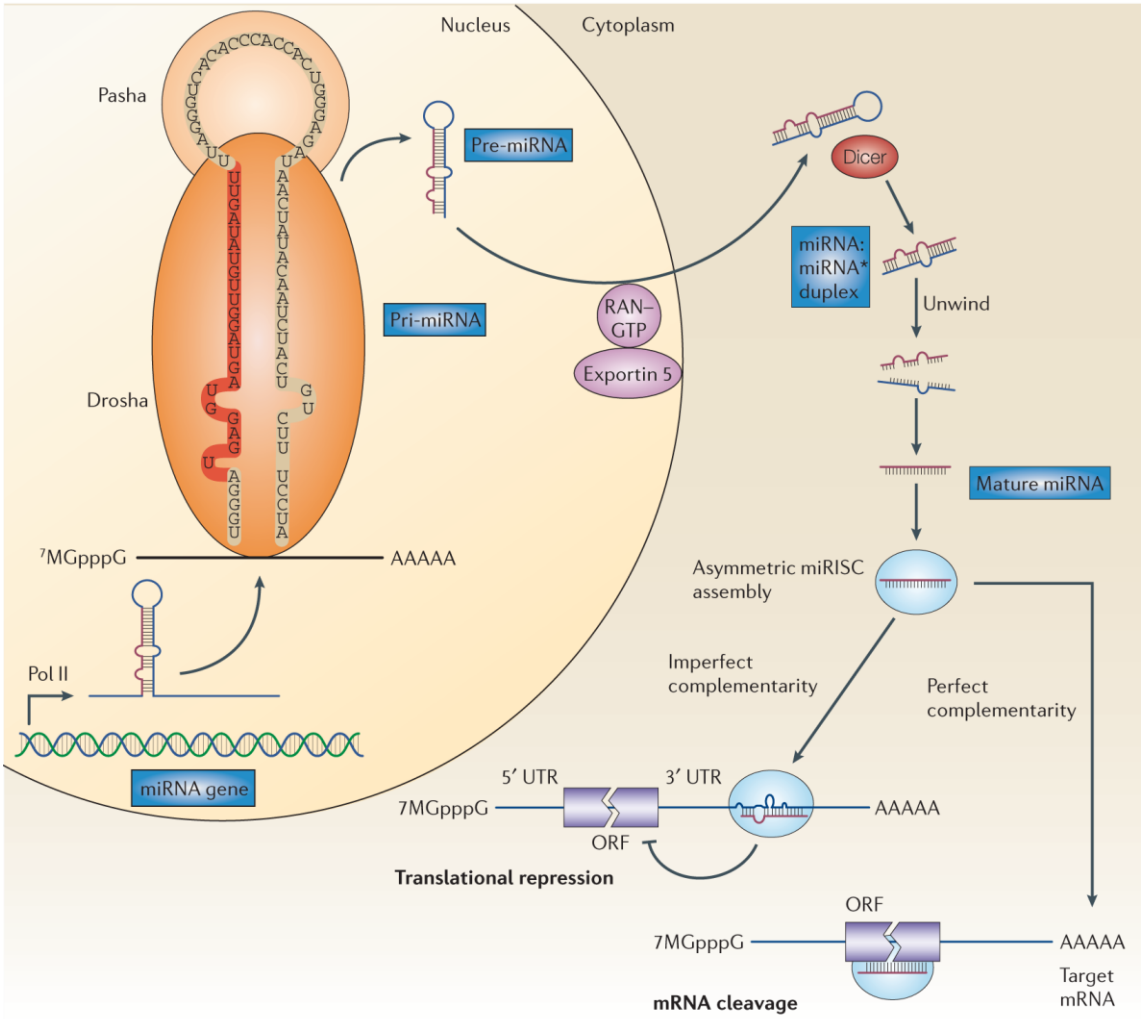


Figure 1.7 MicroRNA biogenesis (Esquela-Kerscher and Slack, 2006). Pri-miRNA is transcribed from the miRNA gene and to pre-miRNA by Drosha in the nucleus. This pre-miRNA is then exported into the cytoplasm by Exportin 5 and cleaved into mature miRNA by Dicer. Mature miRNA is loaded onto RISC/miRISC and delivered to the mRNA where it represses translation and/or results in mRNA cleavage. Pol II: Polymerase II; Pri-miRNA: Primary MicroRNA; Pre-miRNA: Precursor MicroRNA; miRISC: MicroRNA Induced Silencing Complex; ORF: Open Reading Frame; UTR: Untranslated Region; mRNA: Messenger RNA.

According to Bentwich et al, around 50% of all genes are regulated by miRNAs, which makes the investigation of their roles in different diseases very important (Bentwich *et al.*, 2005). In this context, Atarod et al, have reviewed the possible interaction between some miRNA pathways and GvHD using *in silico* approaches and eight microRNAs (miR-146a, miR-155, miR-515, miR-346, miR-143, miR-373, miR-31a and miR-29)

were predicted to impact on different molecules in the GvHD signalling pathway (Atarod and Dickinson, 2013). MiR-155 was one of the first miRNAs to be associated with the regulation of aGvHD. This miRNA is required for the normal function of B and T lymphocytes (Rodriguez *et al.*, 2007). Ranganathan et al showed that miR-155 was up-regulated in patients with intestinal aGvHD, making this miRNA a novel target for therapeutic intervention (Ranganathan *et al.*, 2012).

1.7.3.1 MicroRNA-146a involvement in GvHD

At present, there is extensive knowledge on the cellular mechanisms of GvHD but less is known about the molecular biology of the disease. Molecular studies carried out to date have focused on identifying SNPs (Dickinson and Holler, 2008) and specific genes involved in the development of GvHD (Baron *et al.*, 2007). However, there have been fewer studies focusing on the molecular regulation of GvHD. Recently, the potential role of microRNAs as biomarkers for GvHD has been highlighted (Paczesny *et al.*, 2013).

MicroRNA-146 is increasingly being recognized as a 'fine-tuner' of cell function and differentiation in both innate and adaptive immunity. MiR-146a controls innate immune cell and T-cell responses, and its deficiency was shown to be responsible of autoimmunity (Boldin *et al.*, 2011). MiR-146a is expressed within a family that shares the same seed sequence, but is coded by different loci in the human genome. The miR-146a gene is located on human chromosome 5, corresponding to chromosome 11 in mouse (Garcia *et al*, 2011) (Garcia *et al.*, 2011).

Mechanistically, miR-146a has been shown to directly target two adapter proteins in the nuclear factor (NF) κB activation pathway, tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1), both in innate immune cells and T cells (Taganov *et al.*, 2006b; Boldin *et al.*, 2011; Yang *et al.*, 2012). In addition, the survival and maturation of human plasmacytoid dendritic cells that are involved in GvHD were shown to be regulated by miR-146a (Koyama *et al.*, 2009; Karrich *et al.*, 2013).

MiR-146a gene expression analysis has demonstrated induction in response to microbial components such as LPS which triggers GvHD pathology (Cooke *et al.*, 2001; Taganov *et al.*, 2006b). Upon stimulation with LPS or monocyte activation via

cell surface receptors such as TLR4, miR-146a has been shown, both *in vivo* and *in vitro*, to target IRAK1 and TRAF6 that become associated with the IL-1 receptor upon stimulation and are partially responsible for IL-1-induced upregulation of NF-kB (Figure 1.8) (Boldin *et al*., 2011).

Such binding results in the suppression of the expression of NF-κB target genes such as the interleukins IL-6, IL-8, IL-1β, and TNF-α (Pauley et al., 2008; Tang et al., 2009; Boldin et al., 2011). Taganov et al, established that IRAK1 is regulated by miR-146a (Taganov *et al*., 2006). IRAK1 is considered as a linker of the TLR with the TRAF6 intracytoplasmic activator of transcription factor of NF-κB and is subject to a negative feedback by miR-146a (Figure 1.12) (Chatzikyriakidou *et al.,* 2010).

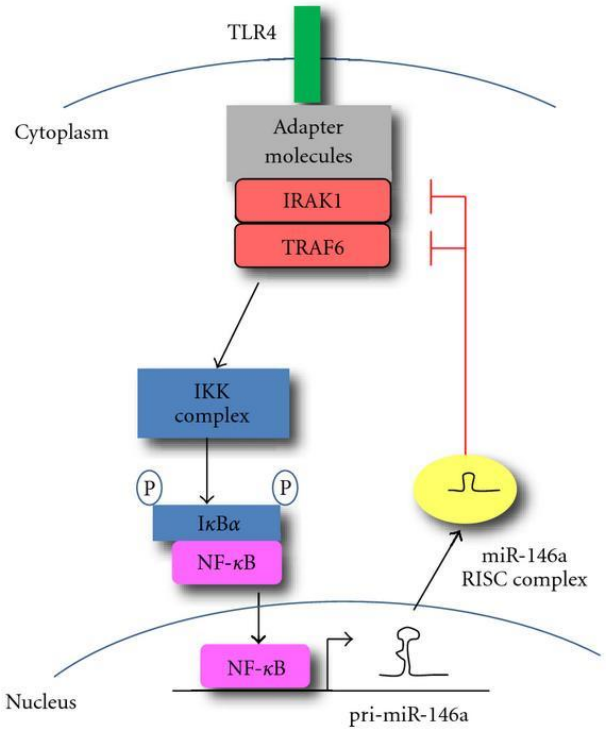


Figure 1.8 MicroRNA-146a and IRAK1 interaction and their association with NF-κB signalling (adapted from Rusca and Monticelli, 2011). MiR-146a negatively regulates signal transduction pathways leading to NF-κB activation. Upon activation of cell surface receptor such as TLR4, a molecular cascade including TRAF6 and IRAK1 leads to IκBα phosphorylation and degradation and to activation and nuclear translocation. NF-κB activation induces transcription of many genes, including pri-miR-146a. Once translocated to the cytoplasm and loaded onto the RISC complex, mature miR-146a contributes to attenuate receptor signalling through the down-modulation of IRAK1 and TRAF6 (Taganov *et al.*, 2006b; Taganov *et al.*, 2007; Rusca and Monticelli, 2011b).

In addition to being studied as potential biomarkers for GvHD, microRNAs also have promising potential to be used in the therapy of different diseases including GvHD (Paczesny, 2013a). As miRNAs are being increasingly studied as key regulators of gene expression, several SNPs in miRNA genes (miRNA-related SNPs) have also been shown to be associated with human diseases by affecting the miRNA mediated regulatory function (Gong *et al.*, 2012).

1.8 Non-invasive biomarkers for HSCT outcome

Unlike the genome, the proteome varies with time and is defined as the proteins present in a single sample at a certain time point. Ideal clinical tests are based on non-invasive collection, which allows for repetitive sample collection from the same patient in short amount of time. Thus, proteins represent ideal biomarkers in the post-transplantation setting and have been widely studied, as detailed in the following sections.

GvHD biomarkers may be produced by several sources such as donor cells, the local or systemic cytokine milieu, or recipient target tissues during disease development. These biomarkers may then be released into a variety of body fluids. For non-invasive tests used in diagnostics, bio-fluids such as plasma, sera (Paczesny *et al.*, 2009; McDonald *et al.*, 2015), or urine, are the preferred samples. Enormous effort has been placed into developing standardized methods for clinical sample collection (Rai *et al*., 2005; Court *et al*., 2011). Plasma and sera are the most frequently analysed bio-fluids. The levels of individual blood proteins represent a summation of multiple, disparate events that occur in every organ system. Plasma and sera contain proteins shed by the affected tissue as well as proteins that reflect secondary systemic changes.

Urine samples represent an alternative to plasma/sera samples for biomarker discovery. Urine has 3 main advantages compared with plasma/sera: (1) it can be obtained in large quantities; (2) the protein mixture is far less complex and the variation in protein abundance is low (Thongboonkerd, 2007) ; and (3) it is more stable (Schaub *et al.*, 2004). However, urine yields better information about diseases in organs that are directly involved in its production and excretion, such as the kidneys (Paczesny, 2013a).

Although blood biomarkers are ideal for use in a clinical setting, one goal of research into the fundamental biology of GvHD is to identify markers that are target-tissue specific. Thus, the ideal sample for discovery of biologically relevant GvHD proteins may be the target tissue itself. However, finding tissue-specific markers has thus far proven difficult because of the cellular heterogeneity of tissues, and the limited material available in biopsies for tissue proteomics. To date, there is no method capable of amplifying the amount of proteins requiring, at best, pooling of several biopsies (Tangrea *et al.*, 2004; Hwang *et al.*, 2007).

1.9 Involvement of the MHC class I chain-related gene A (MICA) in HSCT

Acute GVHD is a serious complication of allo-HSCT and involves tissue damage by the conditioning regimen which induces secretion of proinflammatory cytokines, a critical step for the maturation and activation of host dendritic cells, and for initiation and amplification of donor-derived T-cell-mediated responses (Rocha *et al.*, 2002). Tissue specific expression of stress signals from aGvHD target organs (liver, gut and skin) might contribute to the pattern of clinical pathology (Serrano *et al.*, 2013). In this setting, the human MHC class I chain-related sequence A (MICA) is induced upon cellular distress conditions such as DNA damage, malignant transformation, or intracellular infection (Koreth and Ritz, 2013).

MICA is a non-classical class I gene, located on the short strand of chromosome 6, and encodes a polypeptide of 383 amino acids (Atlas of Genetics and Cytogenetics in Oncology and Haematology). MICA is a member of the MIC gene family containing 2 functional genes, MICA and MICB, and several pseudogenes, MICC to MICG (Bahram and Spies, 1996; Bahram, 2000b; Muro *et al.*, 2014) (Figure 1.9). MICA gene is organized into 7 exons of which exon 5 encodes the transmembrane (TM) region of the MICA molecule (Zou *et al.*, 2007). MICA is by far the most divergent non classical MHC-I gene known sharing only 18, 25 and 30% homology in the α1, α2 and α3 extracellular domains with other MHC-I genes (Bahram and Spies, 1996).

MICA is expressed on the surface of epithelial cells, fibroblasts, keratinocytes and monocytes but not on the surface of CD4+, CD8+ or CD19+ lymphocytes (Hill *et al.*, 2000). MICA engages NKG2D, a C-type lectin-like receptor expressed on effector cells, including natural killer (NK) and T cells. Such engagement triggers NK cells and co-

stimulates T lymphocytes to mount adequate immune responses (Mistry and O'Callaghan, 2007).

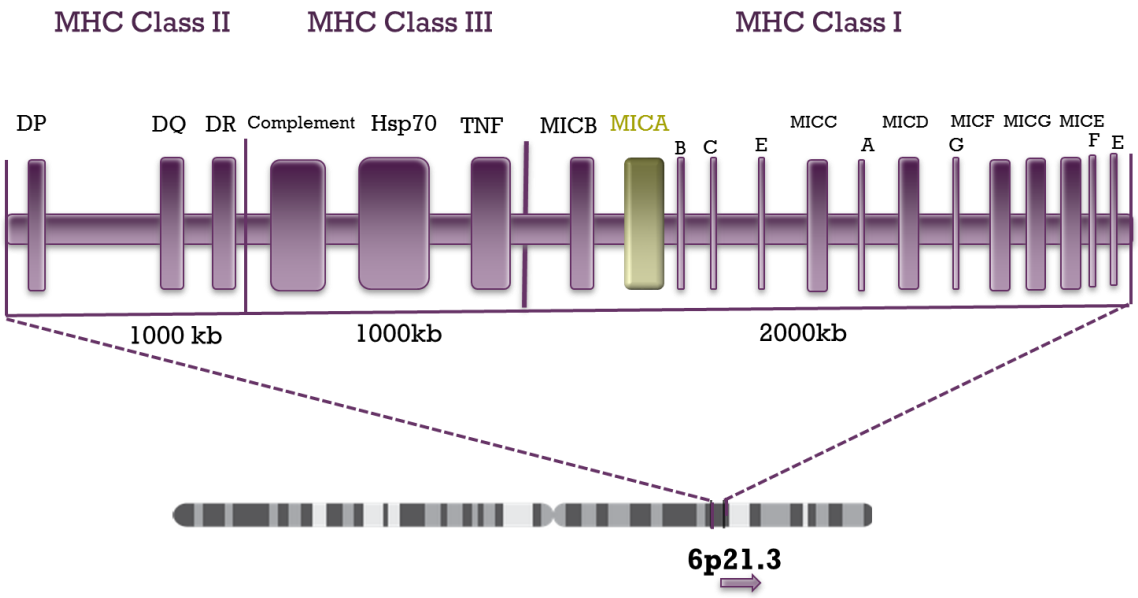


Figure 1.9 Map of the human MHC class I region depicting the location of the MICA gene (adapted from Muro *et al*, 2014). Precisely, the MICA gene is located from base pair 31,399,783 to base pair 31,415,314 (383 AA) on chromosome 6. The MICA gene spans a 11,720-bp stretch of DNA was located 46,445 bp centromeric of the HLA-B locus on the short arm of human chromosome 6 [UniProt] (Muro *et al.*, 2014)

MICA is the most polymorphic non-classical MHC class I gene in humans, sharing only 18%, 25% and 30% homology in the α1, α2 and α3 extracellular domains with other MHC-I genes (other MHC-I whether human, mouse, classical or non-classical have at least 70% homology with each other) (Groh *et al.*, 1999a). The crystal structure of MICA has revealed some unusual characteristics for a MHC class I-encoded molecule (Li *et al.*, 1999). It was confirmed that MICA does not associate with β2-microglobulin and it was observed that the putative peptide-binding groove is too narrow to accommodate a ligand, suggesting that MICA is not an antigen presenting molecule (Li *et al.*, 1999; Li *et al.*, 2001a).

Normally, most cell types do not express MICA but it becomes induced by cellular stress, including virus-infection and malignant transformation. Therefore, it renders stressed cells susceptible to killing by NK cells and allows them, despite being non-

professional antigen presenting cells, to directly activate cytotoxic T cells specific for antigens presented by these cells (Stephens, 2001). Notably, MICA expression was found to be increased in GvHD-affected tissue samples from patients (Gannage *et al*. 2008).

MICA expression can vary for certain MICA alleles (Shafi *et al.*, 2011). The SNP at -1878 (rs2596542) in the promoter region of the MICA gene was described to affect the transcriptional activity (Lo *et al.*, 2013). A polymorphic microsatellite in exon 5 encoding the transmembrane region of MICA modifies its plasma membrane expression (Ashiru *et al.*, 2013). Another SNP within the MICA gene, rs1051792 (further explained in Chapter 3), which leads to an amino acid exchange from valine to methionine at position 129, was investigated for its association with the outcome of HSCT. It was recently shown that the MICA-129 Met variant was associated with an increased overall survival and a reduced risk of death from aGvHD, despite homozygous carriers of the MICA-129 Val allele having an increased risk of developing aGvHD (Isernhagen *et al.*, 2015a).

On the functional level, it has been found that the MICA-129 Met isoform triggered more cytotoxicity and IFN-γ release by NK cells and it activated allo-reactive cytotoxic T cells faster. This variant also induced more rapid and severe down-regulation of NKG2D on NK and cytotoxic T cells (Isernhagen *et al.*, 2015a). The MICA-129 Met variant can therefore initially confer a higher risk of aGvHD, due to a faster activation of allo-reactive cytotoxic T cells (Isernhagen *et al.*, 2015a). However, in the longer perspective, the strong-counter regulation of NKG2D by this variant appears to be associated with a decreased risk of cGvHD and an increased risk of relapse due to lesser GvL effects by cytotoxic T cells and NK cells (Boukouaci *et al.*, 2009). Interestingly, the biological effects of the MICA-129 variants were strongly influenced by MICA expression intensity (Isernhagen *et al.*, 2015a). The MICA-129 Met variant triggered increased NKG2D signals but at low expression intensities, whereas the MICA-129 Val variant elicited more NKG2D-mediated effects at high expression intensities, at which the MICA-129 Met variant already down-regulated NKG2D, leading to an impaired function of this signalling pathway (Isernhagen *et al*. 2015). Thus, the MICA expression intensity could change the biological effect of this SNP, giving an interesting example of the complex functional interactions between SNPs and gene expression.

In a recent study by Isernhagen et al., it has been shown that the MICA-129 Met/Val dimorphism also affects plasma membrane expression. Increased levels of the MICA-129 Met variant were retained intracellularly and if expressed at the cell surface, the MICA-129Met variant was more prone to shedding than the MICA-129 Val isoform (Isernhagen *et al.*, 2015a).

Stern-Ginossar et al., described increased shedding of the NKG2D ligand MICA post infection with several strains of human CMV, due to enhanced activity of ADAM17

(TNF-a converting enzyme) and matrix metalloprotease 14, caused by a reduction in the expression of the endogenous inhibitor of metalloproteases tissue inhibitors of metalloproteinase 3 (TIM3P). In this study, a miRNA encoded by human CMV, miRUS25-2-3p, was shown to bind to a conserved site in the 3' untranslated region of both MICA and MICB and downregulate MICB expression (Stern-Ginossar *et al.*, 2008). This study also showed also that the expression of MICA was decreased by miR-20a, miR-93, miR106b, miR-373 and miR-520d (Stern-Ginossar *et al*., 2008).

Cellular miRNAs have been implicated in controlling MICA expression via post-transcriptional mechanisms (Yadav *et al.*, 2009), although several stress pathways regulate the transcription of the MICA gene (Raulet, 2003). One of these miRNAs is miRNA-520b that once induced by interferon gamma, leads to a reduction in MICA plasma membrane expression intensity (Yadav *et al.*, 2009). Interestingly, miR-520b acted on both the MICA 3'-untranslated region and the promoter region to decrease MICA transcript levels. Yadav et al., transiently transfected MelJuSo and HeLa cells with a luciferase reporter gene construct containing 1 kb of the MICA promoter region in combination with control miRNA, miR-520b, control anti-miR, or anti-miR-520b, and showed a reduction (2- to 3-fold) in luciferase activity in cells transfected with miR-520b as compared with cells transfected with the scrambled control miRNA. Interestingly, the team showed that there was a slight but reproducible increase in MICA promoter activity in MelJuSo, but not HeLa cells transfected with anti-520b compared with control anti-miR (Yadav *et al*., 2009). Taken together, these data indicate that miR-520b inhibits MICA gene expression not only via target sequences in the 3′-UTR, but also by acting on the promoter region.

1.10 Study Aims

The overall aim of this PhD project was to identify and assess genetic finger prints for putative novel molecular biomarkers for acute graft versus host disease (aGvHD). The investigated biomarkers included SNPs in genes and in microRNAs, gene expression patterns and variations of their corresponding protein levels in blood and serum and microRNAs signatures in the gastrointestinal tract of GvHD patients post allogeneic haematopoietic stem cell transplantation (all-HSCT).

In this study, SNP genotyping was performed to investigate 4 SNPs that were demonstrated to be implicated in various disease settings and were reported to be associated with HSCT outcome as well. Two of these SNPs, rs2910164 and rs2431697, are related to miR-146a itself, and a third SNP, rs3027898 which is located in the 3'UTR of Interleukin-1 Receptor Associated Kinase-1 (IRAK-1) a potential target of miR-146a. In addition, a non-synonymous SNP, rs10511792, in the MHC class I polypeptide-related gene A (MICA) was investigated for its association with HSCT outcome.

As the SNP genotyping study for rs10511792 showed that the MICA-129 dimorphism (MICA-129 Met/Val) was significantly associated with HSCT outcomes, the impact of MICA gene expression on HSCT outcome was first investigated and then the possible correlations between MICA-129 dimorphism and MICA gene expression levels were assessed. A comparison between the levels of soluble MICA (sMICA) in patients' sera pre and post transplantation as well as at different time points post HSCT (pre, day -7, day +14, day +28 and day +100) were investigated. The outcome of this part of the study was then correlated with the MICA-129 dimorphism, to better understand how MICA variants (MICA-129 Met and MICA-129 Val) influence the levels of sMICA and thus the incidence of GvHD.

Alongside MICA, gene expression patterns and protein level variations of 8 other non-HLA related genes that were shown to play an important role in generating major immune responses and thus could influence HSCT outcome were investigated. The aim was to determine the expression levels the RNA transcripts and the protein levels of the 8 genes of interest, including C1QTNF7, LGALS7, ANP32A, HTRA1, PIK3AP1, PSTPIPI, MSR1 and CXCL9 in blood samples (PAXgeneTM Blood RNA System) obtained from transplantation patients pre and post-HSCT in order to determine their impact on the outcome of HSCT.

As almost 60% of the human genome is target by microRNAs, these said ones can affect the patterns of expression of various genes playing major roles in the immune system and thus, influence HSCT outcome. For the purpose of discovery of new miRNAs associated with GvHD after HSCT, the nCounter miRNA Expression Assay from Nanostring Technologies was performed to screen for ~800 miRNAs in total RNA samples extracted from GI biopsies of aGvHD patient as intestinal GvHD is particularly important due to its severity and its effect of the general condition of patients. Significantly dysregulated miRNAs discovered from the profiling study were then validated in a separate cohort of samples using Taqman qRT-PCR. Target inspection was also carried for the identified miRNAs thus, investigating their effect on genes related to the pathophysiology of GvHD.

Chapter 2. Materials and Methods

2.1. Patient and donor cohorts

2.1.1. Project ethics

Patients and healthy volunteers were consented for whole blood, sera, gastrointestinal biopsies and gastrointestinal clinical slide collection and molecular testing. The project was approved by the Newcastle and North Tyneside I Research Ethics committee (Trust R&D Project: 6980, Title of the project: Improving haematopoietic stem cell outcome through studies of alloreactivity, immune reconstitution, biomarkers and novel therapies, Project Code: 129780/ 'STEMDIAGNOSTICS' REC REF: 07/H0906/131. Ethical approvals were also acquired from the local ethic committees of the various other centres, including Regensburg, Munich, Paris, Vienna and Prague for the acquisition of DNA samples. Scientific work after 30/09/15 is covered under 'IMPROVING HSCT' REC REF: 14/NE/1136) (Ethics Approval attached).

2.1.2. Clinical information

The different study populations included in this work comprised of allo-HSCT patients collected prospectively with clinical and genotyping data from six European transplant centres (France, Munich, Prague, Regensburg, Vienna and Newcastle). These patients received transplants from a mixture of sibling (SIBs) and matched unrelated donors (MUDs) and had different underlying diseases including mainly, acute myeloid leukaemia (AML), acute lymphoblastic leukaemia (ALL), myelodysplastic syndrome (MSD), Non-Hodgkin's lymphoma (N-HL), Hodgkin's disease (HD), chronic myeloid leukaemia (CML). Cohorts comprised of patients and donors with age range 10-80 years. Monitoring of the patients was performed post-HSCT for assessment of transplantation outcome including: relapse, overall survival (OVS), non-relapse mortality (NRM), aGvHD incidence and severity.

The overall clinical aGvHD grades were diagnosed in accordance with the NIH consensus (Filipovich *et al.*, 2005) . All clinical data were collected from the EuroBank database (www.eurotransplantbank.org). Overall clinical and histopathological gastrointestinal aGvHD grades were assessed using standard criteria (Glucksberg *et al.*, 1974).

2.2. DNA preparation

2.2.1. DNA extraction from peripheral blood and mononuclear cells

For cell concentrations of $5x10^6 - 1x10^7$ (PBMCs or viable cells), cell pellets were suspended in 2 mL of nuclear lysis buffer (400 mM (31.51 g) Tris-HCL, 60 mM (11.17 g) EDTA, 150 mM (4.88 g) NaCL and 1% SDS, all dissolved in 400 mL water, pH 8.0). 0.5 ml of 5 M sodium perchlorate (5 M (70.23 g) sodium perchlorate) was then added to each tube. Samples were mixed by rotation (Blood Tube Rotator SB1, Stuart Equipment, UK) at room temperature for 15 minutes to fully dissolve the pellet, followed by a 30-minute incubation at 65°C. 2 ml of chloroform was added and the subsequent aqueous and organic phases were thoroughly mixed for 10 minutes (Blood Tube Rotator SB1, Stuart Equipment, UK). Once a homogeneous emulsion was formed, samples were centrifuged at 1500 RPM (MSE Muistral 3000i, DJB labcare, UK) for 10 minutes to break the emulsion and a two layered solution was produced (aqueous and organic phases). The DNA containing phase (top layer) was then removed and 2 volumes of absolute ethanol (Fisher Chemicals, UK) were added. Gentle inversion of the tube precipitated the DNA which was spooled onto an inoculating loop (Copan innovation) and let to air-dry for 10 minutes before suspending in 0.2 mL of TE buffer (10 mM Tris (10 mL of 1 M Tris stock, pH 7.5, in 1 L DI water), 0.5 mM EDTA (1 ml of 0.5 M EDTA, pH 8.0, in 1 L DI water)). The DNA samples were then incubated at 65°C for 12-18 hours.

2.2.2. DNA purification

In cases of DNA with suspected contamination during TCR library preparation (investigated by the A260/A280 and the A260/A230 Nanodrop ratios), the genomic DNA samples in question were purified using QIAamp DNA Micro kit (Qiagen, Seattle, WA, USA) following the Manufacture's protocol. Briefly, 100 µl of genomic DNA (containing up to 10 µg DNA) was added to a 1.5 ml microcentrifuge tube (deionized water was added in cases where the volume of the DNA was less than 100 µl) followed by the addition of 10 µl buffer AW1. 250 µl of buffer AW2 was added to the sample and mixed for 10 seconds by gentle agitation. The sample was then transferred to a QIAamp MinElute column and centrifuged at 8000 RPM for 1 minute. After discarding the flow-through, 500 µl Buffer AW2 was added to the QIAamp

MinElute, which was then centrifuged at 6000 x g for 1 minute and the collection tube containing the flow-through was discarded.

Centrifugation at full speed (14,000 RPM) for 3 minutes was then performed to dry the membrane and prevent contamination with carried over ethanol. 100 µl of AE buffer was applied to the QIAamp MinElute column which was then incubated at room temperature for 1 minute before centrifugation at full speed to increase the DNA yield.

2.3. Total RNA extraction

RNA extraction from whole blood (2.5 ml) was collected in PAXgene Blood RNA Tubes (PreAnalytiX GmbH, UK), which contain a reagent that leads to cell lyses and preserves the RNA. Two tubes were collected from each allo-HSCT patient at the following time-points, 7 days pre-transplant and post-transplant at 28 days, 3 months, 6 months, 9 months and 12 months and frozen at -20°C. For healthy volunteers only 2.5 ml of peripheral blood was collected pre-transplantation. Total RNA was extracted using the PAXgene Blood miRNA kit (PreAnalytiX GmbH, UK) (Figure 2.1). PAXgene Blood RNA Tubes were stored at -20°C prior to extraction. PAXgene Blood RNA Tubes were incubated for 12-18 hours at room temperature to increase the RNA yield. The tubes were centrifuged (MSE Muistral 3000i, DJB labcare, UK) at 3500 x g for 10 minutes to obtain a pellet. This pellet was then washed with 4 ml of RNAase-free (PreAnalytiX GmbH, UK) water and centrifuged again at the same conditions. The washing step was repeated twice and the pellet was then re-suspended in 350 µl buffer BM1. 300 µl of the binding buffer BM2 was then added with 40 µl proteinase K (1 mg/mL) to degrade any proteins. The sample was incubated for 10 min at 55°C on a shaker-incubator set at 400-1400 RPM. The lysates were shredded using a needle-syringe (1 ml) to remove any lysate clumps that may contain RNAses that can degrade the RNA and decrease its integrity. The lysate was then transferred into the PAXgene Shredder spin column for further homogenisation and centrifuged for 3 min at 14,000 RPM. The entire supernatant was carefully transferred from the flow-through to a fresh eppendorf and 700 µl of Isopropanol was added to increase binding of the total RNA to the silica membrane. This mix was loaded onto the spin column and then it was centrifuged at 14,000 RPM for 1min. After every spin, the processing tube was discarded and a new one was used. 350 µl of wash buffer BM3 was then added to the spin column and centrifuged for 1 min at 14,000 RPM. DNase 1 mix was made by adding 10 µl of the stock DNAse 1 (1500 Kunitz) to 70 µl DNA

digestion buffer (RDD) per sample. DNase 1 mix was loaded to the spin column and incubated at room temperature for 15 minutes. This treatment ensured that all DNA was digested in order to obtain purified RNA. The reaction was stopped by adding 350 µl of BM3 to the spin column and centrifuged for 1 minute at 14,000 RPM. Then 500 µl of wash Buffer 4 (BM4) (500 µl) was loaded onto the spin column and it was centrifuged for 15 seconds at 14,000 RPM. This step was carried out twice to completely remove any chemicals from the RNA. The spin column was then centrifuged empty for 1 minute at 14,000 RPM to dry the silica membrane. The spin column was transferred to a fresh Eppendorf (1.5 ml) and eluted using 80 µl of elution buffer BR5 and then centrifuged for 1 minute at 14,000 RPM. Total RNA was stored at -80 ºC until further use.

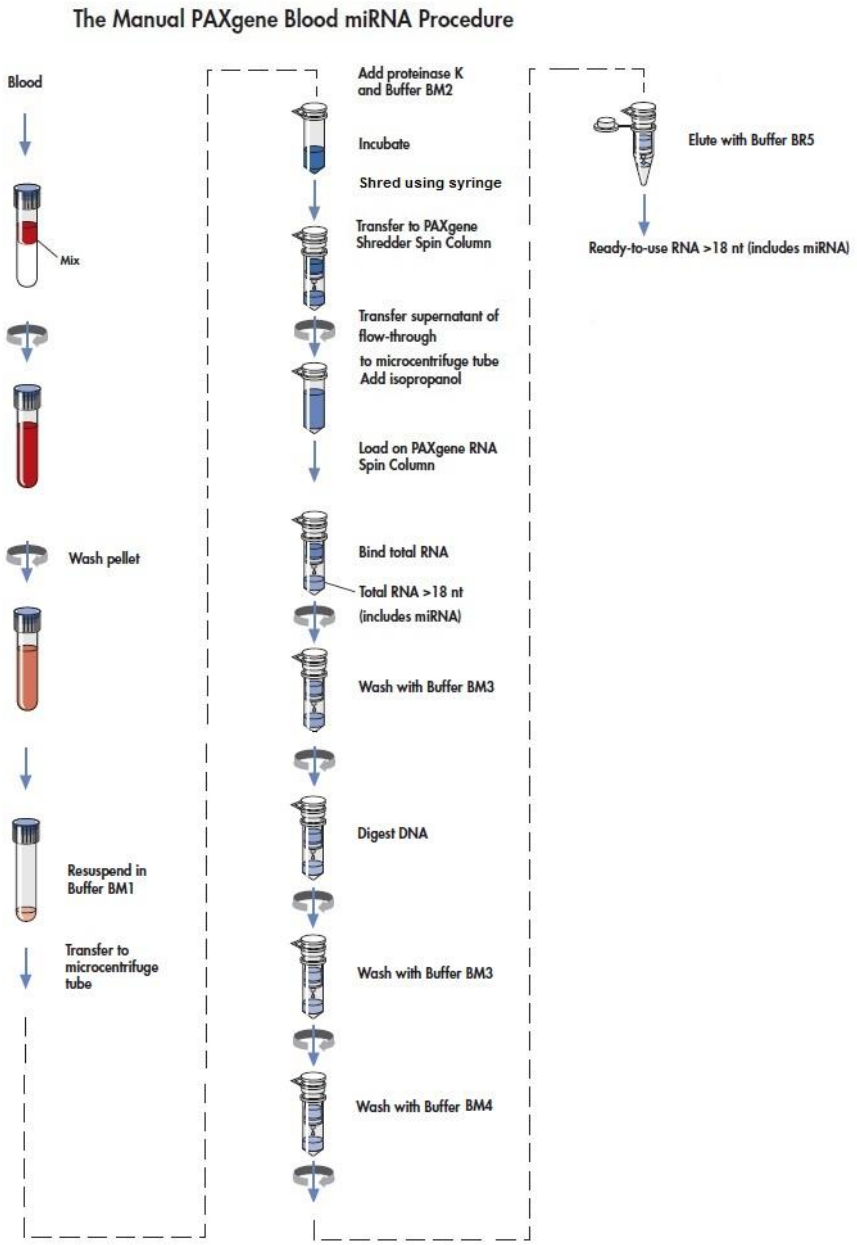


Figure 2.1 Total RNA extraction process from whole blood using the PAXgene Blood miRNA kit (adapted from PAXgene Blood RNA Kit Handbook). The cells were pelleted initially and then resuspended using BM1. Proteins were degraded by incubating samples at 55 ºC with buffer BR2 and proteinase K, respectively. Samples were then homogenized using a 1 ml syringe and loaded onto the shredder column for additional homogenisation. Supernatant was collected into a new eppendorf, isopropanol was added onto the spin column and then centrifuged. BM3 was added in the wash step and centrifuged again. DNAse 1 treatment was performed for 15 minutes. Two wash steps were performed, one with BM3 and twice with BM4, respectively. Purified total RNA was then eluted using buffer BR5.

2.3.1. RNA extraction from clinical gastrointestinal biopsies

Total RNA was extracted from clinical GI biopsies using the mirVana miRNA Isolation kit (Life Technologies, USA) (Figure 2.2). The GI biopsies were initially homogenized on a sterile petri-dish, in 50 µl of Lysis Buffer using a sterile scalpel to cut the tissue to small pieces. The lysate was transferred into an eppendorf tube and Lysis Buffer was added to make the final volume 300 µl. To enhance homogenization, 30 µl of Homogenate Additive was added to each sample, which was incubated on ice for 10 minutes. The organic extraction was performed by adding 300 µl phenol chloroform (Life Technologies, USA) to the samples, vortexing for 60 seconds and centrifuging at 10,000 xg for 5 minutes. The upper phase was carefully transferred to a fresh eppendorf and 375 µl of pure ethanol (Fisher Chemicals) was added to it. The mix was then loaded onto a filter cartridge and centrifuged at 10,000 g for 15 seconds. The flow through was discarded and 700 µl Wash Buffer 1 was added to the filter and the column centrifuged at 10,000 xg for 5 minutes. The flow through was again decanted and total RNA was washed twice with 500 µl of Wash Buffer 2/3 and centrifuged at 10,000 x g for 1 minute. The filter cartridge was air-dried by centrifugation at 14,000 x g for 1 minute to remove any residual chemicals. The filter was transferred to a new eppendorf and 100 µl pre-heated nuclease-free water (95ºC) was used to elute the total RNA with centrifugation at 14,009 x g for 20-30 seconds. The extracted RNA was stored at -80 ºC until further use (Figure 2.2).

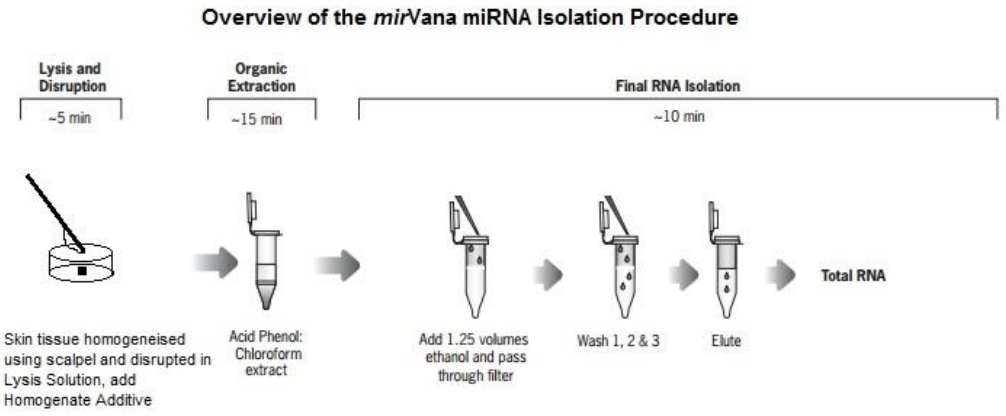


Figure 2.2 Total RNA extraction procedure from clinical gastrointestinal biopsies (adapted from the mirVana miRNA Isolation kit handbook). There are three main steps used in the extraction of total RNA from clinical skin biopsies; (1) Lysis and cell disruption, (2) Organic extraction and (3) Total RNA isolation and elution.

2.4. Evaluation of nucleic acid yield and purity

2.4.1. 2.4.1 Evaluation of the RNA and DNA quality using the NanoDrop.

NanoDrop Spectrometry (NanoDrop 1000, Thermofisher) was utilized for quality control check of previously extracted DNA/RNA samples (Figure 2.3). Pure DNA/RNA preparations were assessed by both the A260/A280 and the A260/230 ratios.

To assess for protein, phenol or other contaminants that absorb strongly at or near 280 nm, the A260/A280 ratios were assessed. Pure DNA/RNA preparations have an A260/280 ratio ≥ to 1.8. As a secondary measure of the nucleic acid purity, the A260/230 ratio was recorded to help evaluate the level of salt carry-over in the purified DNA. As a guideline, the A260/A230 ratio should be greater than 1.5, ideally close to 1.8 (Thermo Scientific) (Figure 2.3).

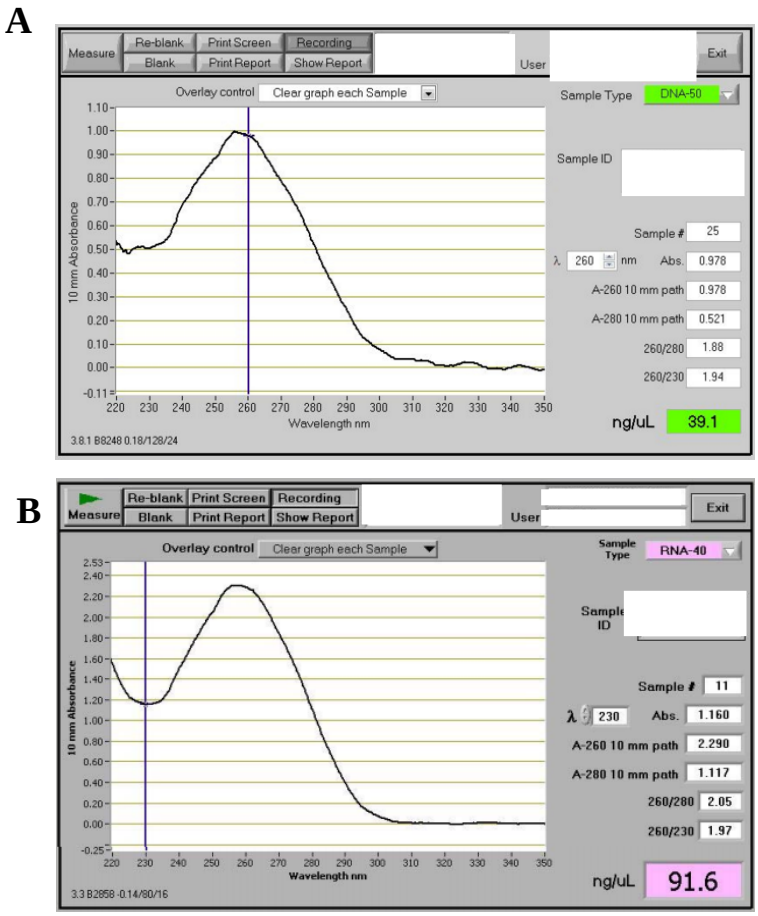


Figure 2.3 Example of NanoDrop traces (obtained from the NanoDrop output). (A) NanoDrop trace for a DNA sample showing a concentration of 39.1 ng/µl, This sample is considered to be a pure preparation with an A260/280=1.88 and A260/230=1.94. (B) NanoDrop trace for an RNA sample with a concentration of 91.6 ng/µl and an A260/280=2.05 and A260/230=1.97.

2.4.2. Agilent 2100 BioAnalyzer for RNA quality control

For miRNA profiling studies, RNA integrity numbers (RIN) were determined using the BioAnalyzer (Agilent 2100 BioAnalyzer) and the Agilent RNA 6000 Nano Kit (Agilent Technologies). Briefly, the chip priming station was first prepared by adjusting the base plate to position C and the syringe clip was adjusted to the top position. On the chip priming station was set, the RNA ladder was denatured for 2 minutes at 70 ºC. The gel was then prepared by adding 550 µl of RNA 6000 Nano gel matrix into a spin filter and centrifuging at 1500 xg for 10 minutes at room temperature. 65 µl of the gel was then filtered using a filter column for further use. RNA 6000 Nano dye concentrate was pulse centrifuged for 10 seconds and 1 µl was added to the filtered gel. The solution was then vortexed and centrifuged at 13,000 xg for 10 minutes. 9 µl of the gel-dye mix was pipetted onto the well marked ‘dark G’ on the RNA 6000 chip. The plunger was set at 1 ml and then the chip priming station was closed and the plunger was pressed until held by the clip. After 30 seconds, the clip was released and slowly pulled back to 1 ml position. The chip priming station was then opened and 9 µl of gel-dye mix was pipetted in the well marked ‘clear G’. 5 µl of the Agilent RNA 6000 Nano Marker was added in to the ‘ladder’ well and 1 µl of each RNA sample were added to all the 12 wells of the RNA Nano chip, which was then vortexed for 1 minute at 2400 RPM using the IKA vortexer (Agilent Technologies). The RNA 6000 Nano chip was then analysed on the Agilent bioanalyzer.

When utilizing the RIN software, sample integrity is no longer determined by the ratio of the ribosomal bands, but by the entire electrophoretic trace of the RNA sample ('The RIN-project., http://www.quantiom.com/RIN,' ; Imbeaud *et al.*, 2005; Schroeder *et al.*, 2006).

The assigned RIN is independent of sample concentration, instrument and analyst therefore is considered as a standard for RNA integrity (Mueller *et al.*, 2004). The RIN software allows the classification of total RNA, based on a numbering system from 1 to 10, with 1 being the most degraded and 10 being the most intact (Mueller *et al.*, 2004) (Figure 2.4). All samples were assessed using their corresponding RIN number and only samples with high integrity (RIN ≥ 7) were considered for further studies.

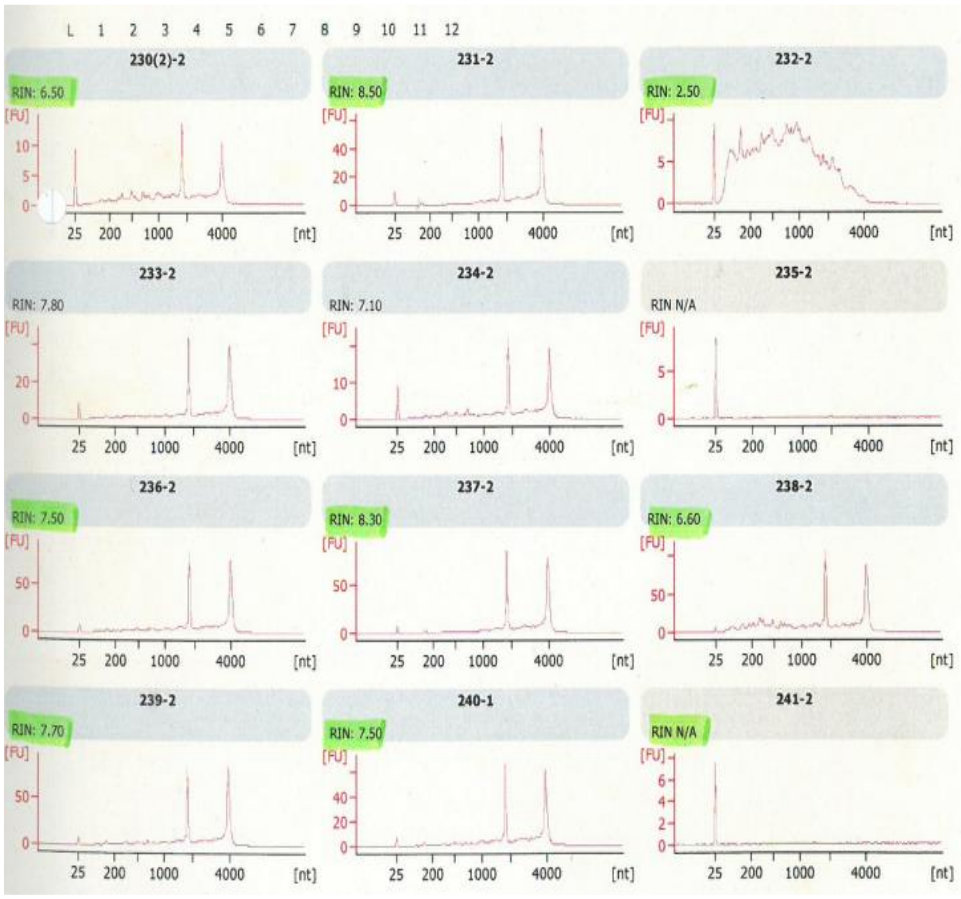


Figure 2.4. Example of BioAnalyzer output for 12 RNA samples. RIN numbers for the 12 samples are highlighted in green. Sample 232-2 showed a very low RIN=2.5 indicating degraded RNA and there was no RIN available for samples 235-2 and 241-2.

2.5. Single nucleotide polymorphism genotyping

2.5.1. Identification of SNPs of interest by gene and SNP databases

Four candidate SNPs novel to GvHD and survival were assessed in DNA samples from HSCT patients and donors. These were rs2910164 = miR-146a (1), rs2431697 = miR-146a (2), rs3027898 = IRAK1 and rs10511792 = MICA (Table 2.1). Information about ancestral and minor alleles in the target SNPs were obtained from the dbSNP Short Genetic Variations database of NCBI (http://www.ncbi.nlm.nih.gov/projects/SNP).

Table 2.1 Details of the targeted SNPs for the genotyping study

Gene	SNP	RefSNP Alleles	Ancestral allele	Minor allele
MIR146A	rs2910164	C/G	G	C
MIR146A	rs2431697	C/T	C	C
IRAK1	rs3027898	A/C	C	A
MICA	rs10511792	A/T	A	T

2.5.2. SNP genotyping method

Genotyping was performed by the LGC genomics company applying a competitive allele specific PCR (KASP) assay (LGC Genomics, UK). As shown in Figure 2.5, during thermal cycling the relevant allele-specific primer binds to the template and elongates, thus attaching the tail sequence to the newly synthesized strand (He *et al.*, 2014). The complement of the allele specific tail sequence is then generated during subsequent rounds of PCR, enabling the FRET cassette to bind to the DNA. The FRET cassette is no longer quenched and emits fluorescence. If the genotype at a given SNP is homozygous, only one of the two possible fluorescent signals will be generated. If the genotype is heterozygous, a mixed fluorescent signal will be generated (He *et al.*, 2014).

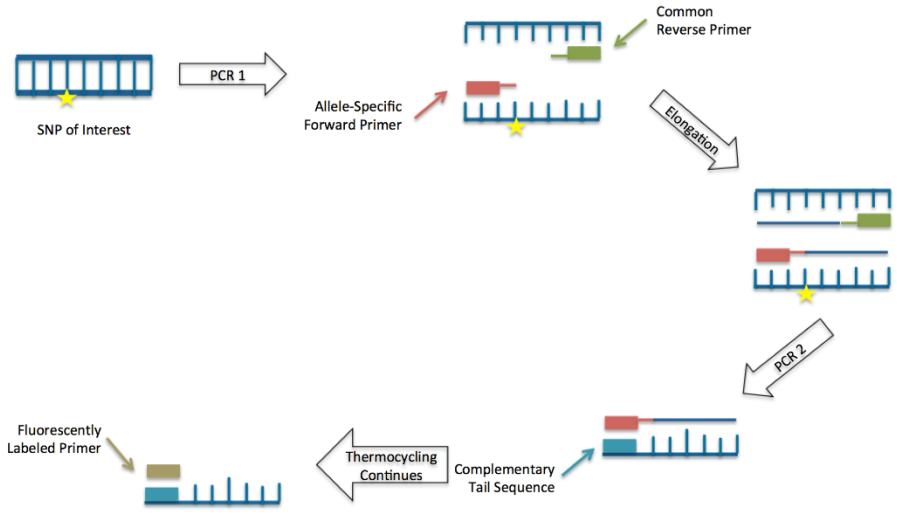


Figure 2.5 Schematic diagram of the KASP method (adapted from Kurnik and Thurnherr, 2012; Semagn *et al*., 2014). In the first round of PCR, specific forward primers bind solely at the SNP of interest allowing DNA polymerase to synthesise the rest of the complementary nucleotide. In the second round of PCR, a compliment to the allele-specific forward primer is generated when the common reverse primer binds to the amplicon formed in the first round of PCR (Kurnik and Thurnherr, 2012; Semagn *et al.*, 2014).

2.5.3. SNPViewer™ for data visualisation

Genotyping outcome included results for the presence or absence of the specific SNPs along with genotypes of both patients and donors. SNPviewer software allowed for data to be viewed as cluster plots (Figure 2.6), but was not used for data analysis or for reporting functionality.

A Cartesian plot was generated using the FAM and HEX fluorescent values; FAM is plotted on the X-axis and HEX is plotted on the Y axis (http://www.lgcgroup.com/). On the SNPviewer software, and for a specific SNP (named assay ID), all genotyped 96-well plates, called master plates, can be viewed at the same time. The list of assays corresponding to the SNPs of interest allow visualisation of how each SNP clusters in relation to each sample, and to the master plates simultaneously (Figure 2.6). Frequencies of each SNP in a specific 96-well pate or in the study cohort were also reported by the SNPviewer software.

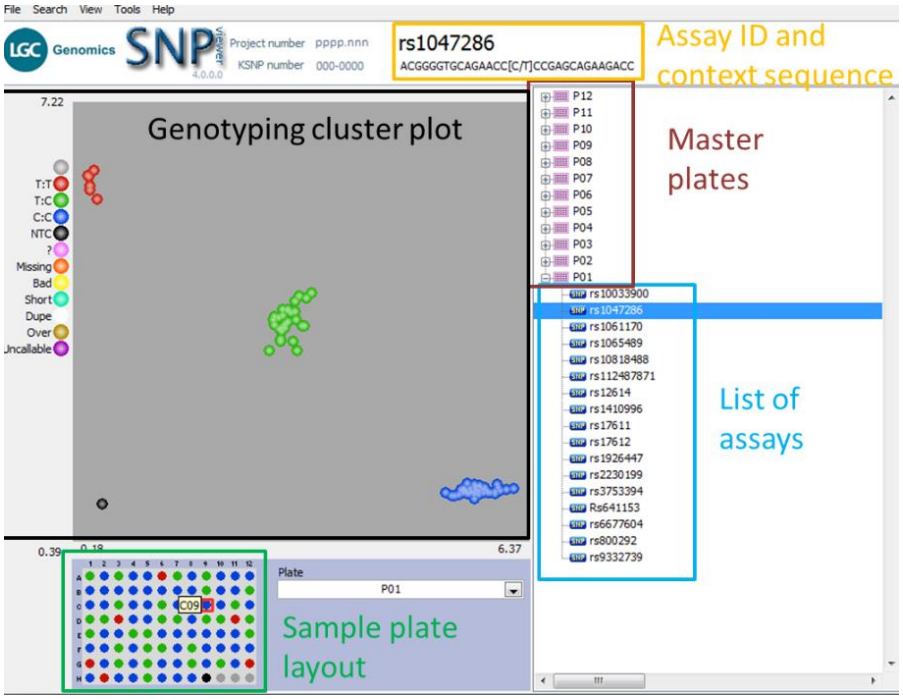


Figure 2.6 SNPviewer window for SNP genotyping outcome visualisation (adapted from www.lgcgroup.com). In the tree on the right hand side, each assay ID (highlighted in the yellow box), list of assays (highlighted in the blue box) and DNA master plate (highlighted in the red box) is listed. Cluster plots, shown in the genotyping cluster plot, are displayed by clicking on the relevant plate in the menu tree. Blue data points are homozygous for the allele reported by FAM, green data points are heterozygous and red data points are homozygous for the allele reported by HEX. The black data points represent the no template controls (NTC). The DNA sample plate layout is shown below the cluster plot.

2.6. Real-time polymerase chain reaction

2.6.1. Taqman qPCR for gene expression analysis

2.6.1.1. cDNA synthesis

For Taqman mRNA specific cDNA synthesis, 1-10 ng of total RNA was reverse transcribed in a 15 µl reaction using Taqman specific RT primers. Briefly, a dNTP stock was made consisting of 50 of µl each dNTP (Thermo Fisher Scientific) and 300 µl of RNAase free water (Thermo Fisher Scientific) (Table 2.2 A).

cDNA master mix (Table 2.2 B) was then made by adding 166.25 µl of the dNTP stock to 332.5 µl of RT-Buffer (Thermo Fisher Scientific), 210 µl of random primers pdN6 (Thermo Fisher Scientific) and 166.25 µl of DTT (Thermo Fisher Scientific). cDNA mix (Table 2.2 C) consisted of an aliquot of 100 µl of cDNA master mix, 7 µl of MMV reverse transcriptase (Thermo Fisher Scientific) and 3.5 µl of Rnasin (Promega) (Table 2.2 C). The final reaction mix (Table 2.2 D) was made by adding an equal volume of the cDNA mix to the pre-heat inactivated RNA (ratio1:1). All steps were performed on ice.

Table 2.2 Reverse transcriptase reaction composition. Reagents and volumes for dNTP stock, cDNA master mix, cDNA mix and the final reaction mix are shown.

A. dNTP stock	**Volume (µl)**
RNAase-free-water	300
dATP	50
dTTP	50
dGTP	50
dCTP	50
B. cDNA master mix	**Volume (µl)**
Buffer (5x)	332.5
dNTP stock	166.25
pdN6	210
DTT	166.25
C. cDNA mix	**Volume (µl)**
cDNA master mix	100
MMV reverse transcriptase	7
Rnasin	3.5
D. Final reaction mix	**Volume (µl)**
cDNA mix	10
Total RNA (50ng/µl)	10

The following settings were set on the thermal cycler (Applied Biosystems, 2720 Thermal Cycler) for cDNA synthesis: total RNA denature was performed at 65°C for 5 minutes. Then cDNA was synthesised by incubating on 37°C for 2 hours and 65°C for 10 minutes in thermal cycler (Applied Biosystems, 2720 Thermal Cycler). The cDNA was stored at -4°C till use.

2.6.1.2. Taqman qPCR gene expression assay

The RT-qPCR mix comprised of 15 µl of nuclease free water, 20 µl of 2X Taqman gene expression Master Mix and 2 µl target specific primer-probes (Life Technologies) (Table 2.3).

The MicroAmp Optical 96-well plates without barcodes were used for all the qRT-PCR steps. The qRT-PCR was performed on 7900HT Fast-Real Time PCR system (Life Technologies). A summery list of all the assays used in the investigations is shown in Table 2.4.

Table 2.3. Quantitative PCR master mix for gene expression assays Reagents and volumes for both the master mix and for each reaction.

qRT-PCR Master Mix	Volume per triplicate (µl)
Taqman gene expression Master Mix	20
Specific Primer Probe	2
Nuclease-free-H_2O	15
cDNA	3
Total Volume	40

Table 2.4 Taqman gene expression primer-probes for qPCR. Gene IDs, names and their corresponding assay ID were provided by ThermoFisher Scientific.

Taqman primer-probe	Gene name	Assay IDs
C1QTNF7	C1q and tumor necrosis factor related protein 7	Hs00230467_m1
LGALS7	Lectin, galactoside-binding, soluble, 7	Hs00170104_m1
ANP32A	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member A	Hs00829953_g1
HTRA1	HtrA serine peptidase 1	Hs01016151_m1
PIK3AP1	Phosphoinositide-3-kinase adaptor protein 1	Hs00381030_m1
PSTPIP1	Proline-serine-threonine phosphatase interacting protein 1	Hs00182777_m1
MSR1	Macrophage scavenger receptor 1	Hs00234007_m1
CXCL9	Chemokine (C-X-C motif) ligand 9	Hs00171065_m1

2.6.2. Taqman qPCR for miRNA expression analysis

2.6.2.1. miRNA specific cDNA synthesis

For Taqman miRNA specific cDNA synthesis, 10 ng of total RNA was reverse transcribed in a 15 µl reaction using Taqman specific RT primers and the Taqman miRNA Reverse Transcription Kit (Life Technologies). Briefly, the RT-PCR master mix (Table 2.5 A) consisted of 6.66 µl nuclease free water, 1.5 µl Buffer, 1 µl reverse transcriptase (Rtase), 0.19 µl RNAse inhibitor and 0.15 µl dNTPs. RT-PCR reaction mix (Table 2.5 B) was made by adding 9.5 µl of the RT-PCR master mix to 3 µl of miRNA specific primer and 2.5 µl of total RNA.

Table 2.5 RT-PCR reaction for miRNA specific cDNA. Reagents and volumes for both the master mix and for each reaction.

A. RT-PCR Master Mix	**Volume (µl)**
Nuclease-free-H_2O	6.66
Buffer (x10)	1.5
RTase (50U/µl)	1
Inhibitor (20U/µl)	0.19
dNTP (100 mM)	0.15
Total Volume	9.5
B. RT-PCR Reaction Mix	**Volume (µl)**
RT-PCR Master Mix	9.5
Primer	3
Total RNA (10 ng)	2.5

cDNA synthesis was performed on the thermal cycler (Applied Biosystems, 2720 Thermal Cycler) with the following program: 16°C for 30 minutes, 42°C for 30 minutes and then 85°C for 5 minutes. cDNA was stored at -20°C until further use.

2.6.2.2. Taqman qPCR for miRNA expression assay

MiRNA specific cDNAs were used for the RT-qPCR step using the miRNA specific Taqman probes. Briefly, the master mix comprised of 2.5 µl microRNA specific cDNA, 13.8 µl nuclease-free water, 1.8 µl Taqman primer-probe sets and 18 µl Taqman Universal Master mix (Table 2.6).

Table 2.6 qRT-PCR reaction for miRNA expression. Reagents and volumes for both the master mix and for each reaction.

qRT-PCR Master Mix	**Volume (µl)**
Taqman Primer-Probe	2
Taqman Universal Master Mix	18
Nuclease-free-H_2O	13.8
cDNA	2.5
Total Volume to equal 40 µl	36.1

2.6.2.3. Identification of a suitable endogenous control for Taqman qRT-PCR gene expression assay

For studying the gene expression of *C1QTNF7, LGALS7, ANP32A, HTRA1, PIK3AP1, PSTPIP1, MSR1* and *CXCL9* in peripheral blood by Taqman qRT-PCR, 6 potential reference genes (Table 2.7) were investigated using the Primerdesign geNorm Reference Gene Selection Kit (Primer Design, UK). This work was realised by an undergraduate student, Matthew Mankarious, under my supervision.

Complementary DNA was produced using the Primerdesign Precision NanoScript 2 Reverse Transcription Kit (Southampton, UK). Using a mix of random nonamer and oligo-dT primers, cDNA was produced in a 10 µl reaction volume following a two-step process; (1) Annealing (Table 2.8 A): 1 µl of 20 ng/µl isolated RNA was incubated at 65˚C for 5 minutes to anneal to 2µl of the primers for extension, followed by immediate cooling on ice. (2) Extension (Table 2.8 B): a mixture of 5 µl of nanoScript2 buffer, 1 µl of dNTP mix, 1 µl of nanoScript2 enzyme and 3 µl RNAse/DNAse free water was added to the samples, mixed by vortexing, centrifuged briefly, and incubated first at room temperature for 5 minutes, followed by 42˚C for 20 minutes. The reaction was incubated at 75˚C for 10 minutes to inactivate the reaction. All the incubations were performed using the Applied Biosystems 2700 Thermal Cycler.

Table 2.7 Reference genes included in the Primerdesign Precision nanoScript 2 reverse Transcription Kit. IDs and functions for the potential reference genes investigated for the Taqman qRT-PCR gene expression assay.

Taqman primer-probe	Gene name	Assay IDs
C1QTNF7	C1q and tumor necrosis factor related protein 7	Hs00230467_m1
LGALS7	Lectin, galactoside-binding, soluble, 7	Hs00170104_m1
ANP32A	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member A	Hs00829953_g1
HTRA1	HtrA serine peptidase 1	Hs01016151_m1
PIK3AP1	Phosphoinositide-3-kinase adaptor protein 1	Hs00381030_m1
PSTPIP1	Proline-serine-threonine phosphatase interacting protein 1	Hs00182777_m1
MSR1	Macrophage scavenger receptor 1	Hs00234007_m1
CXCL9	Chemokine (C-X-C motif) ligand 9	Hs00171065_m1

Table 2.8 cDNA synthesis. Steps and composition for the investigation of potential reference genes for Taqman qRT-PCR gene expression assay.

Annealing step components	**Volume (µl)**
RNA template (2µg)	1.8
RT primer	18
Nuclease-free-H_2O	13.8
Extension step components	**Volume (µl)**
nanoScript2 4X Buffer	5
dNTP mix 10mM	1
Nuclease-free-H2O	3
nanoScript2 enzyme	1

Quantitave RT-PCR used the Primerdesign geNorm Reference Gene Selection Kit with Double-Dye (Hydrolysis) probe. Specific primer and probe sets for the 6 reference genes were provided (Table 2.7). For each gene a mix was made comprising of the following per reaction; 1 µl primer/probe, 10 µl PrecisionPLUS/ Mastermix and 4 µl of RNAse/DNase free water (Table 2.9).

Table 2.9 qRT-PCR reaction for the potential reference gene expression. Reagents and volumes for the reaction are shown.

qRT-PCR Master Mix	**Volume (µl)**
Primer/probe mix	1
Primerdesign 2x PrecisionPLUS™ /PrecisionFAST™ Mastermix	10
Nuclease-free-H_2O	4
cDNA	5

MicroAmp Optical 96-well plates were used for the qPCR steps. The qPCR was performed on the 7900HT Fast-Real Time PCR system (Life Technologies).

2.6.2.4. Taqman qPCR data analysis

Efficiency of the commercial Taqman assays was considered to be 90-100%. Thus, efficiency (E) was set at 2 (RQ= 1/ ECt, when E=2, then RQ= 2-Ct). In relative quantification the Ct value for a gene/miRNA of interest is normalized to the Ct of a reference gene/ miRNA (ΔCt= Ct gene of interest - Ct reference gene). This results in the ΔCt derivative of Ct. The RQ is calculated using the formulae $RQ=2^{-\Delta Ct}$. Since ΔCt, and RQ values were all linear values and qPCR data is non-linear (exponential), the values were log transformed (Log transformation= Log2RQ) using logarithms. Biological data is not usually normally distributed and thus, presents a heterogeneity of variance (McDonald, 2009). In order to use parametric statistical tests on qPCR data, the RQ values must therefore be transformed logarithmically to create a normal distribution of these values. Log transformation also helps when there are outliers present in the data. The higher values are concentrated together while the smaller values are spread (Rieu and Powers, 2009). Statistical analysis and plotting of qPCR data were all performed on Log transformed data.

2.6.3. SYBR Green qPCR for MICA gene expression level investigation in gastrointestinal tissue

2.6.3.1. MICA cDNA synthesis

For the analysis of *MICA* gene expression level in gastrointestinal tissues, cDNA was synthesised as follows. Total RNA was denatured for 5 minutes at 65 °C and reverse transcribed using an equal volume of master mix (ratio1:1) consisting of: 1 µl random decamers (Ambion), 1 µl of dNTPs (Amersham), 13 µl nuclease free water (Amersham), 1 µl of Moloney Murine Leukemia Virus Reverse Transcriptase RNase H Minus. Point Mutant (M-MLV RT (H–)) (Promega).

The reaction mix was incubated at 42°C for 52 minutes and then at 70°C for 15 minutes using the PTC-100 Peltier Thermal Cycler (Table 2.10).

Table 2.10 RT-PCR reaction for the synthesis of cDNA for MICA gene expression investigation. Reagents and volumes for the reaction of cDNA making for *MICA* gene.

RT-PCR Master Mix for *MICA*	**Volume (µl)**
Random Decamers	1
dNTPs (10 pmol/dNTP)	1
Nuclease-free-H_2O	13
Total RNA (1 µg/500 ng)	1

2.6.3.2. Optimal reference for the study of MICA with SYBR GREEN qRT-PCR

In order to set a standard (positive control) for *MICA*, expression patterns of this gene were investigated using the Expression Atlas. This is an integrated database that provides information about gene and protein expression in animal and plant samples of different cell types, organism parts, developmental stages, diseases and other conditions (http://www.ebi.ac.uk/gxa). The Expression Atlas showed that the highest levels of MICA expression were observed in lung tissue, with a value of 17 Fragments Per Kilobase of transcript per Million mapped reads (FPKM) (Figure 2.7).

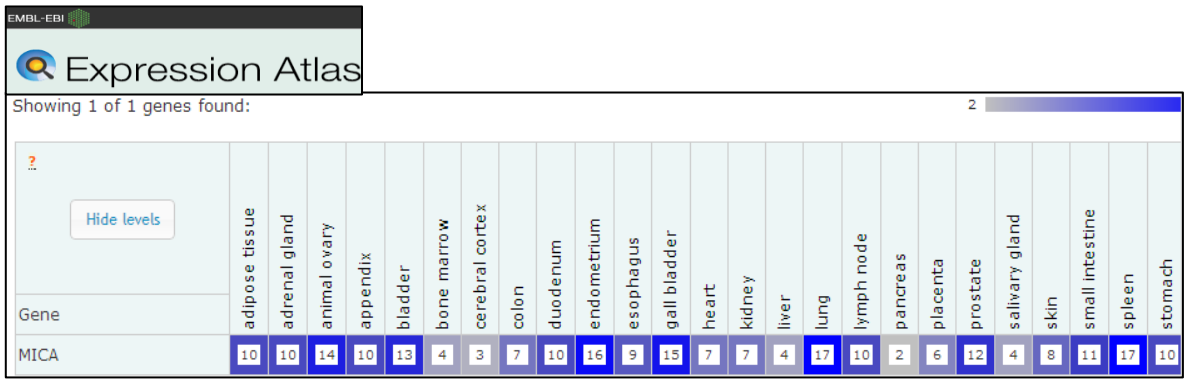


Figure 2.7 MICA expression intensity in different tissue according to the Expression Atlas (adapted from http://www.ebi.ac.uk/gxa). *MICA* expression pattern showed that the highest expression level of this gene was in the lung (17 FPKM), while the lowest expression was noted in the cerebral cortex (3 FPKM).

MICA expression patterns were also investigated using The Gene Atlas of The mouse and Human Protein-Encoding Transcriptomes which is a high-density oligonucleotide array that was made to examine patterns of gene expression on the human genome

scale (http://symatlas.gnf.org). This allowed for an observation of a quantified version of *MICA* expression patterns compared against its median expression in the different tissues (Figure 2.8).

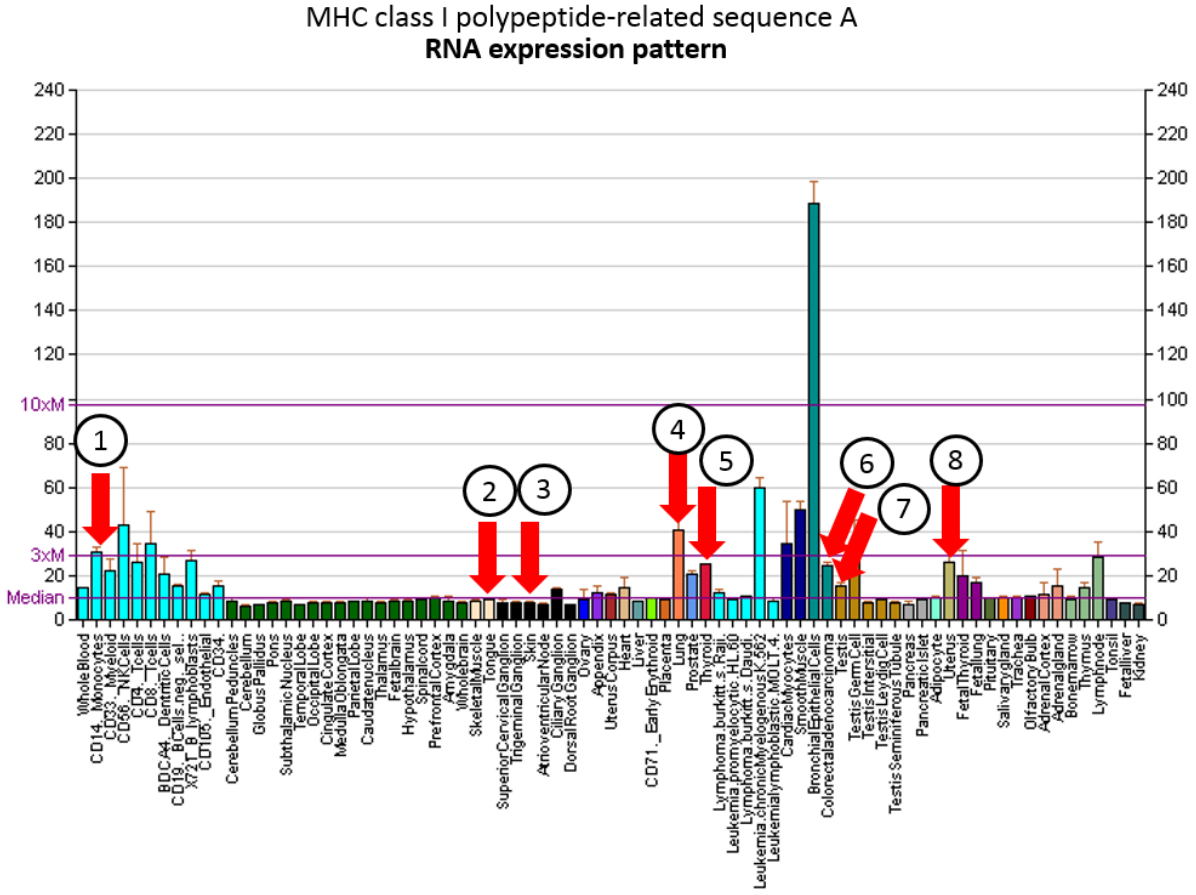


Figure 2.8 MICA expression patterns in different tissues (adapted from http://symatlas.gnf.org). 8 different tissue were considered as potential optimal standards for *MICA* expression levels analysis: (1) monocytes, (2) tongue, (3) skin, (4) lung, (5) thyroid, (6) colorectal carcinoma, (7) testis and (8) uterus.

From these investigations, 8 different tissues were considered as potential standards based on (1) *MICA* expression within these tissue was ≥ median (10 FPKM), and (2) their availability within the laboratory. These tissues were: monocytes cDNA, tongue cDNA, skin cDNA, lung cDNA, thyroid cDNA, colorectal adenocarcinoma cDNA, testis cDNA and uterus cDNA (Figure 2.8).

2.6.3.3. SYBR green qRT-PCR for MICA expression analysis

SYBR Green qRT-PCR was used to test cDNA preparations from the 8 different tissues as potential standards. A standard curve was obtained by creating a serial dilution (undiluted, 1:10, 1:50, 1:100, 1:500, 1:1000 and 1:5000) consisting of known concentration of each tested reference sample.

The QuantiFast SYBR Green PCR Kit (Qiagen, Hilden) was utilised for analysis of *MICA* expression levels in gastrointestinal tissue. The reaction mix, per sample, was prepared by adding 5 µl of SYBR Green mix, 0.5 µl of MICA specific forward primer (MICA_FACTTGACAGGGAACGGAAAGGA, Eurofins MWG Operon, Ebersberg, Germany). 0.5 µl of *MICA* specific reverse primer (MICA_R CCATCGTAGTAGAAATGCTGGGA, Eurofins MWG Operon, Ebersberg, Germany), 3 µl of nuclease free water to which 1 µl of the template cDNA was added (Table 2.11). All SYBR Green qPCR reactions were ran in triplicate on the Mastercycler® Ep Realplex (Eppendorf), using the following cycling conditions: 95°C for 5 minutes, (95°C for 8 seconds followed by 52°C for 20 seconds) x 45 times, 95°C for 15 seconds, 60°C for 15 seconds and finally 95 °C for 20 minutes (Table 2.11).

Table 2.11 SYBR Green qRT-PCR reaction components.
Reagents and volumes for qPCR analysis of *MICA* expression.

SYBR Green qRT-PCR reaction mix	Volume per sample (µl)
SYBR Green mix (2x)	1
MICA forward primer (100 pM)	1
MICA reverse primer (100 pM)	13
Nuclease free water	1
Template cDNA (100ng)	1

Verification of the amplification efficiency was performed via analysis of the melting and standard curves. For all the tested standards, the following parameters were analysed: (1) The Ct value for standard (only Ct values below 37 were considered, (2) No contamination of the negative control, which is nuclease free water, (3) The efficiency (E) of the qPCR reaction, which ideally should be 100% meaning that for each cycle the amount of PCR product doubles. This efficiency is calculated from the slope of the standard curve according to the following formula E = 10(-1/slope)-1

(Bustin *et al.*, 2009). A suitable reaction should have an efficiency between 90% and 100%, which corresponds to a slope between -3.58 and -3.10 (www.thermofisher.com). (4) The standard curve, created by the Mastercycler® EP realplex Software (Eppendorf), based on a serial dilution of the potential standard. Analysis of the standard curve gives important information regarding the performance of a primer set. (5) The performance of a primer set (R^2). R2 is the coefficient of correlation obtained for the standard curve and should be >0.99. (6) The melting curve analysis, for verification of specific amplification of the gene of interest with no contamination.

A comparison of all the qPCR parameters for the 8 standards showed that the optimal results were obtained when using lung cDNA as a standard. Lung cDNA results were as follows: there was no contamination of the negative control, the Ct value was 24.69, the slope Slope= -3.047, the efficiency E = 1.16, the correlation R^2 =0.971, and the melting curve showed no contamination with a mean T_m= 82.79 °C (Figure 2.9).

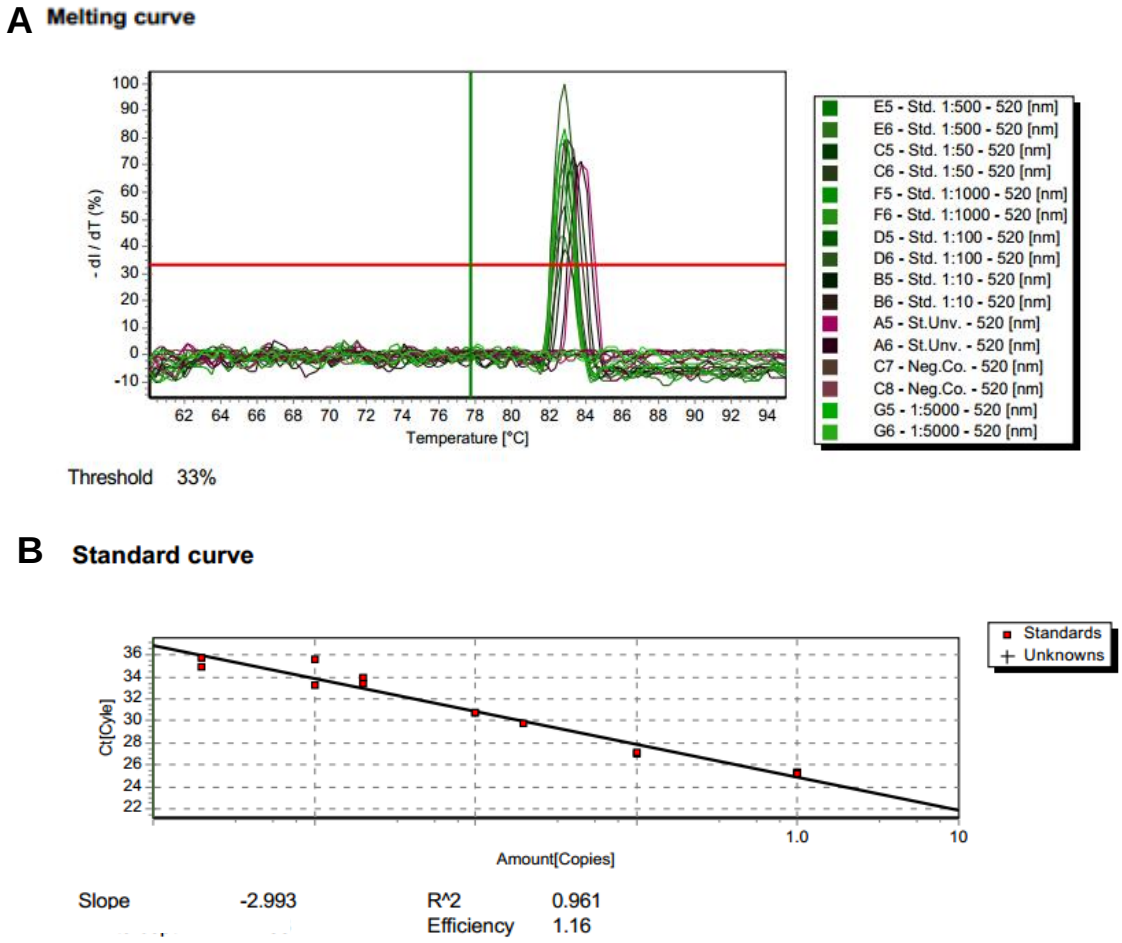


Figure 2.9 Melting curve and standard curve for lung cDNA as a reference standard. (A) Melting curve obtained when 7 concentrations of lung cDNA were tested (undiluted, 1:10, 1:50, 1:100, 1:500, 1:1000 and 1:5000). The melting curve shows a mean T_m of 82.79 °C with no other unspecific amplifications. (B) Standard curve obtained from the Mastercycler® EP realplex Software, corresponding to the lung cDNA with the different dilutions. From this curve, the primer set had a performance of R^2 =0.971 (optimal R^2 =0.99). The efficiency E= 1.16 (optimal E=1) and Slope =-2.993 (optimal is between -3.58 and -3.10).

Along with lung cDNA as a reference standard, 18s was used as an endogenous control gene for normalisation. A set of 7 dilutions were also used to produce a standard curve for 18s (1:5, 1:10, 1:50, 1:100, 1:500, 1:1000 and 1:2000) (Figure 2.16 B). The qPCR parameters for 18s were as follows: there was no contamination of the negative control, the slope= -3.134, the R^2 =0.970, the Efficiency = 1.08, the melting curve showed no contamination and the mean Tm= 88.7 °C (Figure 2.10 A).

For all SYBR Green qRT-PCR reactions, lung cDNA was used as a standard reference and 18s was used as endogenous control against which MICA expression levels were normalised.

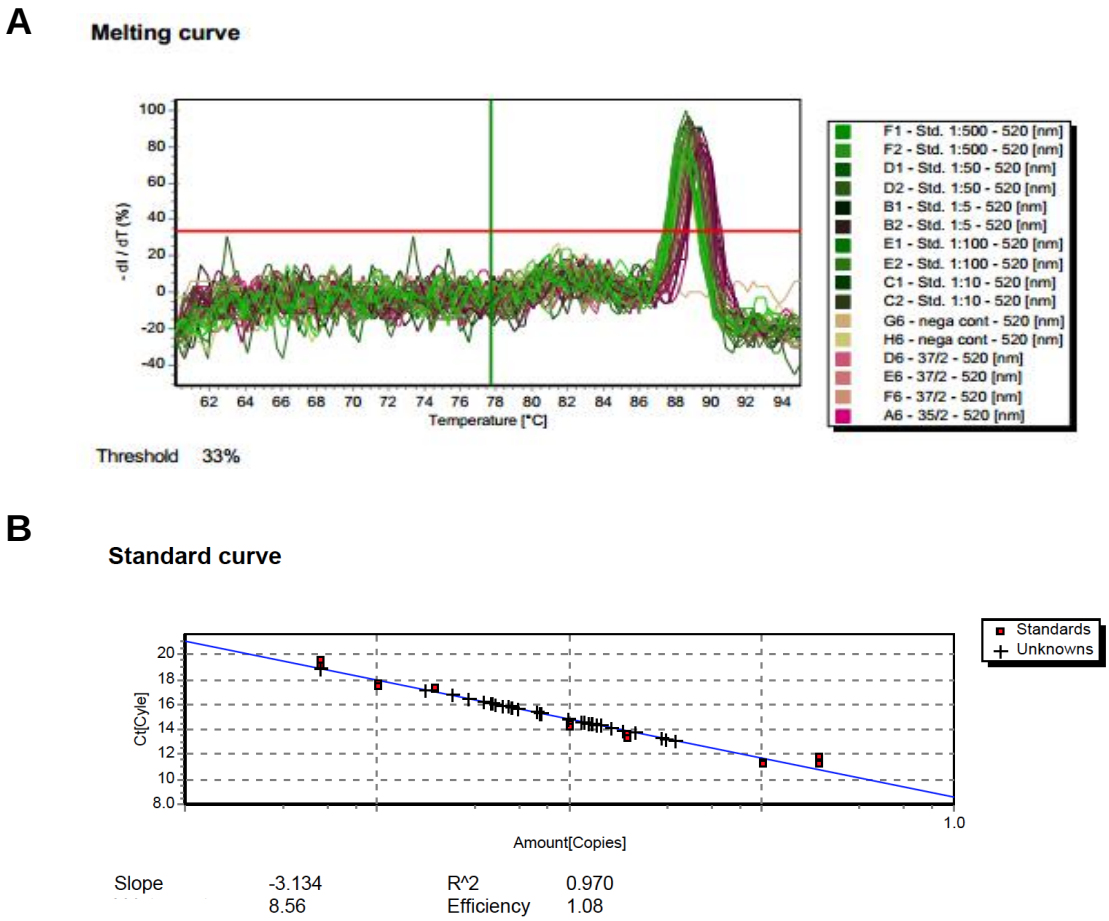


Figure 2.10 Melting curve and standard curve for 18s. (A) Melting curve obtained when 6 concentration of lung cDNA were tested (undiluted, 1:5, 1:10, 1:50 1:100 and 1:500). The mean T_m was 82.79°C. No unspecific amplifications were observed. (B) Standard curve obtained from the Mastercycler® ep realplex Software, corresponding to 18s showing that the slope= -3.134 (optimal is between -3.58 and -3.10), the correlation R^2 =0.970 (optimal R^2 =0.99) and the Efficiency E= 1.08 (optimal E=1).

2.6.3.4. SYBR green qRT-PCR data analysis

MICA gene expression levels were directly obtained from the Mastercycler® EP realplex Software (Eppendorf). These expression levels were automatically normalised against the levels of expression of 18s using the ΔCt method (ΔCt= Ct gene of interest - Ct reference gene). The obtained *MICA* expression values were then utilised for further statistical analysis.

2.7. Investigation of protein levels

2.7.1. Randox protein biochip array

A protein Biochip Array (Evidence Investigator®, RANDOX) was utilized for measuring soluble MICA, CXCL9 and LGALS7 levels in HSCT patient serum samples. The Evidence Investigator analyzer (Randox) is based upon Biochip Array Technology. This technology allows a multi-protein testing platform for the simultaneous quantitative and qualitative detection of target proteins in a single sample and from 48 samples simultaneously.

For the investigation of target protein levels, serum samples were added to the biochip assay reagents according to the manufacturer's protocol. Briefly, on a handling tray, 200 µl of the Randox assay diluent was pipetted per well, to which 100 µl of the Randox calibrator, 100 µl of the Randox control and 100 µl of the serum sample were added. The mixture was then incubated on a thermoshaker (Randox) for 1 hour at 37°C and 370 RPM. The handling tray was incubated at 2°C for 16-20 hours with no shaking. 2 wash cycles were carried out by adding 350 µl of the wash buffer to each well. A further 4 wash cycles, each for 15 seconds, were performed by adding 350 µl of the wash buffer to each well. Then 300 µl of the conjugate was immediately pipetted to each well, followed by an incubation for 1 hour at 37°C and 370 RPM. The reagents were discarded using a sharp, flicking action of the handling tray. Washing was then performed as previously described (2 quick wash cycles, followed by 4 wash cycle each for 15 seconds, using 350 µl of the wash buffer) (Table 2.12).

Table 2.12 Randox protein biochip array components. Reagents and volumes used for investigation of protein levels in serum samples using the Evidence investigator from Randox.

Randox protein biochip array reagents	Volume per sample (µl)
Assay diluent	200
Calibrator	100
Randox control	13
Washing buffer	4550
Serum sample	100

Samples were then left to soak for 30 minutes before being taken to imaging using the Evidence Investigator (Randox). Chemiluminescence signals were automatically quantified and reported by the Evidence Investigator as levels of expression in pg/ml. These values were then used for further statistical analysis.

2.7.2. CusabioTm ELISA

The protein biochip array from Randox did not include: C1QTNF7, HTRA1, PSTPIP1, PIK3AP1, MSR1 or ANP32A and therefore, their serum levels were investigated with a different approach using the Cusabio™ enzyme-linked immunosorbent assay (ELISA) kit (CUSABIO Life science, China).

This assay employs the quantitative sandwich enzyme immunoassay technique. The antibodies specific for the proteins of interest were pre-coated on the microplate provided by the Cusabio™ ELISA kit.

A standard curve was used to estimation the concentration of proteins of interest. In The standard curve is prepared by making serial dilutions of a known concentration of the standard across a range of concentrations near the expected unknown concentration. The concentration of unknown samples is then determined by interpolation of the standard curve.

For the Cusabio™ ELISA, the standard (5000 pg/ml) was reconstituted with 1 ml of sample diluent and a 2-fold dilution series (7 dilutions: 5000 pg/ml, 2500 pg/ml, 1250 pg/ml, 625 pg/ml, 312 pg/ml, 156 pg/ml, 78 pg/ml and undiluted) was created by adding 250 µl of sample diluent each time. The reconstituted standard served as undiluted and the sample diluent served as the zero standard.

The assay was carried out in a 96-well plate (12 x 8 coated microwells). Briefly, 100 µl of standard and samples were added to each well and the plate was incubated for 2 hours at 37°C. The liquid in the well was removed and 100 µl of biotin-antibody was added. The plate was incubated for 1 hour at 37°C. All wells were then aspirated and 2 washing steps of 2 minutes were carried out by adding 200 µl of the wash buffer. This was followed by adding 100 µl of the HRP-avidin to each well, the microplate was covered with an adhesive strip and incubated for 1 hour at 37°C and washed twice as previously described. 90 µl of TMB Substrate was added to each well and

the plate was then left to incubate for 30 minutes at 37°C. The reaction was stopped by adding 50 µl of Stop solution (Table 2.13).

Table 2.13 Cusabio ELISA components. Reagents and volumes used for investigation of protein levels in serum samples using the Cusabio ELISA Kit.

Cusabio ELISA reaction components	Volume per sample (µl)
Standard	100
Biotin-antibody (x100)	100
HRP-avidin (x100)	100
TMB Substrate	90
Wash buffer (x25)	800
Serum sample	100
Sample diluent	100

2.7.2.1. Cusabio ELISA data analysis

The optical density of each well was determined within 5 minutes of stopping the reaction using a microplate reader (Thermo Labsystems, Multiskan Ascent). Wavelenth was set to 450 nm for samples and 570 nm for the background. Readings at 570 nm were subtracted from the readings at 450 nm. This subtraction corrected for optical imperfections in the plate. Readings made directly at 450 nm without correction were higher and less accurate. Protein expression levels were obtained by averaging the wavelength reads of each sample duplicate obtained from the microplate reader.

A representative standard curve was plotted for each protein of interest. The standard curves corresponding to C1QTNF7 and HTRA1 are shown in Figure 2.11. Each point on the graph represents the mean of the duplicates. The standards for each protein of interest were within the linear area of the line of best fit.

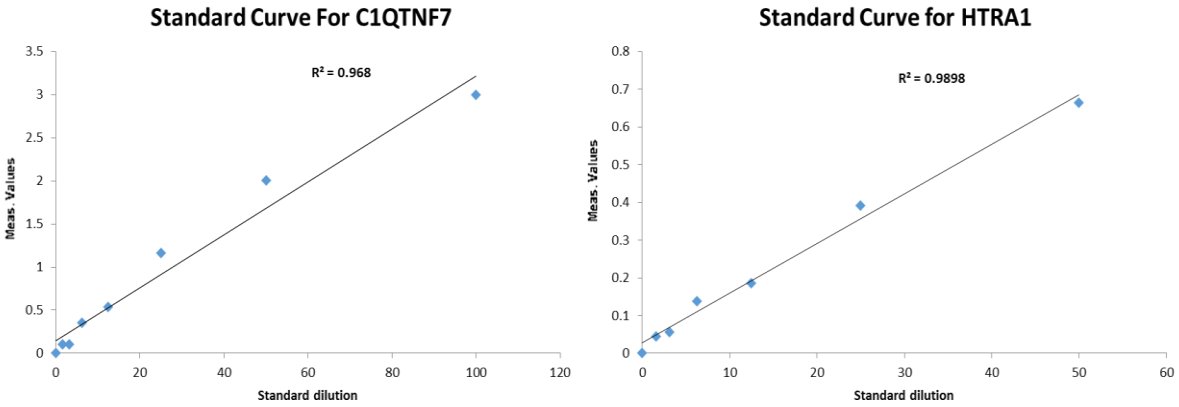


Figure 2.11 Representative standard curve for C1QTNF7 and HTRA1. A 2-fold dilution series for the standard (made of 7 dilution for the following concentration: 5000 pg/ml, 2500 pg/ml, 1250 pg/ml, 625 pg/ml, 312 pg/ml, 156 pg/ml, 78 pg/ml and undiluted) was utilised to create the standard curve for both C1QTNF7 and HTRA1. The correlation values for C1QTNF7 was R^2=0.968 and for HTRA1 R^2=0.989. The standards for each protein of interest were within the linear area of the line of best fit.

2.8. MicroRNA profiling in clinical gastrointestinal biopsies

2.8.1. nCounter chemistry for miRNA expression assays

MicroRNA profiling in clinical gastrointestinal total RNA samples was performed using the nCounter® miRNA Expression Assay (Nanostring Technologies). This assay is a highly multiplexed method for detecting miRNAs in total RNA across all biological levels of expression, without the use of reverse transcription or amplification, using molecular barcodes called nCounter Reporter Probes (Nanostring Technologies). The assay allows for the detection of more than 800 miRNAs within an RNA sample. NanoString technology is based on the direct molecular barcoding and digital detection of target molecules through the use of a color-coded probe pair. The probe pair consists of a Reporter Probe (Figure 2.12), which carries the signal on its 5' end, and a Capture Probe (Figure 2.12) which carries a biotin on its 3' end. The complexity of the colour codes, comprised of four colours in six positions, allows a large diversity of targets present in the same sample to be individually resolved and identified during data collection (Nanostring Technologies).

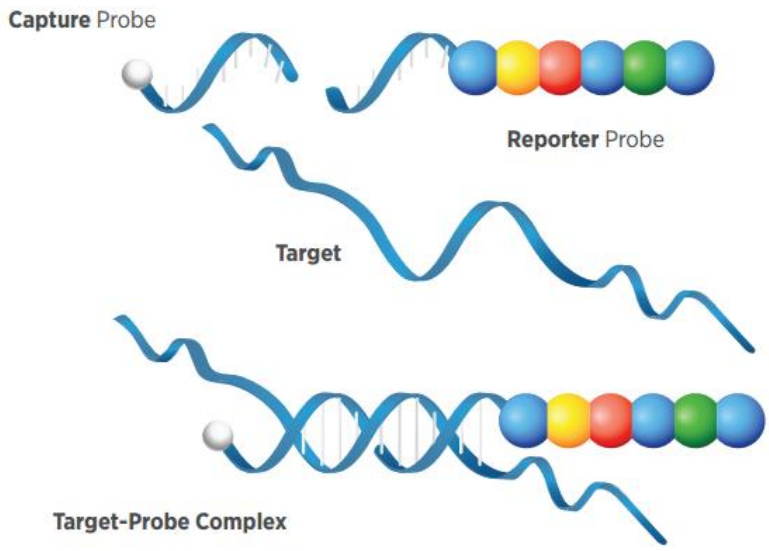


Figure 2.12 nCounter probe pairs for miRNA expression assay (Adapted from the nCounter® miRNA Expression Assay User Manual). The nCounter® miRNA Expression Assay delivers multiplexed measurement of miRNA expression, providing digital readouts of the relative abundance of hundreds of miRNA simultaneously. The nCounter Analysis System is based on miRNA-specific probe pairs that are hybridized to the sample in solution. Capture and Reporter Probes (top) and, Probe pair bound to an RNA target (bottom). The Reporter Probe carries the fluorescent signal; the Capture Probe allows the complex to be immobilized for data collection.

The process of miRNA profiling using the nCounter® miRNA Expression Assay is based on two main steps: (1) RNA sample preparation and (2) CodeSet hybridization and downstream processing.

The sample preparation involves a multiplexed annealing of specific tags to their target miRNA, a ligation reaction, and an enzymatic purification to remove the unligated tags (Figure 2.13). Briefly, total RNA samples were diluted to 33 ng/µl using RNAase free water (Ambion, Thermo Fisher Scientific) and 3 µl (100ng) of each RNA sample was added to a 12x 0.2 ml strip tube. A dilution (1:500) of the miRNA assay controls was prepared by adding 499 µl of RNAase free water to 1 µl of the miRNA assay controls. Preparation of the annealing master mix was performed by combining 13 µl of annealing buffer and 26 µl of nCounter miRNA tag reagent. 3.5 µl of this annealing master mix was added to each tube of the 12x 0.2 ml strip tube.

The strip was placed in a thermocycler (Applied Biosystems, 2720 Thermal Cycler) and the annealing protocol was initiated (94°C for 1 minute, 65°C for 2 minutes, 45°C

for 1 minutes and finally, the reaction was held at 48°C). For the ligation step, a ligation master mix was produced by combining 24 µl of polyethylene glycerol (PEG) and 16 µl ligase buffer. Following completion of the annealing protocol when the thermocycler had reached 48°C, 2.5 µl of the ligation master mix was added to each tube and these were then incubated at 48°C for 5 minutes. Leaving the strip in place on the heat block at 48°C, 1 µl of the ligase was added directly to each tube. Immediately after addition of the ligase to the final tube, the thermocycler was closed and the ligation protocol was launched (48°C for 3 minutes, 47°C for 3 minutes, 46°C for 3 minutes, 45°C for 5 minutes, 65°C for 10 minutes and then the reaction was held at 4°C). After completion of the ligation protocol, 1 µl of ligation clean up enzyme was added to each reaction. Tubes were then returned to the thermocycler and the purification protocol was initiated (37°C for 2 hours, 70°C for 10 minutes and then reaction held at 4°C). After completion of the purification protocol, 40 µl of RNAase free water was pipetted to each sample (Figure 2.13).

Immediately after completion of sample preparation, the CodeSet hybridization step was performed. During this step, probe pairs were present in large excess to target RNAs to ensure that each target finds a probe pair. Briefly, a master mix was created containing 130 µl of the reporter CodeSet and 130 µl of hybridisation buffer. 20 µl of this master mix was added to each of the samples. The final hybridatio n reaction contained the following components: 10 µl reporter CodeSet, 10 µl hybridisation buffer, a 5 µl aliquot from the miRNA sample preparation protocol and 5 µl capture probe set. This hybridisation assay was incubated at 65°C for 12 hours. After hybridization, excess probes were washed away using a twostep magnetic bead-based purification on the nCounter Prep Station (automated processing). Magnetic beads derivatized with short nucleic acid sequences that are complementary to the Capture Probe and the Reporter Probes were used sequentially. First, the hybridization mixture containing target/probe complexes was allowed to bind to magnetic beads complementary to sequences on the Capture Probe. Wash steps were performed to remove excess Reporter Probes and non-target cellular transcripts. After washing, the Capture Probes and target/probe complexes were eluted off the beads and were hybridized to magnetic beads complementary to sequences on the Reporter Probe. An additional wash was performed to remove excess Capture Probes (automated processing).

Finally, the purified target/probe complexes were eluted off the beads and immobilized on the cartridge for data collection.

Data Collection was carried out in the nCounter Digital Analyzer. Digital images were processed and the barcode counts were tabulated in a comma separated value (CSV) format.

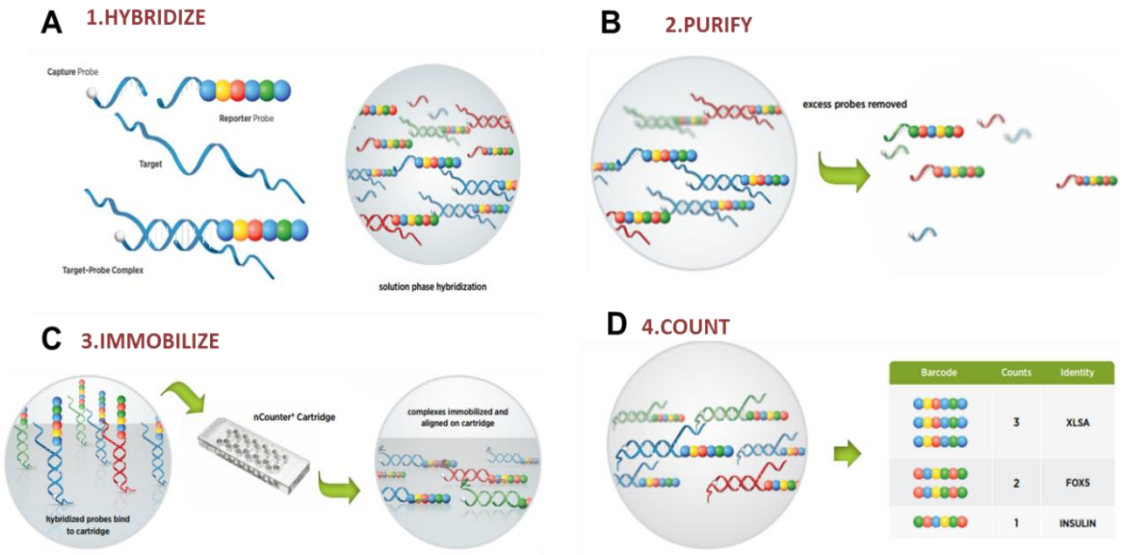


Figure 2.13 The nCounter® miRNA Expression Assay steps (Nanostring Technologies). (A) miRNA-specific probe pairs are hybridized to the sample in solution. The protocol eliminates any enzymatic reactions that might introduce bias in the results. Barcoded probes hybridize directly to a target molecule in solution. (B) After hybridization of the CodeSet with target nucleic acids, samples are transferred to the Prep Station, which contains a fluidic processing system that removes excess probes, unbound targets, and other extraneous material. (C) Purified probe:target complexes are deposited onto a streptavidin-coated imaging surface and immobilized via the biotinylated capture probe. (D) Immobilized reporters are then aligned, stretched, and immobilized again at the other end of each complex in order to create parallel fluorescent barcodes that can be imaged. An automated fluorescence microscope in the Digital Analyzer scans the cartridge, and the ordered fluorescent segments on the attached reporter probe identify each target molecule of interest. The Reporter Probe carries the signal and the Capture Probe allows the complex to be immobilized for data collection.

2.8.2. nSolver ™ Analysis Software and R for miRNA profiling data analysis

The data produced by the nCounter Digital Analyzer is exported as a Reporter Code Count (RCC) file. RCC files are comma-separated text (.csv) files that contain the counts for each gene in a sample. The data for each sample hybridization is contained in a separate RCC file. Prior to comparing data between hybridizations, slight

differences in hybridization, purification, binding efficiency and other experimental variables must be normalized. To accomplish this, a reference (housekeeping gene) normalisation was performed in order to adjust counts of all probes relative to a probe (or set of probes) that are not expected to vary between samples or replicates. Reference gene normalization assumes that some of the target sequences recognized by the CodeSet are consistent in their expression levels (Nanostring Technologies).

Because reference genes are often expressed at different levels, the geometric mean of the reference genes for each lane was utilised to calculate scaling factors. The average of these geometric means across all lanes was used as the reference against which each lane, was normalized. A normalization factor was then calculated for each of the lanes based on the geometric mean of counts for the reference genes in each lane relative to the average geometric mean of counts for the reference genes across all lanes. This normalization factor was then used to adjust the counts for each gene target and controls in the associated lane, calculate the fold change and estimate the significantly dysregulated miRNAs.

Normalised data along with the fold change data were imported into R (The R Project for Statistical Computing, v3.3.0), where data was visualized as volcano plots, dendograms and heat-maps (all scripts and analysis pipelines were developed by Kile Green, Human DC lab, Haematological Sciences Department, Institute of Cellular Medicine, Faculty of Medical Silences, Newcastle University, UK).

2.9. Immunofluorescence staining for MICA in gastrointestinal tissue

2.9.1. Clinical gastrointestinal biopsy slides

As GI GvHD biopsies are very challenging to obtain and rarely available, a set of 4 gastrointestinal biopsy slides obtained from non-transplanted patients suffering from Crohn's disease (long-term condition that causes inflammation of the lining of the digestive system which gives symptoms similar to GvHD in the gut (Galati *et al*., 1993) were used for testing the antibodies and optimizing an appropriate protocol to stain MICA protein in gut biopsies.

2.9.2. Cell preparation for anti-MICA antibody test

Before using the biopsy slides, normal human dermal microvascular endothelial cells (HDMEC) from PeloBiotech were used to test the performance of 2 antibodies; Human MICA Biotinylated Antibody (R&D Systems) and Anti-MICA antibody produced in rabbit (Sigma-Aldrich).
In sterile conditions, HDME cells were added to a 4 chamber culture slide (IBIDI) to which 100ng/ml of lipopolysaccharides (LPS) (from *E.Coli*) were added to stimulate the expression of MICA. The slide was incubated at 37°C for 24 hours. After 24 hours, all media was removed and cells were washed with 1X PBS (0.137 M NaCl, 0.05 M NaH2PO4, pH 7.4) and 400µL of Formaldehyde Fixative Solution (85 mM Na2HPO4, 75 mM KH2P04, 4% paraformaldehyde and 14% (v/v) saturated picric acid, pH 6.9) was added to each chamber of the culture slide for 15 minutes. Cells were then washed again with PBS, and then 200µl of 0.3% Triton X-100 was added to each chamber.

This was followed by an incubation at room temperature for 15 minutes. A final step of washing with PBS was performed for 5 minutes and cells were blocked with blocking buffer (1% horse serum in PBS) for an hour and stored at 4°C.

2.9.3. Immunostaining of HDME cells

HDMECs were stained with both antibodies (MICA Biotinylated Antibody (50 µg/ml), and Anti-MICA antibody (1mg/ml)

Both of the antibodies concentrations were optimised by testing 6 different dilutions (1:10, 1:15, 1:20, 1:25, 1:30 and 1:35). An optimal dilution of 1:30 was chosen for MICA Biotinylated Antibody (R&D) and 1:50 was considered for Anti-MICA antibody (Sigma-Aldrich).

For both antibodies, a volume of 200µ/L of the diluted antibody was added to each culture chamber. After one hour, the cells were washed with PBS 3 times for 2 minutes and then the secondary antibody (Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L) Antibody, Life Technologies) was added. Slides were then observed under the Zeiss Axiovert 200 inverted florescence microscope.

2.9.4. Double immunofluorescence staining of gastrointestinal slides

Duplicates of individual gastrointestinal sections were tested. One of each gastrointestinal section and control was stained with the diluted MICA antibody and the remaining gastrointestinal section and control acted as a verification, incubated with only the secondary antibody.

The slides were bathed in xylene (Thermo Fisher Scientific) for 7 minutes to dissolve the paraffin from the tissue section. This was followed by consecutive soaking in absolute ethanol, 96% ethanol and 70% ethanol each for 5 minutes to dehydrate the tissue section. Antigen retrieval was then performed by soaking the slide into a citrate buffer (2.94 g of sodium citrate (10 mM), 0.5 ml of 0.05% Tween 20,1L distilled, water, pH 6.0) and heating in the microwave at 300 watt for 30 minutes. Slides were then washed with distilled water and PBS and blocked with 20% bovine serum albumin (BSA) for 60 minutes.

The diluted primary antibody (Human MICA Biotinylated Antibody (R&D Systems)), was then added and slides were incubated for 60 minutes at room temperature. The slides were then washed with PBS for 5 minutes and the secondary diluted in 6% BSA was added and slides were incubated for 1 hour.

After the slides were washed again with PBS for 5 minutes and distilled water for 5 minutes, post detection conditioner (Reagent B: DIANOVA) was added and slides were incubated for 5 minutes in the dark at room temperature. Then VECTASHIELD Mounting Medium with DAPI (H1200, Vector Laboratories) was added and slides

were left to air dry and then sealed under a coverslip. An Isotype control was carried out for the confirmation of the antibody specificity.

Slides were observed under the Zeiss Axiovert 200 inverted florescence microscope. Exposure time for both DAPI and Alexa Fluor 488 was set and then kept constant during the imaging process for all the slides in this study.
Quantification of the immunofluorescence intensity for MICA was performed on ImageJ (NIH, v1.49), where MICA expression was translated into relative fluorescence units (RFU) for further statistical analysis and comparison between GvHD grades and MICA expression.

2.9.5. Anti-MICA Antibody specificity analysis in Human Dermal Microvascular Endothelial Cells

MICA is a highly glycosylated cell surface protein which is stably expressed without conventional class I peptide ligands in stress conditions (Groh *et al.*, 1996b). Human dermal microvascular endothelial cells (HDMEC) were cultured and stimulated with LPS to test for the antibodies. Staining with the monoclonal MICA antibody (SIGMA-ALDRICH) did not show any florescence. However, the anti-MICA antibody from R&D was conjugated with the secondary antibody, Alexa 488 (Life Technologies) gave a florescent signal (Figure 2.14).

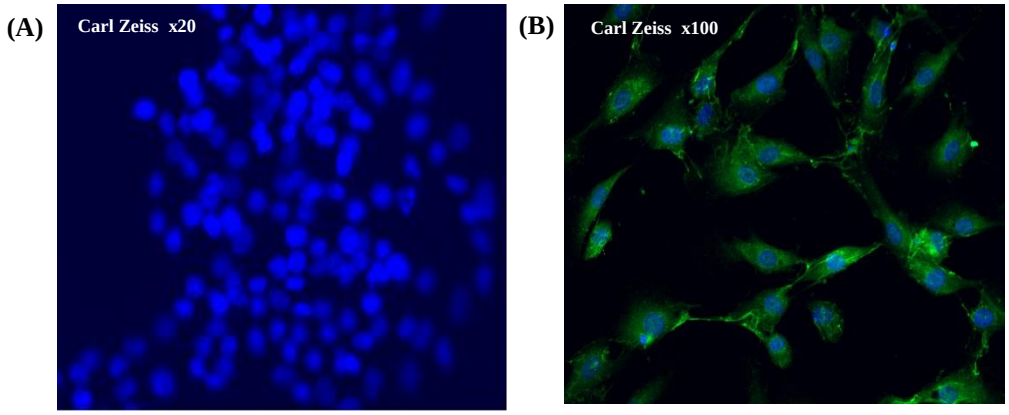


Figure 2.14 Human Dermal Microvascular Endothelial cells staining with the polyclonal Anti-MICA antibody (R&D). (A) HDMECs without the specific anti-MICA antibody. (B) HDMECS with the specific anti-MICA antibody: the cells nuclei coloured in blue and MICA is shown in green.

However, the positive signal obtained with the anti-MICA antibody from R&D did not correspond to a MICA positive cell signal, nor did it correspond to background.

Further optimization of the standard protocol and further investigation of the primary and secondary antibody was required before the staining could be performed (Figure 2.15).

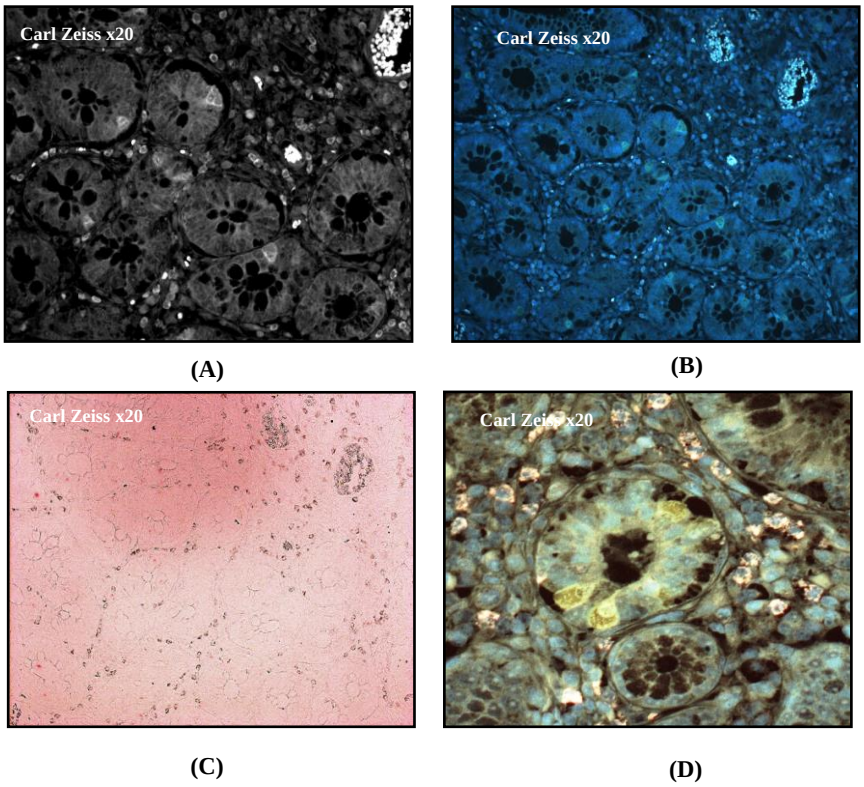


Figure 2.15. **Immunofluorescence staining of GI biopsy slides with the secondary antibody only.** (A) Florescence observation of the gut slide showing a signal on the supposedly positive cells (B) DAPI filter captured image of the cells shown in A. (C) Background filter showing that all signals are derived from cells and no background staining is present. (D) Triple filter image of a magnified cell showing cells with the green signal.

In a third attempt, an anti-MICA antibody from Thermo Scientific ® was tested as a primary antibody for MICA. The same secondary antibody was used (Alexa 488® (Life Technologies). After verification of MICA expression patterns via the EMBL-EBI Expression Atlas (https://www.ebi.ac.uk/gxa/) thyroid tissue was used as a control for proving the antibody and optimising the protocol. Confocal microscopy images showed a positive and specific staining for MICA (Figure 2.16).

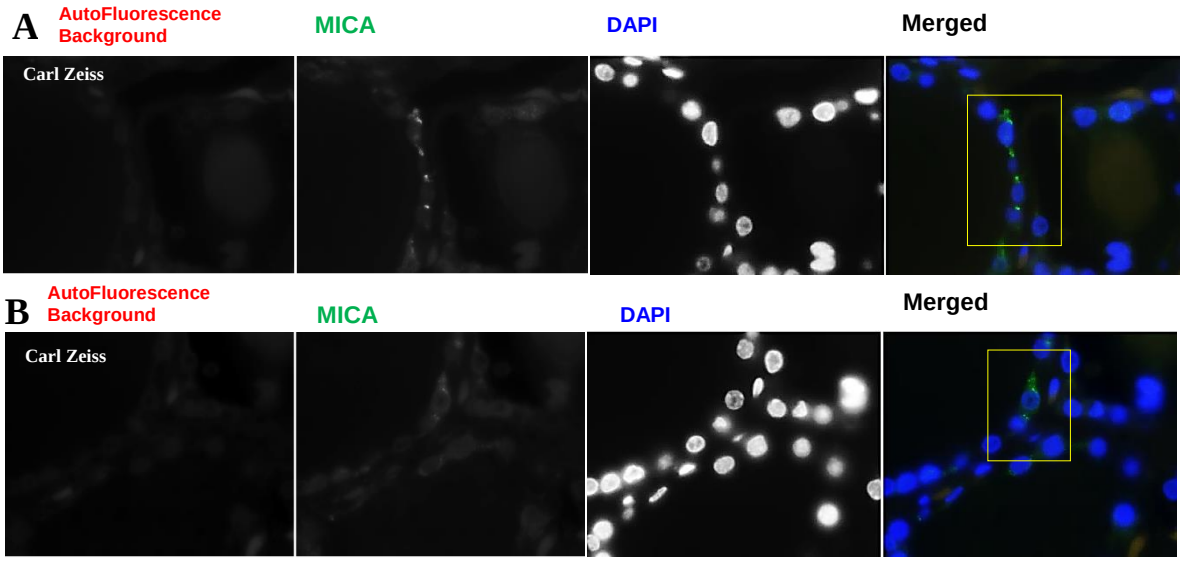


Figure 2.16 Immunofluorescence staining of thyroid slides for MICA detection. (A) A clean background was observed after IF. MICA is shown as green colour in the cytoplasm of cells. DAPI filter captured images of the cells show the nucleus in blue. (B) A clear cytoplasmic expression of MICA is shown as a fading green colour. Images were captured from the same slide in different areas to show the cytoplasmic expression.

MICA expression was lower than expected in the thyroid tissue, but positive staining was observed in the cytoplasm of cuboidal follicular cells (Figure 2.17). Thus, the protocol was optimised and both primary and secondary antibodies along with the isotype control were determined.

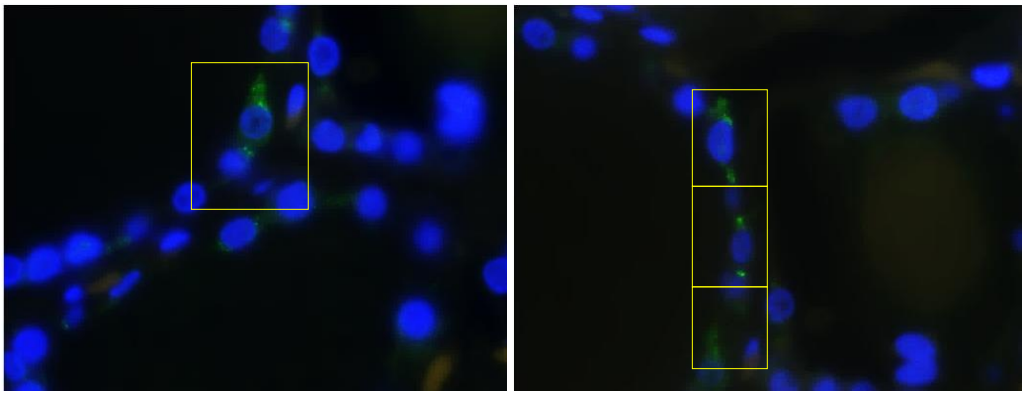


Figure 2.17 Magnification of MICA positive cuboidal follicular cells (shown in yellow boxes) MICA has a cytoplasmic expression. MICA shows low expression in the thyroid, thus the very low intensity of the green colour.

2.10. Statistical analysis

2.10.1. Statistical analysis for the SNP genotyping

After HSCT, both recipient and donor genetic profiles may have a potential effect on treatment response and clinical outcome. Therefore, for the SNP association studies comparisons of genotypes with clinical outcomes were carried out in order to identify the strongest effect.

Genotypes were coded based on the possession of the minor allele alone (recipient and donor separately). For example, if the minor allele is A and the major (ancestral) allele is B, possession of two copies of the minor allele (AA) would be coded 1 while AB and BB would be coded 0 (recessive coding). In the case of the possession of at least one copy of the minor allele (AA, AB) would assign 1 to AA and 1 AB while assigning 0 to BB (dominant coding). Additionally, to assess the additive effect of the minor allele, BB genotypes were coded as 0, AB genotype genotypes were coded as 1 and the AA genotypes were coded as 2 (additive coding) (Chien *et al*, 2012). Data analysis for the SNP genotyping outcome was performed while was taking into consideration various factors including the age, the underlying disease, the relationship between patients and donors and the TCD treatment into the statistical models.

2.10.2. General statistics

In general, statistical analyses were performed with SPSS v.21 software (IBM Analytics), MiniTab (Stata Corporation), R (R Project) packages “cmprsk” (competing risks) (Bob Gray (2014), Subdistribution Analysis of Competing Risks, R package version 2.15.0) and “coxph” (Cox regression to maximize a penalized partial likelihood) (Andersen and Gill 1982) and GraphPad Prism 6 software (GraphPad, San Diego, CA) was used for statistical analysis and generating graphs.

The Mann-Whitney tests were used to determine the statistical significance when there were two groups only, whereas Kruskall-Wallis test was used to determine the statistical significance when there were more than two groups and normality in the groups could not be assumed; $p \leq 0.05$ was considered as statistically significant.

Analysis of Variance (ANOVA) test was used to determine the statistical significance when there were more than two groups compared and normality in the groups could be assumed and $p \leq 0.05$ was considered as statistically significant.

Univariate relationships between potential risk factors for death were assessed via Kaplan-Meier statistics and the Log-Rank tests.

Multivariate associations were determined via Cox (proportional hazard) regression model.

The cumulative incidence of relapse and NRM was assessed via the method of competing risks (Fine and Gray, 1999) with death and relapse taken as competing events. Gray's test was employed to determine the level of statistical associations between potential prognostic factors and outcome (relapse and NRM). All tests were 2-sided, with type I error rate fixed at 0.05.

Chapter 3. Impact of single nucleotide polymorphisms in miR-146a, IRAK1 and MICA on HSCT outcome

3.1 Introduction

Several studies have established the involvement of polymorphisms in non-HLA genes in determining clinical outcome after transplantation (Welniak *et al.*, 2007). SNPs in genes essential for allogeneic immune responses and inflammatory reactions have been described as potential biomarkers for the severity of GvHD (Elmaagacli *et al.*, 2008; Gruhn *et al.*, 2009; Espinoza *et al*., 2011; Elbahlawan *et al*., 2012).

SNPs are an important variation to create diversity among individuals, as well as leading to different phenotypes, traits, and diseases (Shastry, 2009). Since miRNAs are key regulators of gene expression, miRNA-related SNPs including SNPs in miRNA genes and their target sites may function as regulatory SNPs, through modifying miRNA regulation to affect phenotypes and disease susceptibility (Dignam *et al.*, 1983). Moreover, SNPs located in miRs are likely to have a complex influence by affecting miR maturation, functional strand selection and target selection (Dignam *et al.*, 1983). Since 2005, several studies have systematically identified and analysed human polymorphisms in miRNAs and/or miRNA target sites (Iwai and Naraba, 2005; Saunders *et al.*, 2007; Landi *et al.*, 2008; Shastry, 2009; Ryan *et al.*, 2010; Bhartiya *et al.*, 2011).

MiR-146a is an immediate early-response gene induced by various microbial components and proinflammatory mediators. The human genome contains two miR-146 genes (miR-146a and miR-146b) on chromosomes 5 and 10, respectively, and their mature products differ only by 2 nucleotides in the 3′ region (Figure3.1) (Bentwich *et al.*, 2005; Cai *et al.*, 2005).

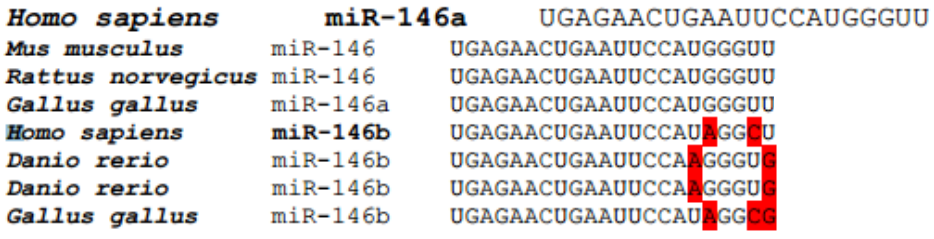


Figure 3.1 Sequence alignment of the miR-146 family of miRNAs (adapted from Griffiths-Jones, 2004). All sequences are taken from the MicroRNA Registry (release 7.1). Variable nucleotides are shown in red (Griffiths -Jones, 2004).

MiR-146a is highly expressed in Treg cells and is induced upon activation of effector T cells and myeloid cells (Lu *et al.*, 2010). In the latter, miR-146a acts as a negative feedback regulator to limit TRAF6 and IRAK1-mediated signaling in inflammatory settings (further explained in Chapter 1, section 1.7.3.1) (Taganov *et al.*, 2006a; Hou *et al.*, 2009), whereas in activated human T cells, miR-146a has been suggested to oppose apoptosis and IL-2 production (Curtale *et al.*, 2010).

miR-146a has been validated to target the expression of at least two genes, *IRAK1* and *TRAF6*, and acts as a negative regulator in TLR and pro-inflammatory cytokine (IL-1) signaling pathway (further explained in Chapter 1, section 1.7.3.1) (Taganov *et al.*, 2006b). *IRAK1* encodes for a key intracellular signaling protein that is activated by ligands of Toll-like receptors. *IRAK1* activation by interleukin-6 results in phosphorylation and activation of the transcription factor STAT3 and consequent transcriptional activation of the gene for C-reactive protein (Zhang *et al.*, 1996). Specifically, IRAK1 plays significant role in TLR/IL-1 receptor (TIR) activation of NF-κB (Chatzikyriakidou *et al.*, 2010). IRAK1 is considered as a linker of the TLR with the TRAF6 intracytoplasmic activator of transcription factor NF-κB, which subsequently increases the expression of many genes related to immunological reactions such as TNF-α and IL-8 (Dunne and O'Neill, 2003; Janssens and Beyaert, 2003). Subsequently, IRAK1 is subjected to negative feedback control by miR-146a, expression of which is also NF-κB dependent, leading to a concerted immunological response (Chatzikyriakidou *et al.*, 2010).

Activation and nuclear translocation of NF-κB transcription factors is medicated by the TCR and Natural Killer Group 2D (NKG2D) receptor stimulation (Rajasekaran *et al*., 2011). Such stimulation is mediated by one of the most polymorphic NKG2D-ligands, MICA (Spear *et al*., 2013) (further explained in Chapter 1, section 1.9).

Thus, miR-146a, IRAK1 and MICA all participate in a network controlling diverse biological process. This complex network is further complicated by the presence pf SNPs in the miR-146a, IRAK1 and MICA encoding loci. A common polymorphism in *pre-miR-146a*, designated rs2910164, causes a G to C change at position +60 relative to the fist nucleotide of *pre-miR-146a* (Jazdzewski *et al*., 2008). This SNP leads to a miss-paired hairpin sequence within the precursor of miR-146a, which affects processing of the miRNA and consequently, lowers expression of the mature sequence (Onnis *et al.*, 2012).

MiR-146a rs2910164 has been previously investigated for its association with the severity of GvHD in allo-HSCT patients, where it was shown that the CC genotype is associated with severe aGvHD (Stickel *et al*., 2014). A study by Shen et al, showed that among 42 patients with familial breast cancer and 82 patients with ovarian cancer, those with at least one rs2910164(C) SNP tended to be diagnosed at an earlier age than those with only (G) alleles (Shen *et al*. 2008). Xu et al., also suggested that a functional polymorphism in the pre-miR-146a gene is associated with prostate cancer risk and mature miR-146a expression *in vivo*. The author reported that patients with the CC genotype of this SNP were at decreased risk for prostate cancer compared with those carrying the GG/GC genotype. In addition, the team also reported that the G-to-C change in the precursor of miR-146a resulted in reduced expression of mature miR-146a in prostate cancer tissue (Xu *et al*. 2010).

Another SNP in miR-146a is rs2431697. This occurs at position 5q35.1 of the miR-146a gene and causes a T to C transition, resulting in the miR-146a (2) variant (SNPedia). Investigation of 20 patients with non-HLA psoriasis showed that miR-146a (2) is associated with susceptibility to psoriatic arthritis and psoriasis vulgaris in the Chinese population (Yang *et al.* 2013). There is currently no published information about the association of miR-146a (2) and HSCT outcome. However, a pilot study within our laboratory showed that presence of the T allele is associated with the incidence of relapse in a RIC cohort of HSCT patients with aGvHD grades II-IV (unpublished data).

Rs3027898 is a SNP in the 3'-UTR of the IRAK1 gene, which occurs at position Xq28. This SNP encodes an A->C tansversion. Because the gene is located on the X chromosome, men are more likely than woman to show an association between this SNP and diseases. Several studies have shown an association between both the A and C alleles with numerous diseases, such as atherothrombotic cerebral infarction (Yamada *et al*. 2008), rheumatoid arthritis (Chatzikyriakidou *et al*. 2010) and lupus erythematosus (Zhai *et al*. 2013).

There are currently more than 100 alleles known which encode for 79 protein variants for MICA (http://www.ebi.ac.uk/ipd/imgt/hla/). Interestingly, a SNP at position 454 (A→G, rs1051792) leads to an amino acid substitution of methionine by valine (Met→Val) at position 129 in the α2 domain of the MICA protein, that categorizes the

MICA alleles into strong (MICA-129 Met) and weak binders (MICA-129 Val) binders of NKG2D (Raache *et al*,. 2012) (Figure 3.2).

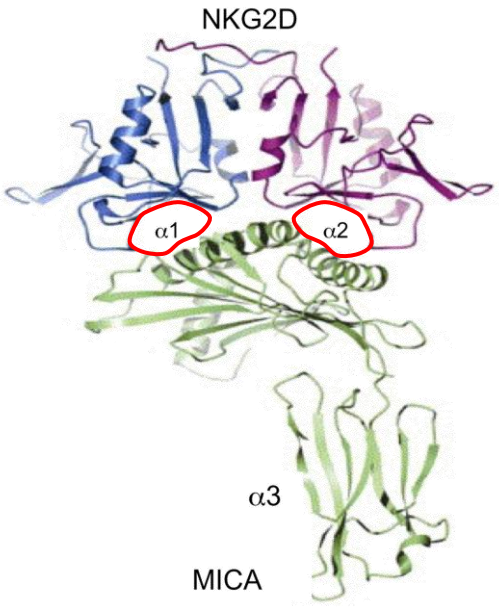


Figure 3.2 Ribbon diagram showing crystal structures of NKG2D bound to MICA (adapted from Li et al., 2001). The NKG2D homodimer is colored in blue and magenta, MICA is green with domains labeled. NKG2D recognizes the alpha1 and alpha2 domains of MICA. rs1051792 occurs in the the α2 domain which is a binding site for NKG2D (Li *et al.*, 2001b).

Thus, it is important to pinpoint SNP-mRNA-miRNA regulatory network alterations and their contribution to the risks associated with HSCT. Such investigation will help elucidate the consequences of the interaction between these three genetic elements, deciphering the genetic risks of HSCT.

3.2 Study aims

This study aimed to investigate rs2910164 and rs2431697 in miR-146a, rs3027898 in IRAK1, a potential target of miR-146a and rs1051792 in MICA for their association with HSCT outcome in a study cohort of n=817 patient and donor pairs. All results were subsequently considered for validation in a cohort of n= 576 patient and donor pairs.

3.3 Results

3.3.1 Study cohort results

3.3.1.1 Clinical characteristics of the study cohort

The study cohort comprised of n=817 donor and patient pairs who underwent allo-HSCT between 1984 and 2014 and for whom SNP genotyping data was available for the 4 SNPs of interest. The genotyping was performed using genomic DNA samples collected pre- and post-transplantation. The study cohort was recruited from two different transplantation centres including the Newcastle Upon Tyne NHS Foundation Trust, Newcastle upon Tyne, United Kingdom and the Transplantation Centre, University Clinic of Regensburg, Germany (Sample collection and usage is elaborated in Chapter 2, section 2.1.1) (copy of ethics in Appendix).

Various clinical variables were investigated: patient and donor ages, patient and donor genders, underlying disease, patient survival, the frequency of patients developing aGvHD and/or cGvHD, the conditioning regimen, the T cell depletion treatment and the relationship between the patients and donors. All the clinical characteristics for the study cohort are shown in Table 3.1.

Table 3.1 Clinical characteristics of the study cohort (n=817)

Characteristics		**N (%)**
Patients	Age range (years)	10-67
	Female	309 (37.8)
	Male	508 (62.2)
Donors	Age range (years)	10-74
	Female	251 (30.7)
	Male	509 (62.3)
	Missing	57 (7)
Underlying disease	Acute Myeloid Leukaemia	139 (17.0)
	Acute Lymphoblastic Leukaemia	52 (6.4)
	Chronic Myeloid Leukaemia	44 (5.4)
	Non-Hodgkin's lymphoma	43 (5.3)
	Hodgkin's Disease	17 (2.10
	Other diagnoses	69 (8.44)
	Missing	456 (55.8)
Adult		408 (50.1)
Relationship	Haploidentical	1 (0.1)
	Matched unrelated donors	200 (24.5)
	Siblings	215 (26.3)
	Missing	401 (44.5)
T cell depletion		164 (20.1)
Reduced Intensity conditioning		251 (30.70)
Female to Male ratio	Valid	136 (16.60
	Missing	43 (5.3)
Source of transplant	Bone Marrow	167 (20.4)
	Cord blood	3 (0.4)
	PBMCs	249 (30.5)
	Missing	398 (48.71)
Acute GvHD	Grade 0	145 (17.7)
	Grade 1	102 (12.50
	Grade 2	91 (11.1)
	Grade 3	35 (4.3)
	Grade 4	16 (2.0)
	Missing	428 (52.4)
Chronic GvHD	Valid (all grades)	148 (18.11)
	Missing	548 (67.1)
Relapse	Yes	123 (15.1)
	No	299 (36.6)
	Missing	395 (48.3)
Deceased	Yes	231 (28.3)
	No	202 (24.7)
	Missing	384 (47.0)

The genotype frequencies of rs2910164 and rs2431697 in miR146a, rs3027898 in IRAK1 and rs1051792 in MICA for the patients and the donors within the study cohort are described in Table 3.2.

Table 3.2 Genotype frequencies for the SNPs on interest for the patients and donors within the study cohort

SNP of interest	Genotype	N (%)
Patient genotypes for rs2910164 in miR-146a	GG	358 (43.8)
	GC	239 (29.3)
	CC	40 (4.9)
	Missing	180 (22.1)
Donor genotypes for rs2910164 in miR-146a	GG	387 (47.4)
	GC	204 (25)
	CC	33 (4)
	Missing	193 (13.6)
Patient genotypes for rs2431697 miR-146a	TT	205 (25.1)
	TC	300 (36.7)
	CC	121 (14.8)
	Missing	191 (24.4)
Donor genotypes for rs2431697 miR-146a	TT	195 (23.9)
	CA	298 (36.5)
	CC	116 (14.2)
	Missing	208 (25.4)
Patient genotypes for rs3027898 in IRAK1	AA	358 (43.8)
	CA	57 (7)
	CC	61 (7.5)
	Missing	341 (40.8)
Donor genotypes for rs3027898 in IRAK1	AA	357 (43.7)
	CA	56 (6.9)
	CC	57 (7)
	Missing	347 (42.5)
Patient genotypes for rs1051792 in MICA	AA	49 (6)
	GA	234 (28.6)
	GG	351 (43)
	Missing	183 (22.4)
Donor genotypes for rs1051792 in MICA	AA	43 (5.3)
	GA	221 (27.1)
	GG	351 (43)
	Missing	202 (24.7)

The genotype frequencies of rs2910164 and rs2431697 in miR146a, rs3027898 in IRAK1 and rs1051792 in MICA described above are in coherence with the standard Caucasian genotype frequencies and obey the Hardy–Weinberg principle (HWP) ($p<0.6$) (The HapMap project, http://hapmap.ncbi.nlm.nih.gov/; the 1000genome, www.1000genomes.org/).

The previously described frequencies correspond to the complete study cohort comprising both patients and donors.

Various HSCT outcomes were investigated for their association with rs2910164 and rs2431697 in miR146a, rs3027898 in IRAK1 and rs1051792 in MICA. These included relapse, NRM, OVS, aGvHD and cGvHD. Statistical analysis was carried out for each SNP separately correcting for various variables including disease, relationship, the female to male ration and the TCD treatment.

3.3.1.2 Association between rs2910164 and rs2431697 in miR146a and HSCT outcome

Statistical analysis was performed using Grey's test. In patients, there was no significant association between rs2431697 in miR-146a (2) and HSCT outcome including relapse, NRM, OVS, aGvHD and cGvHD. However, the presence of the 'C' allele in rs2910164 in patients showed a borderline significance for its relation with the NRM. Patients carrying the 'C' allele, thus the CC or CG genotype, had a tendency toward an increased NRM (p=0.054) (Figure 3.4). There was no significant association between rs2910164 in miR-146a and relapse, OVS, aGvHD and cGvHD. Based on donor genotypes, no significant association was observed between rs2431697 in miR-146a (2) and any HSCT outcome.

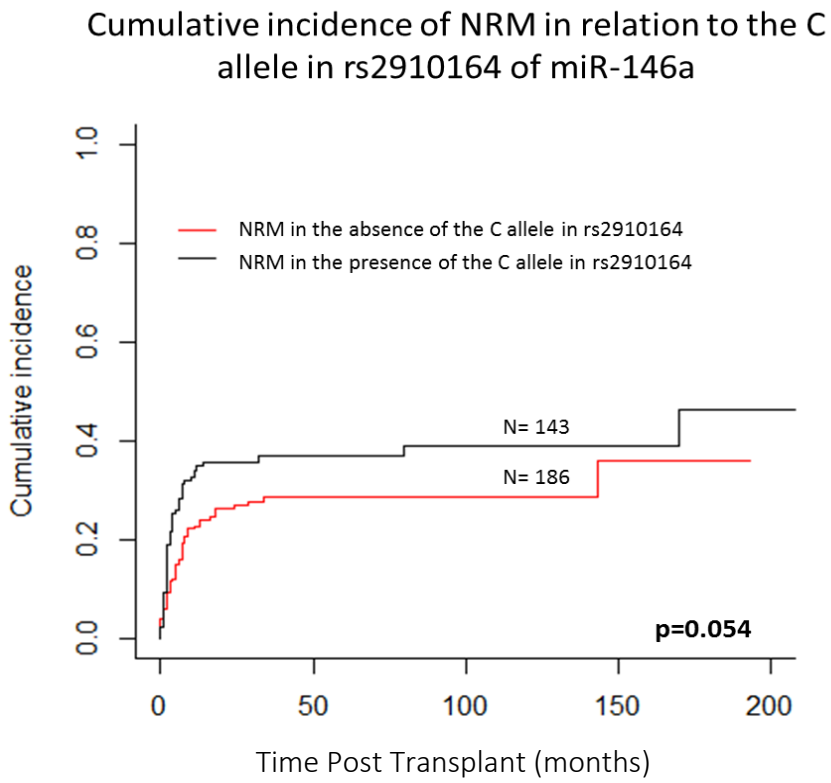


Figure 3.3 Association between the C allele in rs2910194 of miR-146a and non-relapse mortality. In patients, statistical analysis using Grey's Test showed that the presence of the C allele (GC or CC genotypes) had a border line significance for its association with NRM. Patients who carried at least one copy of the C allele showed an increased NRM over time post-HSCT.

3.3.1.3 Association between rs3027898 in IRAK1 and HSCT outcome

Statistical analysis for investigation of the impact of rs3027898 in IRAK1 on relapse and NRM were performed on the complete study cohort. This revealed that in patients who suffered relapse, presence of at least one copy of the C allele in rs3027898 was significantly associated with a decreased risk of relapse (p=0.035) (Figure 3.5 A). When carrying two copies of this allele, heterozygous patients showed an even improved outcome with even lower level of relapse (p=0.001) (Figure 3.5 B).

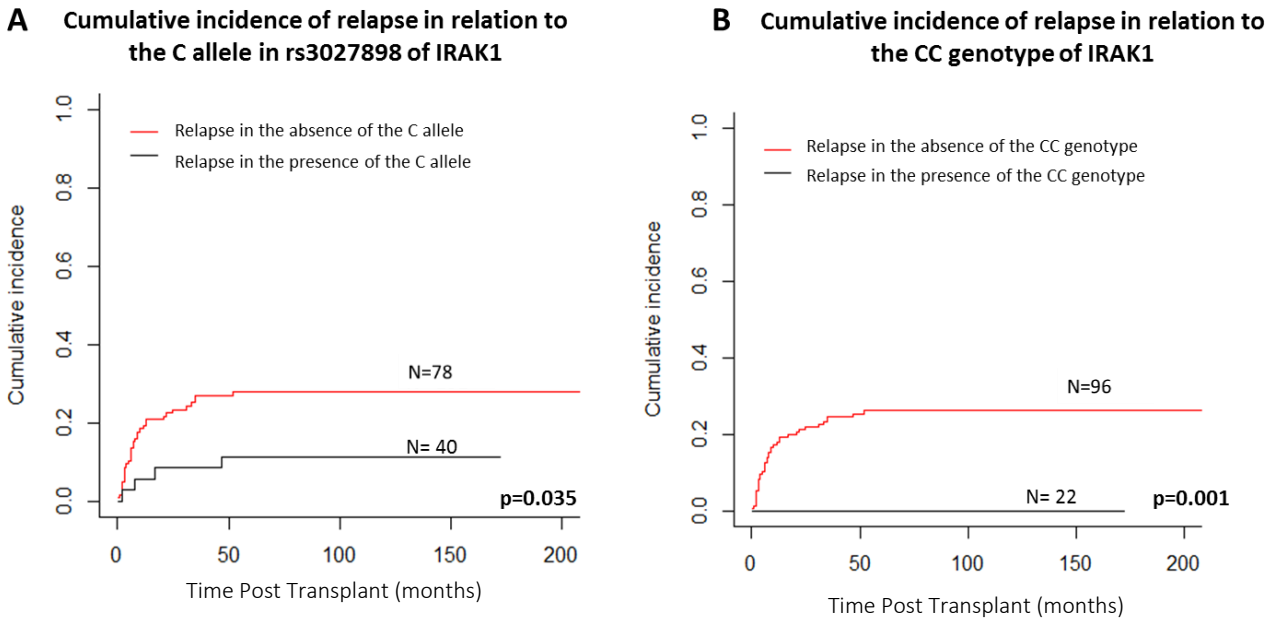


Figure 3.4 Association between rs3027898 of IRAK1 and relapse. In patients, statistical analysis for the association between rs3027898 and relapse, using Grey's Test, revealed that: (A) the presence of the C allele (GC or CC genotypes) in IRAK1 was significantly associated with lower incidence of relapse in patients (p=0.035). (B) Patients who carried the CC genotype had no relapse (p=0.001)

Conversely, in patients, the CC genotype of IRAK1 was significantly associated with an increased NRM (p=0.020) (Figure 3.6). This outcome strengthens the previous results as it shows that in patients, there was an increased risk of mortality which was not due to relapse, and indeed, Figure 3.5 shows the CC genotype was associated with less relapse.

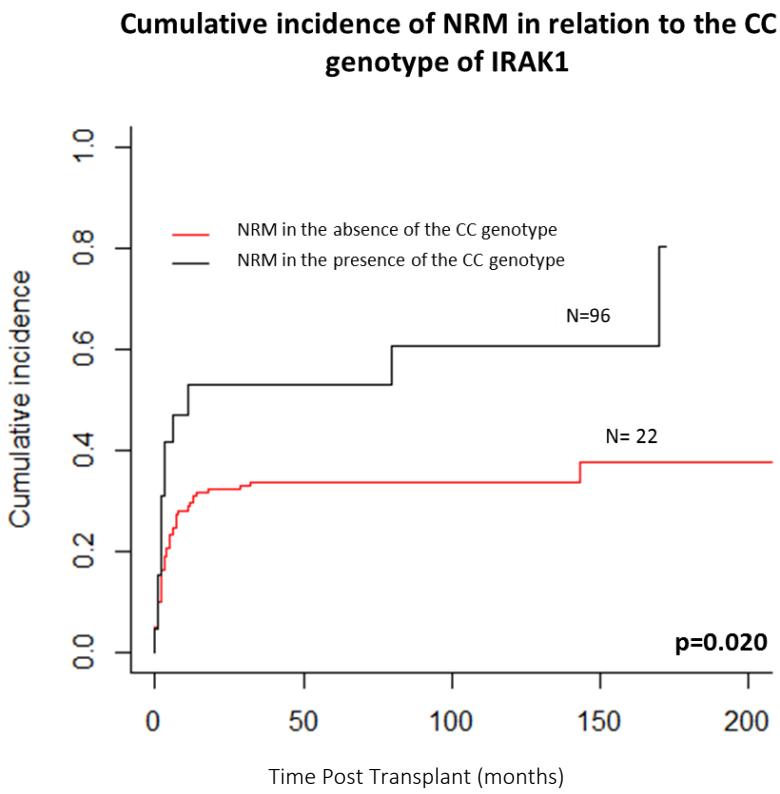


Figure 3.5 Association between the CC genotype of IRAK1 and NRM. In patients, Grey's Test analysis showed that the presence of CC genotype of IRAK1 was significantly associated with an increased NRM (p=0.020).

There was no significant association between rs3027898 in IRAK1 and OVS, aGvHD or cGvHD post-HSCT in patients. No significant association between rs3027898 in IRAK1 and any HSCT outcome was observed for donor genotypes.

Since IRAK1 is located on chromosome X, analysis was also performed based on the gender of the patients (females in comparison to males). Statistical analysis showed that in female patients, no significant association was observed between carrying the C allele in rs3027898 in IRAK1and HSCT outcome. In male patients however, Grey's test showed that there was a significant association between carrying the C allele in rs3027898 and a reduced risk of relapse post HSCT (p=0.005) (Figure 3.6).

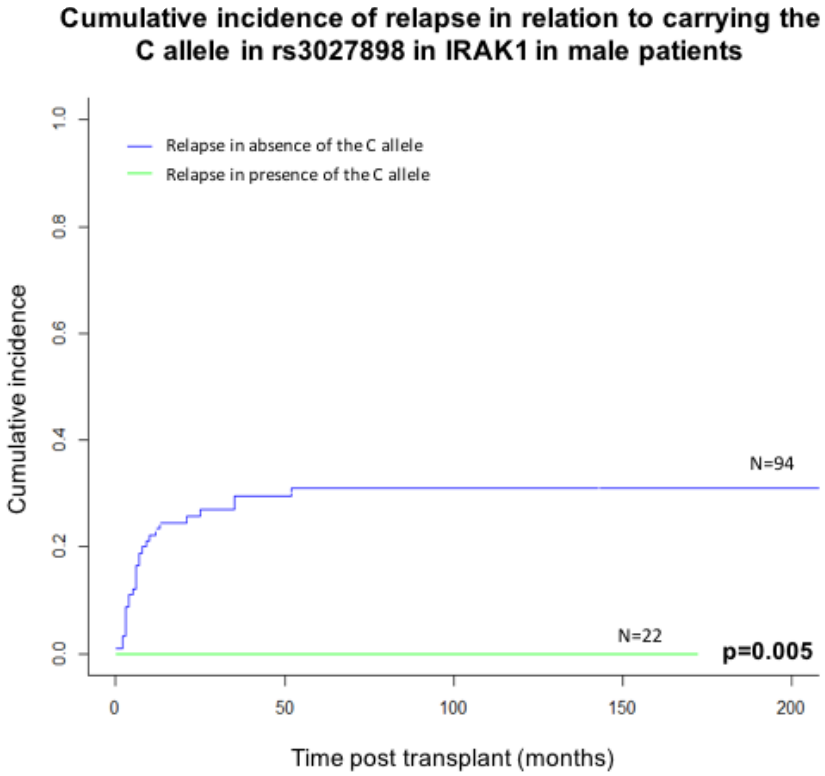


Figure 3.6 Association between carrying the C allele is rs3027898 in IRAK1 within male patients and the risk of relapse after HSCT. In male patients, Grey's Test analysis showed that the presence of C allele in rs3027898 was significantly associated with a lower incidence of relapse after HCST (p=0.005).

Analysis performed using Grey's test showed that in the same sub-cohort of male patients, carrying the C allele in rs3027898 in IRAK1 was significantly associated with an increased risk of NRM after HSCT (p=0.017) (Figure 3.7).

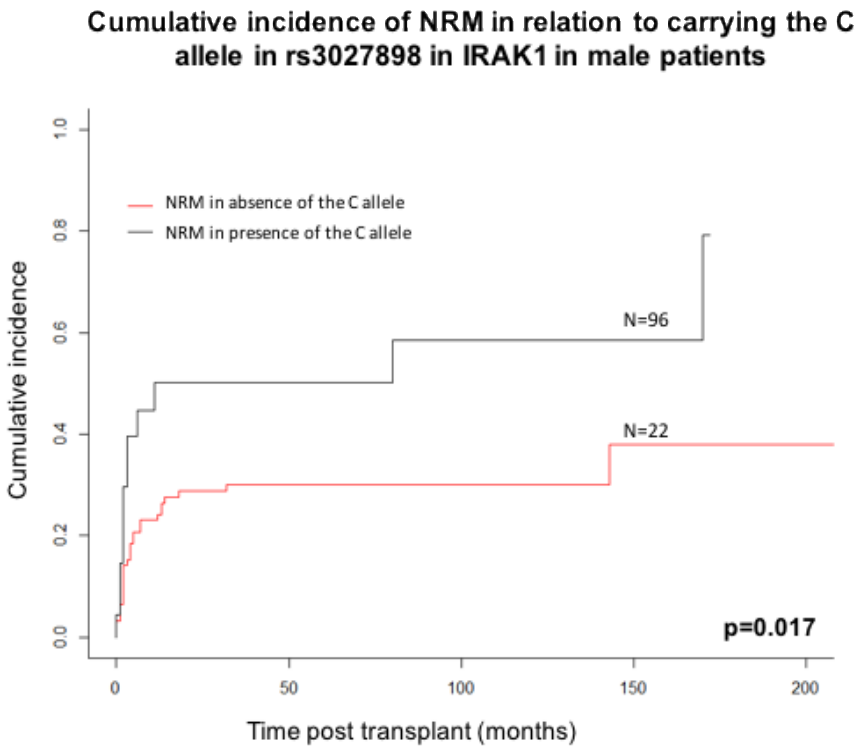


Figure 3.7 Association between carrying the C allele is rs3027898 in IRAK1 within male patients and the risk of NRM after HSCT. In male patients, Grey's Test analysis showed that the presence of C allele in rs3027898 was significantly associated with an increased risk of NRM after HCST (p=0.017).

3.3.1.4 Association between rs1051792 in MICA and HSCT outcome

For rs1051795 in MICA, Grey's test analysis showed in patients, the presence of the MICA-129 Met allele was significantly associated with an increased risk of relapse (p=0.028) (Figure 3.8).

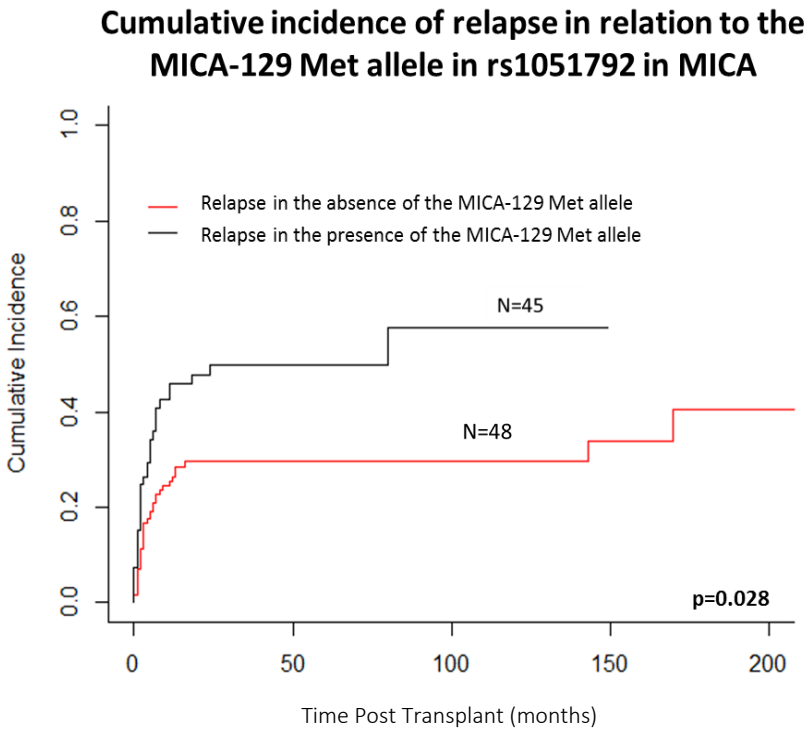


Figure 3.8 Association between the MICA-129 Met allele and relapse of patients port HSCT. In patients, Grey's test analysis showed that the presence of MICA-129 Met allele was significantly associated with an increased risk relapse (p=0.028).

There was, no significant association between rs1051795 in MICA and NRM of patients post HSCT.

For the association with OVS, survival analyses were performed using the Kaplan-Meier estimator. Results showed that the strong NKG2D binder, MICA-129Met allele, was significantly associated with a decreased OVS post-HSCT (p=0.041) (Figure 3.9 A). Carriage of the MICA-129 Met allele in the donors was also associated with reduced OVS (p=0.019) (Figure 3.9 B).

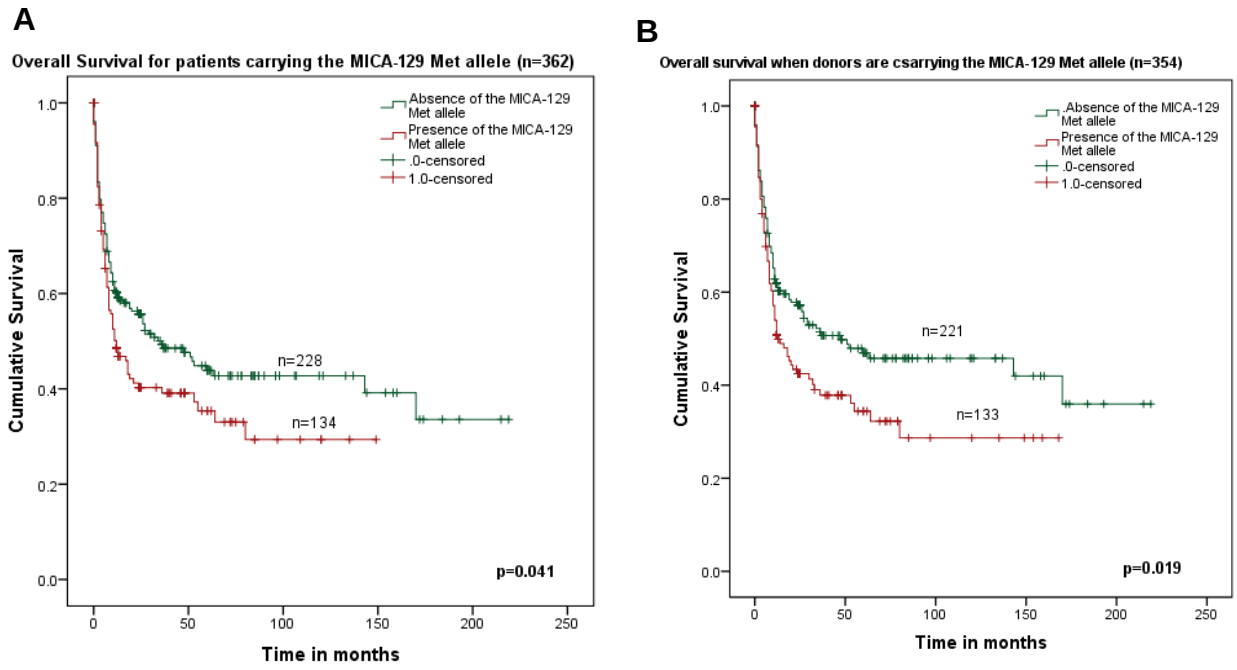


Figure 3.9 Association between MICA-129 Met allele and OVS post HSCT. Survival analysis were performed using the Kaplan-Meier test. (A) Analysis showed that patient carrying the MICA-129 Met allele had a lower OVS (p=0.041). (B) Presence of the MICA-129 Met allele is donors was significantly associated with a decreased OVS post-HSCT (p=0.019).

The impact of MICA-129 Met allele on OVS in patient was significantly affected by the TCD treatment. Stratification of the data based on T cell depletion treatment showed that in the group of patients who received a T cell depleted allo-graft, no significance was observed between MICA-129 Met and OVS (Figure 3.10 A). When taking into consideration the group of patients who received non-TCD treatment, it was revealed that there was significant decrease in the OVS post-HSCT for patients carrying the MICA-129 Met allele (Figure 3.10 B).

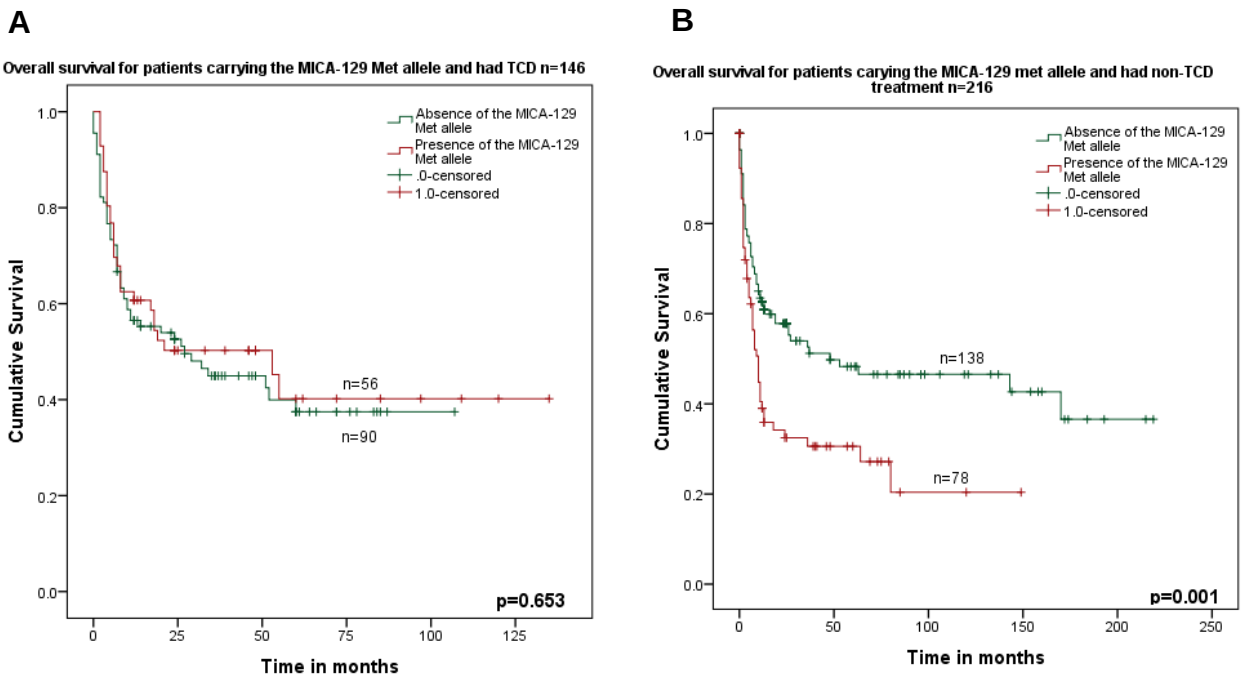


Figure 3.10 Comparison between the effects of MICA-129 Met allele on the OVS in patients who received a TCD treatment vs. patients who did not. (A) Survival analysis showed that patient in the case of the patient who received TCD, no significant association between carrying the MICA-129 Met allele and OVS was observed. (B) In the case of patients who received a non-TCD treatment, carriage of the MICA-129 Met was significantly associated with an increased OVS (p=0.001).

The presence of the MICA-129 Met allele, was shown to be responsible for the decrease of the overall survival in patients (p=0.004) (Figure 3.11).

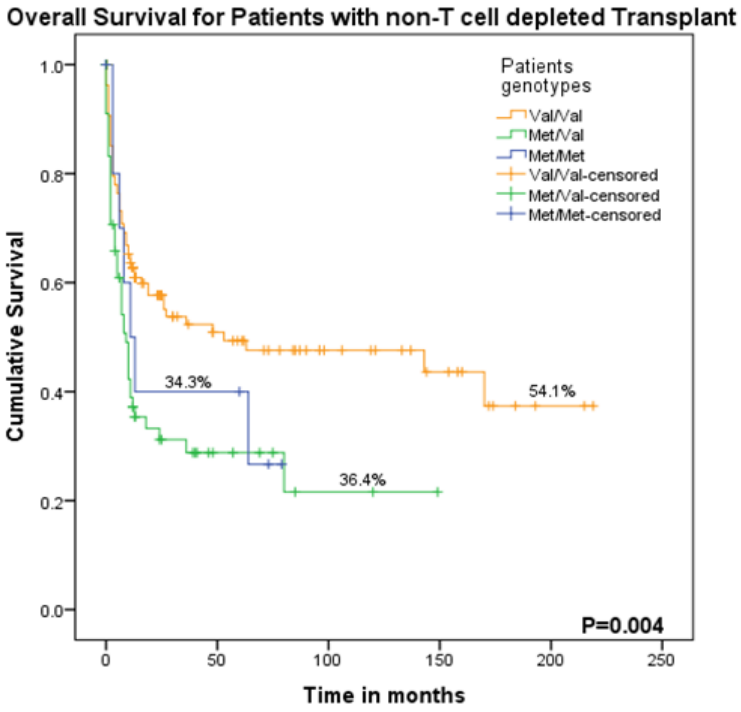


Figure 3.11 Comparison of the effect of different genotypes on the OVS for patients treated with a non-T cell depleted transplant. The major decrease of the OVS in patients was mainly caused by the carriage of the Met/Met genotype (p=0.004).

Cox regression analysis for the association of MICA-129 Val with aGvHD showed that patients receiving non-TCD treatment and carrying the Val allele had a higher risk of developing aGvHD (grade 2-4) (p=0.044). No significant association was observed between rs1051795 in MICA and aGvHD or cGvHD in patients receiving TCD treatment. No association between the MICA-129 Val and HSCT outcome in donors.

3.3.2 Validation cohort results

In order to confirm results of the SNP genotyping analysis, all results were considered for a validation study. A validation cohort of n=576 patient and donor pairs were recruited for this purpose. DNA samples were collected from 5 Transplantation centres including France, Prague, Vienna, Munich and Regensburg. Sample collection and usage was all covered by the CellEurope Project ethics (See Chapter 2, section 2.1.1) (ethic approval is attached as Appendix).

All the frequencies for the various genotypes of patients and donors along with all the clinical characteristics for the validation cohort are stated in Table 3.3.

The described frequencies are in coherence with the standard Caucasian genotype frequencies and obey the Hardy–Weinberg principle (HWP) (The HapMap project, http://hapmap.ncbi.nlm.nih.gov/; the 1000genome, www.1000genomes.org/) (Table 3.3)

Table 3.3 Clinical characteristics of the validation cohort (n=576)

Characteristics		**N (%)**
Patients	Age range (years)	10-67
	Female	226 (39.2)
	Male	350 (60.8)
Donors	Age range (years)	11-74
	Female	200 (34.7)
	Male	352 (61.1)
	Missing	20 (4.1)
Underlying disease	Acute Myeloid Leukaemia	82 (14.2)
	Acute Lymphoblastic Leukaemia	41 (7.1)
	Chronic Myeloid Leukaemia	28 (4.9)
	Non-Hodgkin's lymphoma	11 (1.9)
	Hodgkin's Disease	2 (0.3)
	Other diagnoses	48 (8.33)
	Missing	344 (59.7)
Adult		205 (35.6)
Relationship	Haploidentical	17 (3)
	Matched unrelated donors	115 (20)
	Siblings	91 (15.8)
	Missing	342 (61.28)
T cell depletion		45 (7.8)
Reduced Intensity conditioning		93 (16.1)
Female to Male ratio	Valid	104 (18.1)
	Missing	17 (3)
Source of transplant	Bone Marrow	93 (16.14)
	Cord blood	5 (0.9)
	PBMCs	126 (21.9)
	Missing	352 (61.11)
Acute GvHD	Grade 0	69 (12)
	Grade 1	51 (8.9)
	Grade 2	62 (10.8)
	Grade 3	19 (3.3)
	Grade 4	18 (3.1)
	Missing	357 (62)
Chronic GvHD	Valid (all grades)	71 (12.32)
	Missing	440 (76.4)
Relapse	Yes	67 (11.6)
	No	161 (28)
	Missing	348 (60.4)
Deceased	Yes	135 (23.4)
	No	98 (17)
	Missing	343 (59.5)

The genotype frequencies of rs2910164 and rs2431697 in miR146a, rs3027898 in IRAK1 and rs1051792 in MICA for the patients and the donors within the validation cohort are described in Table 3.4.

Table 3.4 Genotype frequencies for the SNPs on interest for the patients and donors within the validation cohort

SNP of interest	Genotype	N (%)
Patient genotypes for rs2910164 in miR-146a	GG	299 (51.9)
	GC	171 (29.7)
	CC	30 (5.2)
	Missing	76 (13.19)
Donor genotypes for rs2910164 in miR-146a	GG	301 (52.3)
	GC	191 (33.2)
	CC	30 (5.2)
	Missing	54 (9.4)
Patient genotypes for rs2431697 miR-146a	TT	152 (26.4)
	TC	243 (42.2)
	CC	90 (15.6)
	Missing	91 (15.8)
Donor genotypes for rs2431697 miR-146a	TT	159 (27.6)
	TC	255 (44.3)
	CC	90 (15.6)
	Missing	72 (12.5)
Patient genotypes for rs3027898 in IRAK1	AA	357 (62)
	CA	61 (10.6)
	CC	77 (13.4)
	Missing	81 (14.06)
Donor genotypes for rs3027898 in IRAK1	AA	371 (64.4)
	CA	66 (11.5)
	CC	82 (14.2)
	Missing	57 (9.89)
Patient genotypes for rs1051792 in MICA	AA	57 (9.9)
	GA	195 (33.9)
	GG	228 (39.6)
	Missing	96 (16.66)
Donor genotypes for rs1051792 in MICA	AA	43 (5.9)
	GA	221 (30.6)
	GG	351 (48.5)
	Missing	108 (14.93)

Statistical analysis for investigation of the association between rs2910164 and rs2431697 in miR146a, rs3027898 in IRAK1 and rs1051792 in MICA with HSCT outcome was performed in the same manner as per the study cohort. Association analysis were performed for relapse, NRM, OVS, aGvHD and cGvHD.

Comparison between the study cohort (n=817), containing samples collected from patients recruited at Newcastle and Regensburg, and the validation cohort (n=576), containing samples collected from patients recruited at Vienna, Paris, Prague, Munich and Regensburg, is shown in Table 3.5. There was no significant difference between the two cohorts except for the T cell depletion treatment, where data showed that there were more patients who underwent a T cell depletion treatment in the study cohort compared to the validation cohort (Table 3.5).

Table 3.5 Comparison between the study and the validation cohort

		Study cohort (n=817)	Validation cohort (n=576)	p val*
		n (%)	n (%)	
Gender	Female Patients	309 (37.8)	226 (39.2)	0.544
	Male patients	508 (62.2)	350 (60.8)	
	Female donors	251 (30.7)	200 (34.7)	0.898
	Male donors	509 (62.3)	352 (61.1)	
Age (years)	Patients	10-67	10-67	0.133
	Donors	10-74	11-74	0.305
Underlying disease	Acute Myeloid Leukemia	139 (17.0)	82 (14.2)	0.356
	Acute Lymphoblastic Leukimea	52 (6.4)	41 (7.1)	
	Chronic Myeloid Leukemia	44 (5.4)	28 (4.9)	
	Non-Hodgkin's Disease	43 (5.3)	11 (1.9)	
	Hodgkin's Disease	17 (2.10	2 (0.3)	
	Other diagnosis	69 (8.44)	48 (8.33)	
Adult		408 (50.1)	205 (35.6)	0.104
Relationship	Haploidentical	1 (0.1)	17 (3)	0.091
	Matched Unrelated donors	200 (24.5)	115 (20)	
	Siblings	215 (26.3)	91 (15.8)	
T cell depletion		164 (20.1)	45 (7.8)	**0.001**
Reduced intensity conditionning		251 (30.70	93 (16.1)	0.102
Acute GvHD		389 (47.61)	219 (38.80)	0.660
Chronic GvHD		148 (18.11)	71 (12.32)	0.051
Relapse		123 (15.1)	67 (11.63)	0.157

* For contious data an indenpendent samples t-test was perfomred while a Chi-squared test was perfomed in the case of categorical data

3.3.2.1 Association between rs2910164 and rs2431697 in miR-146a and HSCT outcome in the validation cohort.

Statistical analysis was performed using Grey's test. In the validation cohort, a trend was observed between the presence of the C allele in rs2910164 in patients and an increased NRM (p=0.06) (Figure 3.12 A). A trend was also observed between carrying the T allele in rs2431697 and an increased NRM (p=0.08) (Figure 3.12 B).

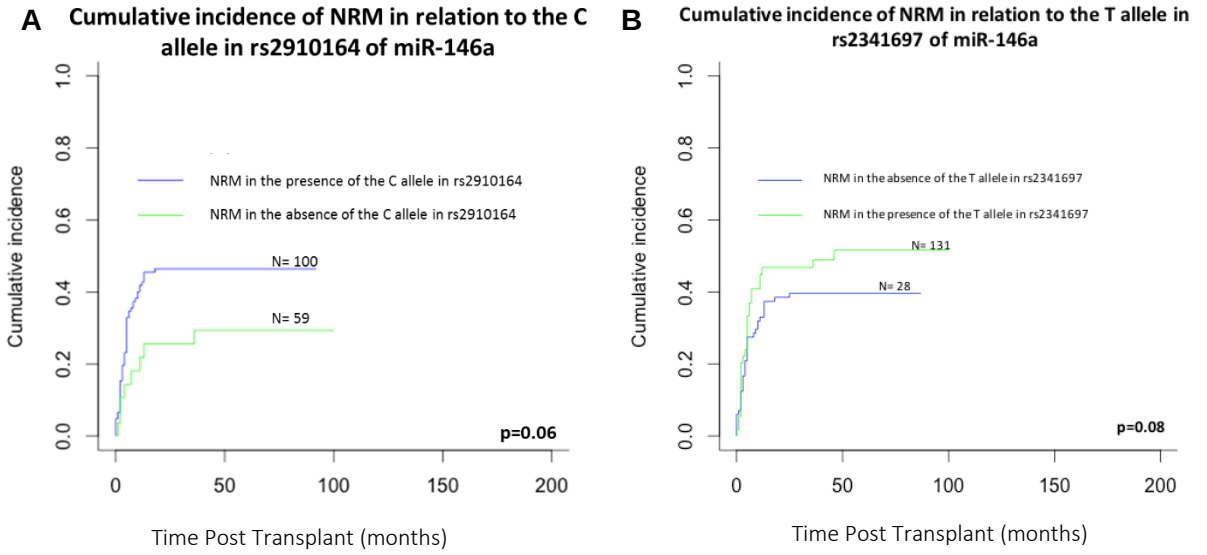


Figure 3.12 Association between rs2910164 and rs2341697 in miR-146a and HSCT outcome. Statistical analysis was performed using the competing risk on R (v2.15.0). (A) a trend (p=0.06) was observed between carrying the C allele in rs2910164 and an increased NRM in patients post HSCT. (B) A trend was also observed between carrying the T allele in rs2341697 and an increased NRM in patients post HSCT.

No significant association was observed between rs2910164 and rs2341697 in miR-146a and the incidence of relapse, OVS, aGvHD or cGvHD in patients post HSCT. In donors, no significant association was observed between rs2910164 and rs2341697 in miR-146a and HSCT outcome.

3.3.2.2 Association between rs3027898 in IRAK1 and HSCT outcome in the validation cohort.

Statistical analysis for investigation of the association between rs3027898 in IRAK1, relapse and NRM in the complete cohort were performed using Grey's test.
Results revealed that, in patients, the presence of the C allele in rs3027898 was significantly associated with a decreased risk of relapse (p=0.007) (Figure 3.13).

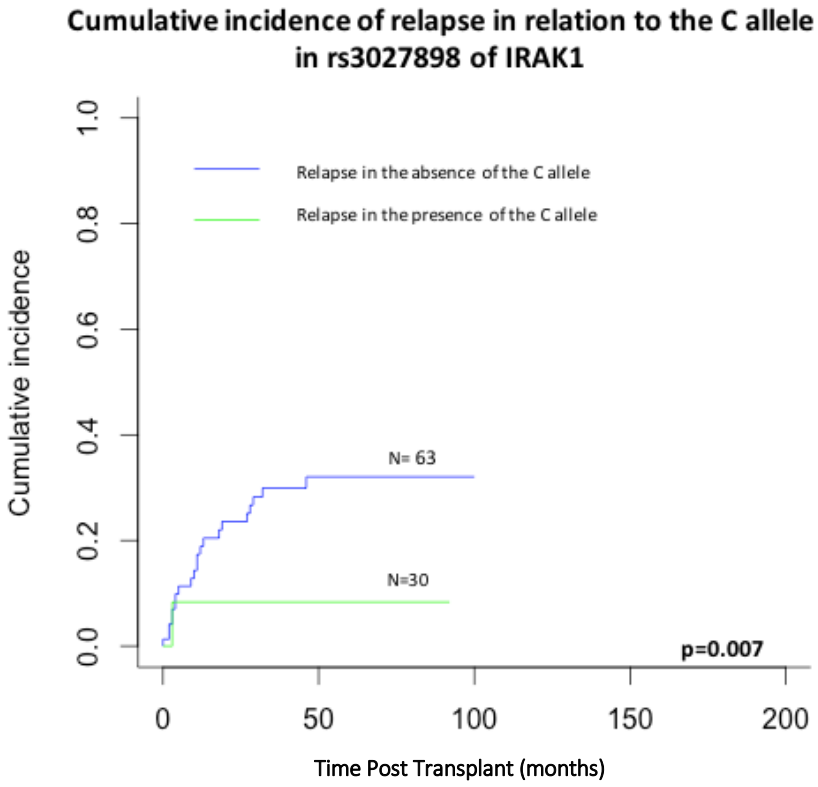


Figure 3.13 Association between rs3027898 in IRAK1 and the incidence of relapse post HSCT. Statistical analysis using Grey's test (R v 2.15.0, cmprsk package) showed that patients carrying the C allele had lower risk of relapse post HSCT (p=0.007).

Unlike the discovery cohort, there was no significant association between rs3027898 in IRAK1 and NRM in the validation cohort. No significant association was also observed between rs3027898 in IRAK1 and aGvHD or cGvHD. No significance was observed between rs3027898 in IRAK1 and HSCT outcome in donors.
Statistical analysis was also performed after stratification of the validation cohort based on the gender of the patients. Within female patients, no significant association was

observed between carrying the C allele in rs3027898 and HSCT outcome and thus confirming the finding of the study cohort.

Within male patients, statistical analysis using Grey's test showed that was trend towards a lower incidence of relapse when patients carried the C allele in rs3027898 (p=0.092) (Figure 3.14).

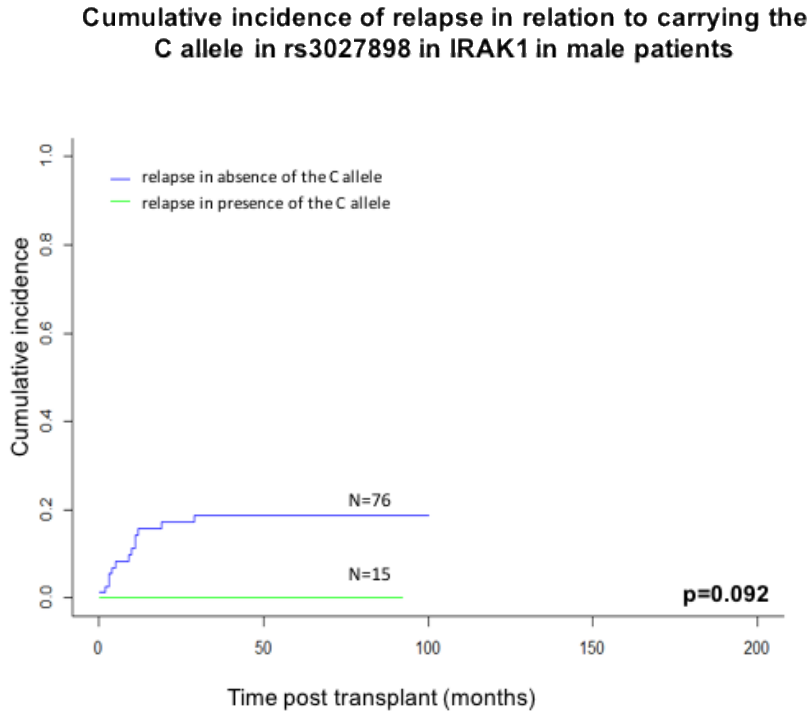


Figure 3.14 Association between carrying the C allele in rs3027898 within male patients and the incidence of relapse post HSCT. Statistical analysis using Grey's test showed that patients carrying the C allele showed a trend towards a lower risk of relapse post HSCT (p=0.092).

No significant association was observed between carrying the C allele in rs3027898 in IRAK1 within male patients and NRM post HSCT.

3.3.2.3 Association between rs1051792 in MICA and HSCT outcome in the validation cohort.

Investigation for the association between rs1051792 in MICA and relapse and NRM were performed using R (v 2.15.0). For the association between rs1051792 in MICA and OVS, aGvHD and cGvHD SPSS (v21, IBM Analytics) was utilized.

Grey's test analysis revealed that the presence of the MICA-129 allele in patients was associated with an increased risk of relapse (p=0.046) (Figure 3.15).

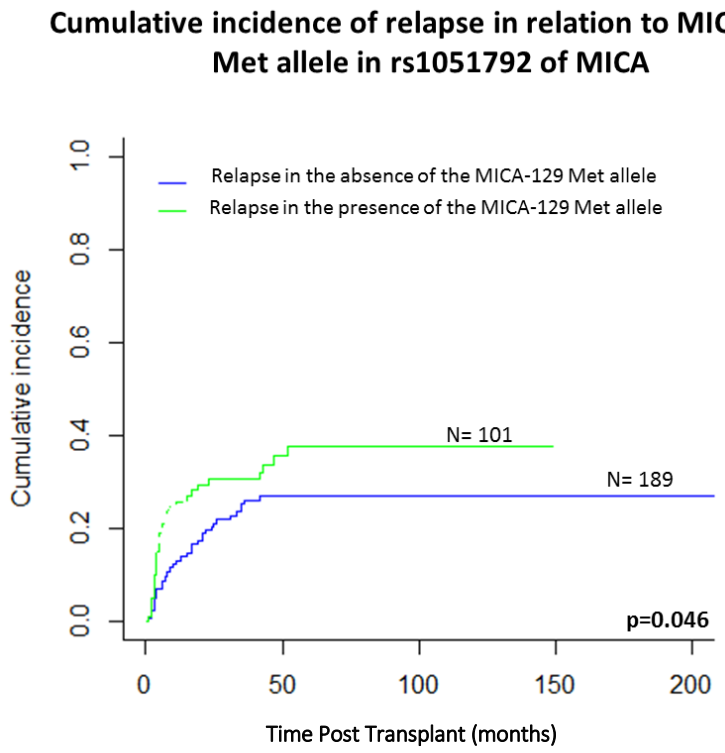


Figure 3.15 Association between the presence of MICA-129 Met and the incidence of relapse. Statistical analysis showed that patients carrying the MICA-129 allele was associated with an increased risk of relapse post HSCT (p=0.046).

There was no significant association between NRM and MICA-129 Met allele in the complete validation cohort.

Stratification of the data based on TCD treatment showed that patients receiving a non T cell depleted allo-graft and carrying the MICA-129 Met allele had significantly increased NRM (p=0.018) (Figure 3.16).

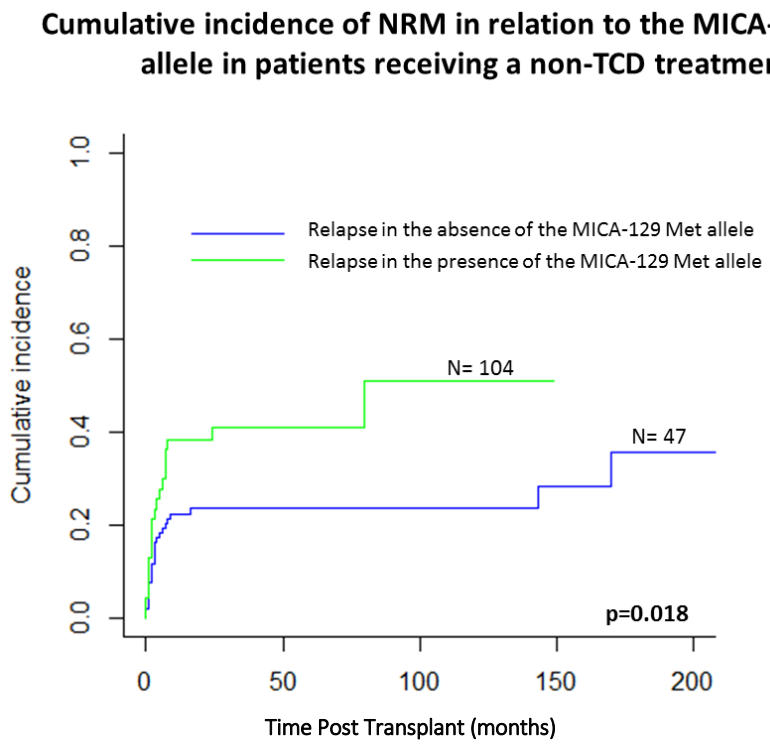


Figure 3.16 Association between presence of MICA-129 Met and NRM in patients post HSCT. Grey's test analysis showed that patients carrying the MICA-129 allele in patients who received non-TCD treatment had an increased risk of NRM post-HSCT (p=0.018).

Survival analysis using the Kaplan-Meier estimator (SPSS v 21, IBM Analytics) showed that there was a significant association with carriage of the MICA-129 Met allele and decreased OVS in the group of patients receiving a non-TCD transplant (p=0.027) (Figure 3.17 A). No significance was observed when the complete cohort was considered for the analysis.

In the same validation sub-group (patients receiving non-T cell depleted allo-grafts), there was a trend towards a decreased OVS when donors carried the MICA-129 Met allele (p=0.058) (Figure 3.17 B).

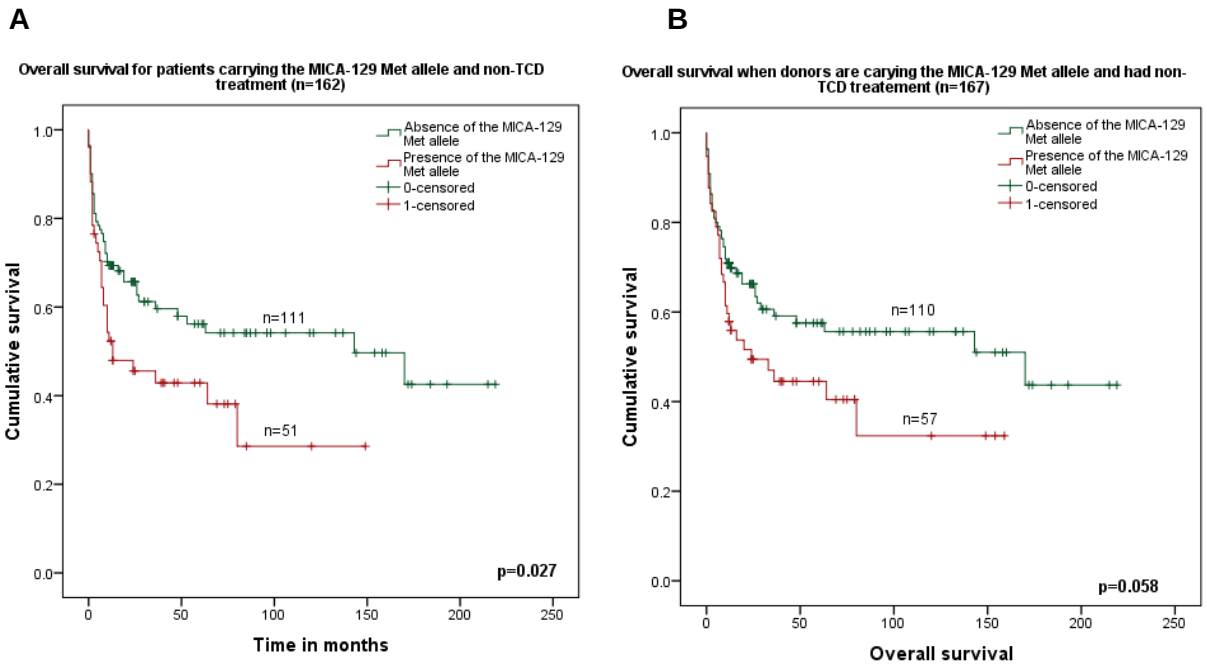


Figure 3.17 Association between MICA-129 Met allele and HSCT outcome in the group of patients receiving a non-TCD allo-grafts. Survival analysis were performed using the Kaplan-Meier estimator (SPSS v 21, IBM Analytics). (A) There was significant association between the presence of the MICA-129 Met allele and a decreased OVS post HSCT. (2) When donors carried the MICA-129 Met allele, a trend towards a decreased OVS was observed in patients post-HSCT.

There was no significant association between rs1051792 in MICA and the incidence of aGvHD or cGvHD in patients post-HSCT.

Summary of results from both the study and the validation cohort is showed in Table 3.5.

Table 3.5. Summary of results for patients from both the study and the validation cohort. Only allele that were significant associated with HSCT outcome are shown in this table.

	Study cohort (n=817)				Study cohort (n= 576)			
	aGvHD	**relpase**	**OVS**	**NRM**	**aGvHD**	**relpase**	**OVS**	**NRM**
miR-146a rs2910164 C allele	×	×	×	↑	×	×	×	↑
miR-146a rs2431697	×	×	×	×	×	×	×	×
IRAK1 rs3027898 C allele	×	↓	×	↑	×	↓	×	×
MICA rs1051795 Met allele	×	↑	↑	×	×	↑	↑	↑
MICA rs1051795 Val allele	↑	×	×	×	×	×	×	×

↑ The specific HSCT outcome was singnificantly uprefulated in the presence of the allele

↓ The specific HSCT outcome was singnificantly downregulated in the presence of the allele

× No significant association was observed between the specific HSCT outcome and the allele

3.4 Discussion

Acute GVHD occurs in 30% to 75% of allo-HSCT recipients and is associated with significant morbidity and mortality, representing a major barrier toward the wider and safer application of this potentially curative approach to hematologic malignancies (Ferrara *et al.*, 2009). aGVHD develops when allogeneic donor T cells destroy HLA-mismatched host tissues by secreting inflammatory cytokines (IL-1, TNF-α, and IFN-γ) and/or inducing a direct cytotoxic cellular response (Ferrara *et al.*, 2009; Socié and Blazar, 2009). Recent studies indicate that miRNAs play critical roles in the development and function of the immune system (Haasch *et al.*, 2002; Rodriguez *et al.*, 2007; Thai *et al.*, 2007; Xiao *et al.*, 2008; Banerjee *et al.*, 2010). In particular, miR-146a is essential for Treg function (Lu *et al.*, 2010). TRAF6 and IRAK1 are validated miR-146a targets and their expression is regulated by a negative feedback-loop via the TLR-4 signaling pathway and NFκB activation (Taganov *et al.*, 2006a; Taganov *et al.*, 2007). IFN regulatory factor 5 (IRF5) and signal transducer and activator of transcription 1 (STAT1) are also known targets of miR-146a (Tang *et al.*, 2009). MiR-146a negatively regulates signal transduction pathways leading to NF-κB activation. Upon activation of a cell surface receptor such as TLR4, a molecular cascade including TRAF6 and IRAK1 leads to IκBα phosphorylation and degradation and to NF-κB activation and nuclear translocation (Taganov *et al.*, 2006a; Taganov *et al.*, 2007). NF-κB activation induces transcription of many genes, including pri-miR-146a. Once translocated to the cytoplasm and loaded onto the RISC complex, mature miR-146a contributes to attenuate receptor signaling through the down modulation of IRAK1 and TRAF6.

IRAK1, one of the established miR-146a targets, is a member of the serine-threonine kinase family consisting of IRAK1, IRAK2, IRAKM and IRAK4 (Singh *et al.*, 2014). IRAK1 plays a pivotal role in the Toll/IL-1 receptor (TIR) family signaling cascade (Kanakaraj *et al.*, 1998). IRAK1 plays an important role in IL-1R/TLR signaling, although there is a small amount of NF-κB activation in its absence (Kanakaraj *et al.*, 1998; Thomas and Blume, 1999; Swantek *et al.*, 2000). Upon ligand activation of TIR family members, IRAK1 is recruited to the receptor complex (Akira and Takeda, 2004). At the receptor, IRAK1 associates with Tollip, MyD88 and TRAF6, and phosphorylation by IRAK4 triggers IRAK1 autophosphorylation (Jiang *et al.*, 2002; Li *et al.*, 2002; Lye *et al.*, 2004). IRAK1 hyperphosphorylation results in disassociation from Tollip and

release from the receptorMyD88 complex (Jiang *et al.*, 2002; Burns *et al.*, 2003). This leads to the formation of a new protein complex consisting of hyperphosphorylated IRAK1 and TRAF6, a prerequisite for TRAF6-mediated NF-B activation and induction of an inflammatory response (Kollewe C 2004).

MICA molecules interact with the NKG2D-activating receptor on human NKT cells, $CD8^+$ cytotoxic T cells, γδ T cells, and under certain conditions $CD4^+$ T cells and elicits a very powerful immune response (Champsaur and Lanier, 2010). *MICA* encodes for a polypeptide of 383 amino acids that is expressed on the cell surface and resembles the domain organization of the α chain of MHC class I molecules, however, MICA does not associate with β2-microglobulin (Groh *et al.*, 1996b; Zwirner *et al.*, 1997). MICA is not expressed by resting T or B lymphocytes but phytohemagglutinin (PHA)-activated CD4+ and CD8+ T cell blasts express MICA (Zwirner *et al.*, 1997). This expression can also be triggered by stimulation with allogeneic PBMCs, and involves TCR/CD3 engagement and co-stimulation through CD28 (Molinero *et al.*, 2002a), involving different cytoplasmic mediators and NF-κB (Molinero *et al.*, 2003).

The main focus of this study was to investigate the association between rs2910164 and rs2431697 in miR-146a, rs3027898 in IRAK1, rs10511792 in MICA with HSCT outcome. rs2910164 in miR-146a concerns a G>C nucleotide substitution which results in a change from a G:U pair to a C:U mismatch in the stem region of miR-146a precursor (Jazdzewski *et al.*, 2008). rs2431697 is a C>T located on 5q33.3 (SNPedia). rs3027898 in IRAK1 and concerns an A > C transition in the 3'-UTR. rs1051792 is an SNP at position 454 (A→G, rs1051792) of *MICA* leading to an amino acid substitution of methionine by valine (Met→Val) at position 129 in the α2 domain of the MICA protein, categorizing the MICA alleles into strong (MICA-129 Met) and weak binders (MICA-129 Val) binders of NKG2D (Groh *et al.*, 1999a).

Investigation of the association between rs2910164 in miR-146a and HSCT outcome in n=817 patient donor pairs showed that there was a borderline significant association between carriage of the 'C' allele and increased NRM in patients post-HSCT (p=0.054). The finding was observed again in the validation cohort (n=576 patient and donor pairs), where a trend towards an increased NRM was observed for patients carrying the C allele (p=0.06). Such outcome was expected for the C allele of rs2910164. Indeed, in a study by Stickel *et al.,* it was demonstrated that the CC genotype within rs2910164, was linked with a higher risk for severe GvHD (grade III-IV) in allo-HSCT recipients

(Stickel *et al.*, 2014). The same study also revealed a trend toward overall higher GvHD severity in patients when the HSCT donor carried the CC genotype (Stickel *et al.*, 2014). It is well known that development of aGvHD after HSCT is associated with relevant morbidity and mortality and represents the most common cause of long-term NRM (Socié *et al.*, 1999). However, the presence of GvHD also decreases disease relapse and it could improve post-transplant outcome, depending on its severity and the success of a graft-vs-malignancy effect (Weiden *et al.*, 1981; Sullivan *et al.*, 1989; Horowitz *et al.*, 1990; Baron *et al.*, 2005). Since 63.37% of the patients recruited for the study cohort experienced aGvHD and since aGvHD has been previously reported to be responsible for higher NRM and therefore not always associated with improved progression-free survival (Kanda *et al.*, 2004; Baron *et al.*, 2005; Valcárcel *et al.*, 2008; Ringden *et al.*, 2012) along with the observation reported by Stickel *et al.,* in relation to the C allele of rs2910164, are all in agreement with the outcome reported in this study, whereby patients carrying the C allele had an increased NRM. Consistent with this findings, an anti-inflammatory role for miR-146a was shown by several SNP studies in which SNPs that lower miR-146a expression were associated with disease activity in the case of Crohn's disease (Gazouli *et al.*, 2013), gastric cancer (Wei *et al.*, 2015; He *et al.*, 2016; Xia *et al.*, 2016), breast cancer (Bansal *et al.*, 2014; Upadhyaya *et al.*, 2016), rheumatoid arthritis (Amal *et al.*, 2013; Zhou *et al.*, 2015) and prostate cancer (Nikolić *et al.*, 2014).

For rs2431697 in miR-146a, no significant association with the outcome of HSCT was observed in the study cohort. In the validation cohort (n=576), the presence of the T allele was associated with an increased NRM post-HSCT. This is the first study to relate rs2431697 to HSCT outcome. The different outcome between the study and the validation cohort may be due to the fact that different approaches of conditioning regimens and GvHD prophylaxis are practiced at different transplantation centres. There was a significant difference between the study and the validation cohort when regarding the TCD treatment (p=0.001). This shows that there is a different approach to treat GvHD patients between both cohorts along with different HSCT outcome. rs2431697 in miR-146a was reported in many studies as related to the susceptibility to conditions such as psoriasis (Yang *et al.*, 2013), systemic lupus erythematosus (Löfgren *et al.*, 2012; Park *et al.*, 2016) and ankylosing spondylitis, (Park *et al.*, 2016).

Polymorphisms affecting miRNA expression, maturation, or mRNA recognition may represent an important risk determinant of disease susceptibility (Li *et al.*, 2015). Thus,

it is important to test whether miR-146a gene polymorphisms, rs2910164 and rs2431697, act as modifiers of GvHD course or affect other HSCT outcome. In fact, the C allele of the rs2910164 polymorphism of miR-146a was shown to cause miss-pairing within the miR-146a hairpin, decreased expression of its mature form, and declined expression of its target genes, TRAF6 and IRAK1 (Su *et al.*, 2011). It was previously demonstrated that the C allele in rs2910164 was significantly associated with increased expression of miR-146a in patients with multiple sclerosis (Li *et al.*, 2015). Several studies have examined the association between the miR-146a rs2910164 polymorphism and autoimmune diseases, including RA (Jiménez-Morales *et al.*, 2012), systemic lupus erythematosus (Jiménez-Morales *et al.*, 2012; Lofgren *et al.*, 2012) and multiple sclerosis (Fenoglio *et al.*, 2011). Although previous studies have suggested that many autoimmune diseases share common predisposing factors, the results are inconsistent (Chen *et al.*, 2013).

For rs3027898 in IRAK1, this study showed in n=817 patients and donor pairs, that the C allele was associated with a decreased risk of relapse in patients (p=0.035) which was more apparent when patients were homozygous for the C allele or carried the CC genotype (p=0.001). This outcome was validated in n=576 patient and donor pairs where the C allele was shown to be associated with a reduced risk of relapse post-HSCT (p=0.007). An increased risk of NRM was also observed when patients carried the C allele (p=0.020). However, this outcome was not replicated in the validation cohort. This could be due to the difference between the study and the validation cohort. Since rs3027898 in IRAK1 in located on chromosome X, analysis based on the gender of the patients showed that significance observed when taking in to consideration the full cohort was driven by the males carrying the C allele in rs3027898 in both the study and the validation cohort when in the case of NRM and relapse.

The present study is also the first to relate the C allele of rs3027898 in IRAK1 to HSCT outcome. There is an increasing body of data to suggest that IRAK1 signaling may be important to the development and progression of cancer (Kutikhin and Yuzhalin, 2015), IRAK1 activation may also be important for cross talk between cancer cells and other cell populations present in the tumour microenvironment (Jain *et al.*, 2015). IL-1β release by lingual squamous cell carcinomas causes upregulation of the IL-1R and increased levels of p-IRAK1 in cancer associated fibroblasts. This results in nuclear

translocation of NF-κB and induction of genes important for tumour progression including IL-6, Cox-2, BDNF, and IRF-1 (Dudás *et al.*, 2011; Jain *et al.*, 2015).

IRAK1 plays an important role during inflammation and thus may play an important role in the pathophysiology of GvHD. It has been shown to promote Th17 development by mediating IL-1β-induced upregulation of IL-23R and subsequent STAT3 phosphorylation, thus enabling sustained IL-17 production (Heiseke *et al.*, 2015). Moreover, it was shown that IRAK1 signaling fosters Th1 differentiation by mediating T-bet induction and counteracts regulatory T cell generation. Furthermore, in mice, IRAK1 expression in T cells was shown to be essential for T cell accumulation in the inflamed intestine and mesenteric lymph nodes (Heiseke *et al.*, 2015). Decades of basic and clinical research have demonstrated that T cells are the principal orchestrators of both GvHD and GvL, as IRAK1 was revealed to promote T cell development and cytokine production, this later may play a critical role during HSCT.

IRAK1 represents one of miR-146a targets (Chatzikyriakidou *et al.*, 2010). Indeed, miR-146a is involved in innate immunity by regulating the acute inflammatory response after pathogen (bacteria l rather than viral components) recognition by TLRs on monocytes or macrophages (Taganov *et al.*, 2006a). Pro-inflammatory cytokines such as TNF-α and IL-1β were reported to target miR-146a expression (Nakasa *et al.*, 2008). Then, miRNA-146a targets the expression of IRAK1 and TRAF6, and therefore acts as a negative regulator in the TLR and pro-inflammatory cytokine (IL-1) signaling pathway. Specifically, IRAK1 plays significant role in TLR／TIR activation of NF-κB. IRAK1 is considered as a linker of the TLR with the TRAF6 intracytoplasmic activator of transcription factor NF-κB, which subsequently increases the expression of many genes such as TNF- α and IL-8 related to immunological reactions (Dunne and O'Neill, 2003; Janssens and Beyaert, 2003). Subsequently, IRAK1 is subjected to a negative feedback by miR-146a, the expression of which is also NF-κB dependent.

This leads eventually to a concerted immunological response. This is consistent with the findings of this study, where it was revealed that rs2910164 in miR-146a was associated with an increased risk of NRM, while rs3027898 in IRAK1 was associated with a decreased risk of NRM and relapse. Although the previously mentioned studies reported the association of miR-146a and IRAK1's genotypes with the susceptibility to several diseases, little is known about the cellular and molecular mechanisms that underlie the transition from the SNP to the molecular mechanism that occurs during

the course of HSCT. Further gene expression pattern analysis of the effect of having these variants in GvHD target tissues is necessary to explain the interaction between the possession of these genotypes and HSCT outcome.

In the case of rs1051792 in MICA, this study showed that the MICA-129 Met variant was significantly associated with low OVS post-HSCT (p=0.018), which was more apparent in the group of patients receiving non-TCD treatment (p=0.001). This result was then confirmed in the validation cohort n=576 (p=0.027). This study also revealed that the presence of the MICA-129 Met allele in patients was significantly associated with an increased risk of relapse (p=0.028), which was again validated in n=576 patient and donor pairs (p=0.046). In fact, Kitcharoen K et al, demonstrated that patients who were matched for the HLA-D, HLA-Cw and for MICA had a significantly improved survival post transplantation (Kitcharoen *et al.*, 2006). MICA is only active when bound to the activating NKG2D receptor, it has been reported that the MICA-129 Met isoform is characterized by stronger NKG2D signaling, triggering more NK-cell cytotoxicity, IFN-γ release and faster co-stimulation of $CD8^+$ T cells (Nausch and Cerwenka, 2008). The MICA-NKG2D system acts as an initial defence against infections and malignant transformation (Groh *et al.*, 1996a; Groh *et al.*, 2003). It was previously reported that carriers of the MICA-129 Met/variant had an increased risk relapse (Isernhagen *et al.*, 2015a). As the presence of GvHD decreases disease relapse increases (Weiden *et al.*, 1981; Sullivan *et al.*, 1989; Horowitz *et al.*, 1990; Baron *et al.*, 2005), the previous is consistent with the observation here that the presence of MICA-129 Met variant in GvHD patients increased the risk of relapse post-HSCT. For this study, patients carrying the MICA-129 Met allele, and not receiving a T cell depleted graft had better OVS. In general, TCD techniques can be classified as *in vitro,* if the stem cell manipulation is performed exclusively *ex vivo*, normally by column adsorption. In contrast, *in vivo* techniques are based on a partial or complete depletion of donor lymphocytes in the patient after transplanting the stem cell product using ATG or the monoclonal antibody alemtuzumab (Chakrabarti *et al.*, 2004; Maeda *et al.*, 2005; Rizzieri *et al.*, 2007). In was reported by Marek et *al.,* that *in vivo* TCD caused more profound lymphocyte suppression early after HSCT (Marek *et al.*, 2014). Thus, the cohort of patients not receiving TCD treatment and carrying the MICA-129 Met variant had better NKG2D binding and thus better NK cells and cytotoxic T cell activation and proliferation and leading to improved survival post HSCT. It was also revealed in this study that the presence of the MICA-129 Val variant in patients is significantly

associated with an increased risk of developing of aGVHD post-HSCT (p=0.044). This agrees with previous findings, where it has been shown that induction of MICA contributed to tissue damage and increased the risk of aGvHD (Gannage *et al.*, 2008). Interaction between the weak binder MICA-129 Val variants and the NKG2D receptor may alter NK cell and cytotoxic T lymphocyte activation and/or co-stimulation (Kim *et al.*, 2005). The inability of MICA-129 Val allele to induce the activation of NK cells substantiates the study that showed that transplantation with higher numbers of NK-cells is inversely correlated with the occurrence of aGvHD (Kim *et al.*, 2005). Indeed, it has been previously reported that the MICA-129 Val variant was associated with a failure to activate NK cells and considered as a risk factor for early onset of nasopharyngeal carcinoma in patients predisposed to viral/environmental factors (Douik *et al.*, 2009). Since both variants of MICA are able to mediate different effects on NK cells and T cells after engaging with NKG2D, and since MICA is a stress induced molecule mostly abundant in the gastrointestinal tract (GI), further investigation of the gene expression patterns of MICA in this tissue may help elucidate the molecular mechanism through which MICA is affecting HSCT outcomes.

In conclusion, this study has considered the combination of both rs2910164 and rs2431697 in miR-146a, rs3027989 in IRAK1 and rs1051792 in MICA and identified associations with NRM, OVS, relapse and aGvHD post HSCT. This study has explored a cohort of n=817 patient and donor pairs and then validated findings in an independent cohort of n=576 patient and donor pairs. The outcome of this study supports the statement that there is increasing evidence indicating that non-HLA polymorphisms influence HSCT outcome (Paczesny *et al.*, 2013). The data reported demonstrates that SNP genotyping can be translated easily into donor selection. With over 18 million unrelated donors registered worldwide (Foeken *et al.*, 2010), the potential to benefit future patients in need of a life-saving transplant is anticipated to be significant. In 1998, the first International meeting on SNPs and complex genome analysis was held in Sweden (Syvaanen *et al.*, 1999). Since then, SNP technology has become more widely adopted. Clinical expectations remain high for diagnostic and pharmacogenomic uses of SNPs. However, clinicians are confronted with the same issue as mHA disparities; donor selection according to SNP genotyping is still not performed clinically, although it may be available in the near future as recently reported by Petersdorf et al. The transplant barrier is comprised of classical HLA loci as well as non-HLA variation within the gene-dense MHC region. Two new genetic markers are informative for disease-

free survival and acute GvHD after HLA-matched unrelated donor transplantation. The identification of MHC resident transplantation determinants provides clinicians with tools to lower post-transplant risks through comprehensive donor matching, and identify patients at highest risk for complications who might benefit from directed preventive measures that include optimization of GvHD prophylaxis. This study provides the foundation for future fine-mapping approaches to identify the specific nature of the genes and their mechanisms in health and disease (Petersdorf *et al.*, 2012).

Chapter 4. Assessment of MICA mRNA levels, protein expression in clinical gastrointestinal tissue post-HSCT and soluble MICA levels in patients sera pre and post-HSCT

4.1 Introduction

The MHC complex comprises of a cluster of genes mapping to the short arm of chromosome 6. Most of them encode polypeptides mainly involved in antigen presentation to T lymphocytes. In 1994, the MHC class I chain related (MIC) gene family was first described and was noted to map within the MHC class I region was described (Bahram *et al.*, 1994). This family comprises 2 functional genes, MICA and MICB, and several pseudogenes, MICC to MICG (Figure 4.1) (Bahram and Spies, 1996; Bahram, 2000a; Muro *et al.*, 2014). MICA has an overall homology of 83% with MICB, but their homology with the classical MHC class I genes is quite low, being between 15 and 35% (Bahram *et al.*, 1994).

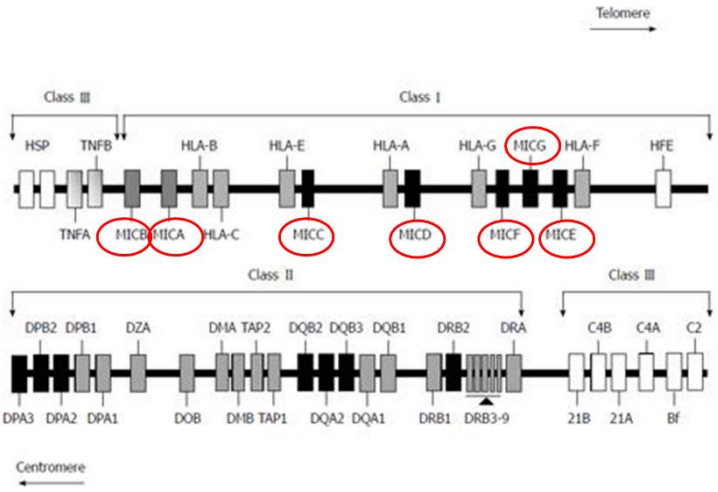


Figure 4.1 Map of the human MHC class I region showing the location of the MIC genes (adapted from Muro *et al,* 2014). In red circles are the MIC genes located with the MHC class I region. Within the MIC gene family, there are 7 member including 2 functional genes, MICA and MICB and 5 pseudogenes, MICC, MICD, MICE, MICF and MICG (Muro *et al.*, 2014)

Typically, MICA encodes for a polypeptide of 383 amino acids that is expressed on the cell surface of different cells and resembles the domain organization of the α chain of MHC class I molecules (one leader peptide encoded by exon 1, three extracellular globular domains encoded by exons 2 to 4, one transmembrane domain encoded by exon 5 and a cytoplasmic tail encoded by exon 6). However, MICA does not associate with β2-microglobulin (Groh *et al.*, 1996a; Zwirner *et al.*, 1997) (further elaborated in Chapter 1, section 1.9).

MICA equivalent genes are present in different species but not in the mouse genome (Bahram *et al.*, 1994; Steinle *et al.*, 1998). However, two putative orthologous genes to MICA and MICB have been described in the mouse genome (Kasahara *et al.*, 2002). Like the other MHC class I genes, MICA is co-dominantly expressed (Molinero *et al.*, 2002b).

MICA transcripts were first detected in human epithelial and fibroblast cell lines (Bahram *et al.*, 1994). When antibodies (Ab) against MICA became available, it was demonstrated that MICA was further expressed by freshly isolated human endothelial cells and fibroblasts (Zwirner *et al.*, 1999), tumours of different histotypes (Groh *et al.*, 1999a), some melanomas and T cell leukaemia cell lines (Pende *et al.*, 2001), in thymic medulla (Hüe *et al.*, 2003), and in gastrointestinal epithelium (Groh *et al.*, 1996b). Expression of MICA was also observed in human keratinocytes (5), which showed no expression of this molecule on the cell surface (Zwirner *et al.*, 1999; Tay *et al.*, 2000). The detection of MICA in tumours suggested that its expression might be related to the process of neotransformation (Zwirner *et al.*, 2006).

MICA is not expressed by resting T or B lymphocytes, but PHA-activated CD4+ and CD8+ T cell blasts express MICA (Zwirner *et al.*, 1997). This expression may also be triggered by stimulation with allogeneic PBMCs, and involves TCR/CD3 engagement and co-stimulation through CD28 (Molinero *et al.*, 2002a; Molinero *et al.*, 2003), involving different cytoplasmic mediators (18) and NF-κB (Molinero *et al.*, 2004). These results suggest that MICA can be induced not only upon neotransformation, but also during cell activation, two cellular processes coincidentally regulated by NF-κB (Kuo and Leiden, 1999; Karin and Greten, 2005). However, low surface expression of MICA was observed on activated T lymphocytes (Molinero *et al.*, 2002a).

The functional implications of MICA have been investigated in many clinical settings, including transplantation in cases both of kidney and heart transplant, where the presence of MICA antibodies was shown to be associated with decreased survival (Kato *et al.*, 2006). In a study by Zou et al., MICA antibodies generated by mismatched amino acids in transplant recipients have been found against MICA in organ transplants that are rejected (Zou *et al.*, 2007). In solid organ transplant, immune

responses in recipients were shown to be mounted against different protein forms of MICA alleles (Luo *et al.*, 2014).

MICA has also been investigated for its implication in a HSCT setting and MICA mismatching has been associated with increased risk of GvHD (Askar *et al.*, 2014), and with allogeneic transplanted T-cells or NK cells targeting mismatched MICA proteins in the GI tract of the transplant recipient (Askar *et al.*, 2014).

MICA is up-regulated by different stress conditions such as heat-shook oxidative stress, neoplasic transformation and viral infection (Allegretti *et al.*, 2013). In fact, MICA molecules function as stress sentinels which interact with the NKG2D-activating receptor on human NK and $CD8^+$ αβ T cells and γδ T cells, triggering the cytolysis of virally infected cells or transformed cells (Hue *et al.*, 2004). Particularly, MICA is expressed in enterocytes where it can mediate enterocyte apoptosis when recognised by the activating NKG2D present on intraepithelial lymphocytes (Allegretti *et al.*, 2013). MICA-NKG2D ligand-receptor plays a significant role in induction of innate and adaptive responses against epithelial pathological conditions, especially those occurring in the gastrointestinal tract (Wagsater *et al.*, 2003). This mechanism was suggested to play a major pathogenic role in active GIGvHD (Boukouaci et al., 2013; Isernhagen et al., 2015a).

Various non-MICA-related features can also influence its expression, e.g. CMV state, GvHD prophylaxis, underlying disease and treatment procedures (Isernhagen *et al.*, 2015b). Steroids are considered the treatment of choice for aGVHD (Kobbe *et al.*, 2001). In this regard it was previously reported that steroids can affect several functions in different systems, by altering expression of genes that are relevant for cell-to-cell communication, cell structure and differentiation including the MICA-NKG2D system (Kawata *et al.*, 1994). Steroids can also regulate gene expression post-transcriptionally, by altering the stability of mRNA (Ing, 2005).

In this study, the effect of steroid dose treatment on MICA expression was investigated and associated with the outcome of gastro intestinal GvHD (GIGvHD). The main focus was to investigate steroid dose-dependent effects on MICA gene expression.
In the previous Chapter (Impact of SNPs on HSCT outcome), MICA genetic polymorphisms were shown to have an impact on HSCT outcome, however, additional MICA-related features, including MICA protein expression and soluble MICA levels

may also influence the cycle of MICA expression and thus, may influence the incidence of GvHD after allo-HSCT.

A soluble isoform of MICA (sMICA) is generated by the proteolytic shedding of membrane-bound MICA (Groh *et al.*, 2002b). This can result in a tumour immune escape, mediated by immunosuppressive sMICA (Groh *et al.*, 2002a; Salih *et al.*, 2002; Chitadze *et al.*, 2013). sMICA can induce NKG2D downregulation by rapid endocytosis and partial lysosomal degradation, resulting in the impairment of NK cell cytotoxicity (Roda-Navarro and Reyburn, 2009) and the co-stimulation of $CD8^+$ αβ T cells via NKG2D (Groh *et al.*, 2001). MICA is cleaved at the cell surface by members of the family of matrix metalloproteases (MMPs) and the "a disintering and metalloproteinase" (ADAM) family, including ADAM10 and ADAM17 (Groh *et al.*, 2002a; Salih *et al.*, 2002; Kaiser *et al.*, 2007; Waldhauer *et al.*, 2008). The α3 domain of MICA forms a complex with the disulphide isomerase/chaperon endoplasmic reticulum protein 5 (ERp5) on the surface of tumour cells, which induces a conformational change enabling the proteolytic cleavage of MICA (Chitadze *et al.*, 2013). Shedding of NKG2D ligands has been reported for many types of cancers and some haematopoietic malignancies (Chitadze *et al.*, 2013). In addition, tumour-derived exosomes contain MICA (Clayton *et al.*, 2008) and may also contribute to a downregulation of NKG2D (Isernhagen *et al.*, 2015a).

4.2 Specific study aims

The main focus of this study was to establish the effect of MICA mRNA levels in GI tract on the HSCT outcome. The association between MICA expression by intestinal epithelial cells and the different grades of GIGvHD was also investigated. This was performed using SYBR GREEN qPCR and immunofluorescence confocal microscopy. Another aim of this study was to investigate the impact of MICA serum levels on HSCT outcome. For this purpose, a comparison between the levels of sMICA in patients pre and post transplantation as well as at different time points post HSCT (pre, day -7, day +14, day +28 and day +100) were investigated for their association with HSCT outcome.

4.3 MICA mRNA levels and protein expression assessment in clinical gastrointestinal tissue

4.3.1 Clinical information for the study cohort

A cohort of n=180 GI biopsies collected from n=96 patients who underwent transplantation at the Transplantation Centre, University Clinic of Regensburg, Germany, were utilised in this study. Clinical characteristics of the study cohort are described in Table 4.1. A

Table 4.1 Clinical characteristics of patient and donor recruited for MICA expression investigation in the GI tract (n=96)

Clinical characteristics		**N (%)**
Patient Gender	Female	64 (66.66)
	Male	32 (33.33)
Patients age range (y)		17-70
Donor Gender	Female	67 (69.79)
	Male	28 (29.16)
Donors age range (y)		15-66
Female to male ratio		11.66
Patient CMV positivity		46 (47.91)
Donor CMV positivity		36 (37.5)
Disease	Acute Myeloid Leukaemia	37 (38.54)
	Myelodysplastic syndrome	7 (7.29)
	Non-Hodgkin's Lymphoma	7 (7.29)
	Chronic Lymphocytic Leukaemia	9 (9.37)
	Hodgkin's Disease	2 (2.08)
	Acute Lymphoblastic Leukaemia	3 (3.12)
	Chronic Myeloid Leukaemia	2 (2.08)
	Missing	29 (30.2)
Alive (at the time of study)		43 (44.79)
aGvHD	Grade 0	34 (35.41)
	Grade 1	16 (16.66)
	Grade 2	22 (22.91)
	Grade 3	19 (19.79)
	Grade 4	5 (2.77)
cGvHD		66 (68.75)
Relapse		24 (25)
TCD		66 (68.75)
RIC		72 (75)
MUD		66 (68.75)
SIB		26 (27.08)
Apoptotic score	Yes Apoptosis (Score=1)	54 (56.25)
Active GIGvHD		57 (59.37)
Steroid treatment	≤ 20mg/kg	89 (92.70)

Abbreviations: CMV: Cytomegalovirus, aGvHD: acute GvHD, cGvHD: chronic GvHD, TCD: T cell depletion treatment, RIC: reduced intensity conditioning, MUD: Matched unrelated donor, SIB: Sibling donor, GIGvHD: gastrointestinal GvHD, MICA: MHC class I chain-related gene A

Within the study cohort, data regarding the MICA-129 genotype was only available for n=89 patients at the MICA gene expression investigation was performed independently after the MOCA-129 genotyping study. Available MICA-129 genotypes for patients and donors are shown in Table 4.2.

Table 4.2 Available MICA-129 genotypes for the patient and donors within this study

MICA-129 genotype		**N (%)**
Patient's MICA-129 Genotype	Val/ Met [GA]	40 (41.66)
	Val/ Val [GG]	39 (40.62)
	Met/ Met [AA]	11 (11.45)
	Missing	7 (7.29)
Donor's MICA-129 Genotype	Val/ Met [GA]	40 (41.66)
	Val/ Val [GG]	39 (40.62)
	Met/Met [AA]	7 (7.29)
	Missing	10 (10.41)

4.3.2 Association between MICA mRNA levels and HSCT outcome

MICA mRNA levels were investigated using SYBR Green qRT-PCR (SYBR Green qRT-PCR chemistry and protocol are described in Chapter 2, section 2.6.5). Various clinical variables were investigated for their association with the expression of MICA mRNA in the GI tract including the incidence of aGvHD, cGvHD, histologically active GIGvHD, relapse, NRM and OVS.

Statistical interpretation of the data was performed using Cox regression analysis for investigation of the association between MICA mRNA levels with the incidence of acute and chronic GvHD, Kaplan–Meier survival analysis was utilised to inspect the impact of MICA mRNA pattern on the OVS of patients post-HSCT and Grey's test served to examine the impact of MICA gene expression on relapse and NRM.

Statistical analysis considering the complete cohort showed that there was no significant association between MICA mRNA levels in the GI tract and aGvHD. No association was observed also between relapse, OVS, NRM or cGvHD and HSCT outcome. In order to investigate the effect of steroid dose treatment on MICA expression in the GI tract, patients were stratified based on a cut-off value of 20mg/kg

of steroid treatment as previously defined by Holler et al. This refined cohort was assessed for association between MICA mRNA expression and HSCT outcome.

4.3.3 MICA expression in relation to active GIGvHD and the apoptotic score

aGvHD manifests primarily as skin, gut and liver disease with the GI tract being the most commonly affected visceral organ (Martin *et al.*, 2004). A clinical diagnosis of GIGvHD is frequently confirmed by finding apoptosis on a mucosal biopsy (Ross and Alousi, 2012). Histologically active GIGvHD (grade 1) is marked by the presence of apoptosis (apoptotic score=1). Histologic grade 1 GIGvHD (apoptosis) is the most common finding for patients with GIGvHD, being present in 90% of patients with aGvHD of the lower GI tract as compared with only 11–14% of negative controls (Epstein *et al.*, 1980).

The apoptotic scores for the clinical GI biopsies along with the active GIGvHD were assigned by the pathology department (Transplantation Centre, Regensburg Clinic, Regensburg, Germany). Apoptotic score of 0 = absence of apoptosis, apoptotic score of 1= presence of apoptosis, active GIGvHD was coded as 1 and non-active GIGvHD was defined as 0.

Taking into consideration only patients treated with low doses of steroid treatment (≤20mg/kg), statistical analysis using One-way ANOVA (SPSS v21.0, IBM Analytics), revealed that high levels of MICA mRNA were significantly associated with absence of apoptosis in GI tract of patients post-HSCT ($p=0.044$) (Figure 4.2 A) and no active GIGvHD ($p=0.046$) (Figure 4.2 B).

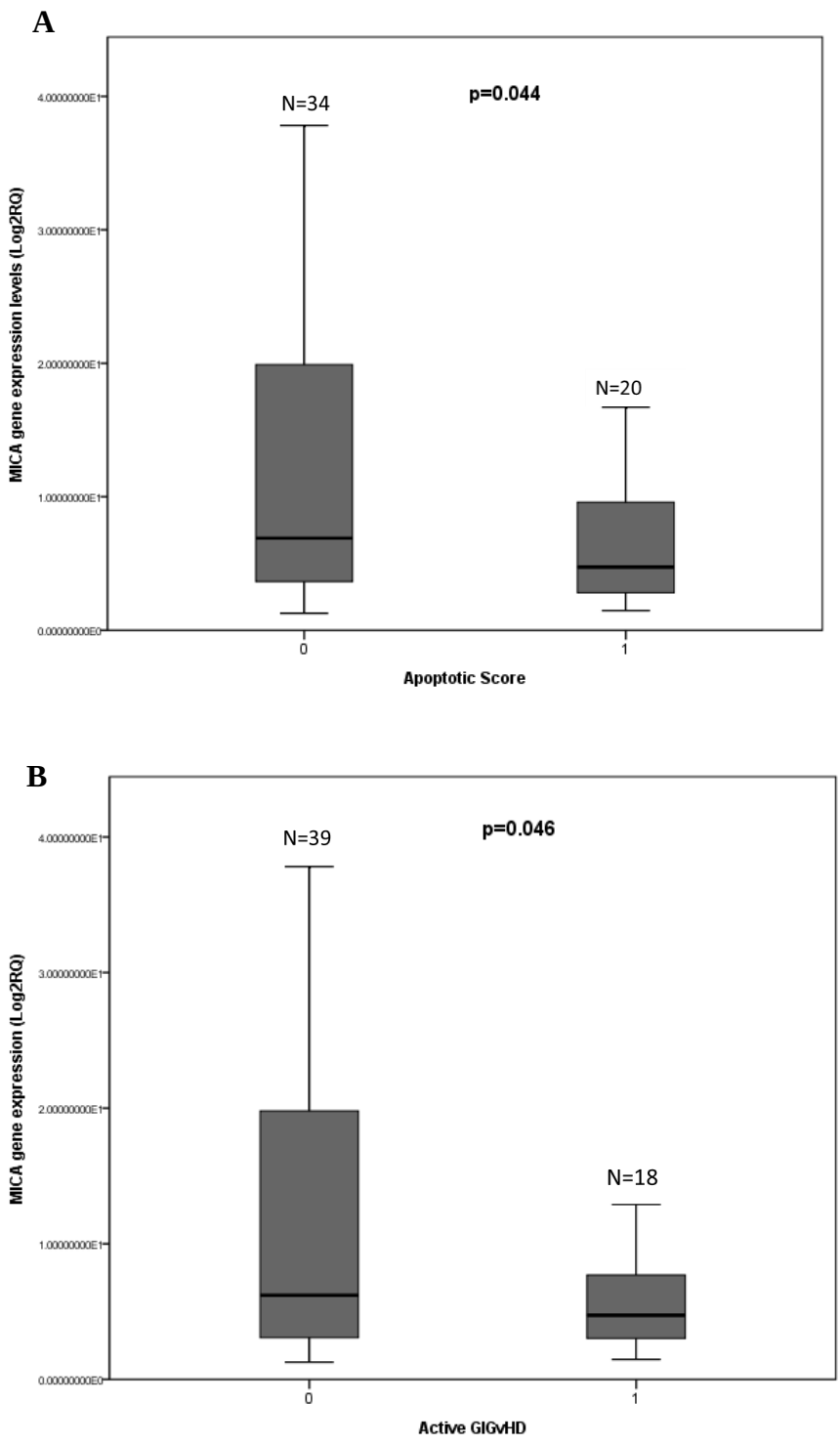


Figure 4.2 Association between MICA mRNA levels, the apoptotic score and the active GIGvHD. Statistical analysis using One-way ANOVA for the investigation of the impact of MICA gene expression on apoptosis and active GIGvHD in patients treated with low dose steroid treatment (≤20mg/kg) showed that (A) high level of MICA expression were significantly associated with the absence of apoptosis in the GI tract of patients post-HSCT (p=0.044). (B) High levels of MICA expression was also associated with less active GIGvHD (p=0.046).

4.3.4 MICA expression in relation to OVS post-HSCT

In order to investigate the association between MICA expression levels and OVS of patients post-HSCT, MICA gene expression was dichotomised as high or low using a cut-off value of 1.06E+01 Log_2 RQ. This value was chosen as it was noted as the start point for the exponential increase of MICA gene expression in patient (Figure 4.3) (Boukouaci *et al.*, 2009).

Based on the defined cut-off point, 63.5 % of the patients had a MICA expression level <1.06E+01 Log_2 RQ, while 36.5% had a MICA expression level >1.06E+01 (Log_2 RQ).

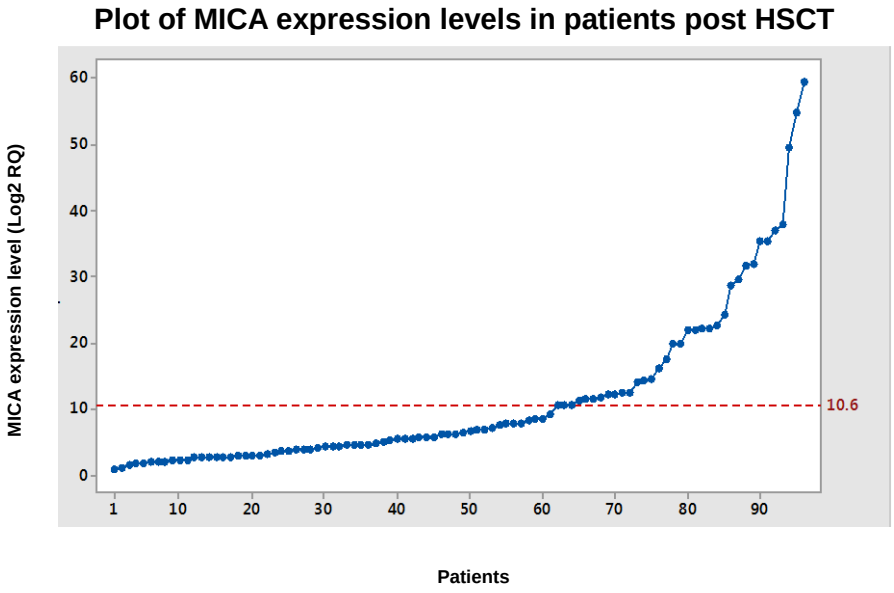


Figure 4.3 Distribution profile for MICA expression levels in patients post HSCT. A cut-off value at 1.06E+01, marked by the red dotted line, was defined as the first level at which MICA gene expression levels started to augment exponentially (Boukouaci *et al.*, 2013). Based on this cut-off point, 63.5 % of the patients had MICA expression levels higher than 1.06E+01 Log_2 RQ and 36.5% had MICA levels lower than 1.06E+01 Log_2 RQ.

Survival analysis using the Kaplan-Meier test for patients showed that there was a tendency towards an improved overall survival for patients who had MICA expression levels >1.06E+01 Log2 RQ (p=0.058) (Figure 4.5).

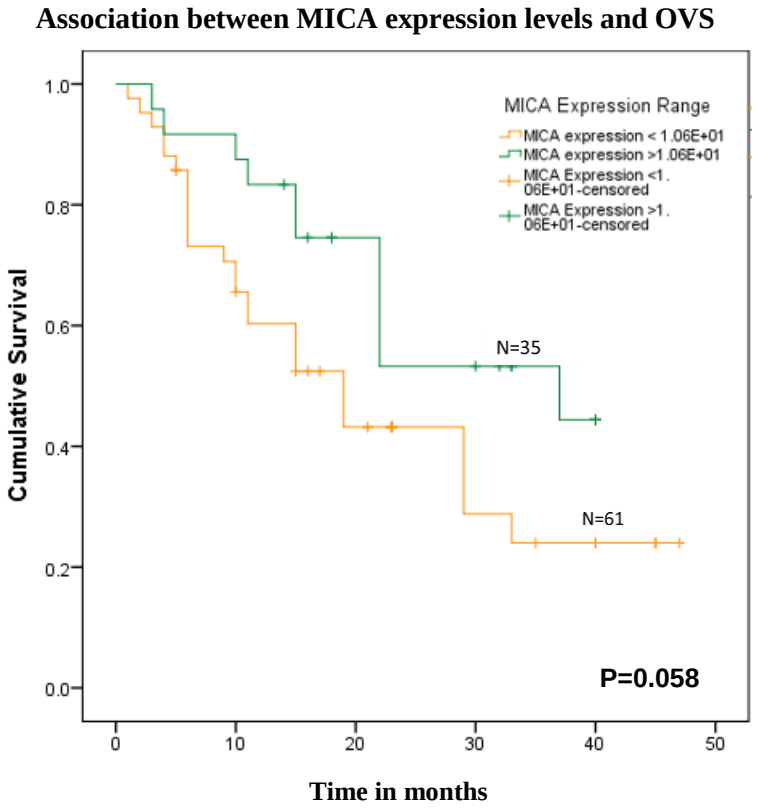


Figure 4.5 Overall survival in patients in relation to MICA expression levels. Kaplan-Meier analysis showed that patients who had a MICA mRNA level > 1.06E+01 Log_2 RQ had a tendency towards an improved OVS (p=0.058) compared to patients who had low MICA mRNA levels.

4.3.5 MICA-129 dimorphism (rs10511792) and association with MICA gene expression and functions

Within the study cohort of this study, 43.80% of the patients were carriers of the Val/Val genotype. Interestingly, relating MICA mRNA expression in the GI tract back to the MICA-129 dimorphism, it was found that the highest levels of MICA mRNA expression observed were in patients with the Met/Met genotype (p=0.022) (Figure 4.6).

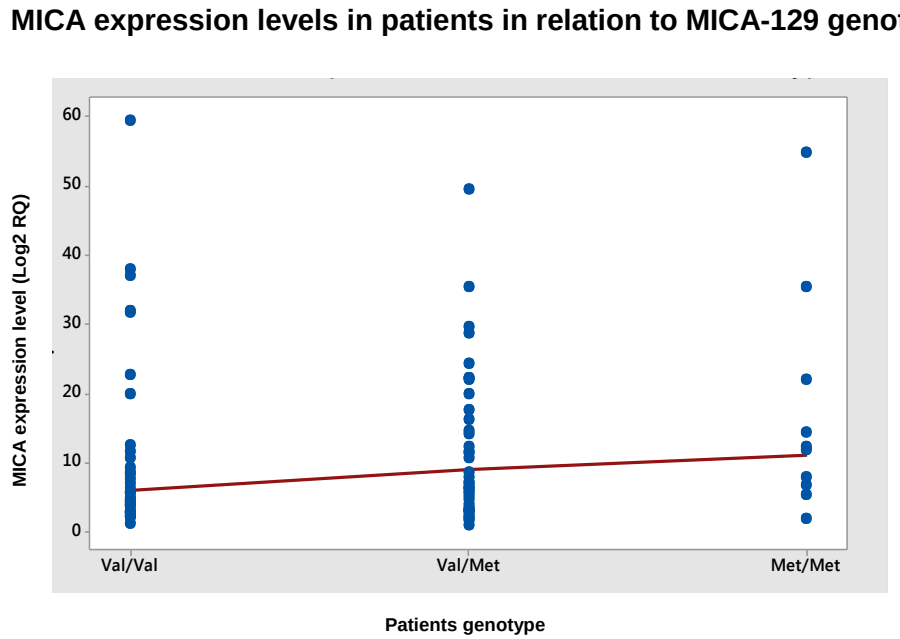
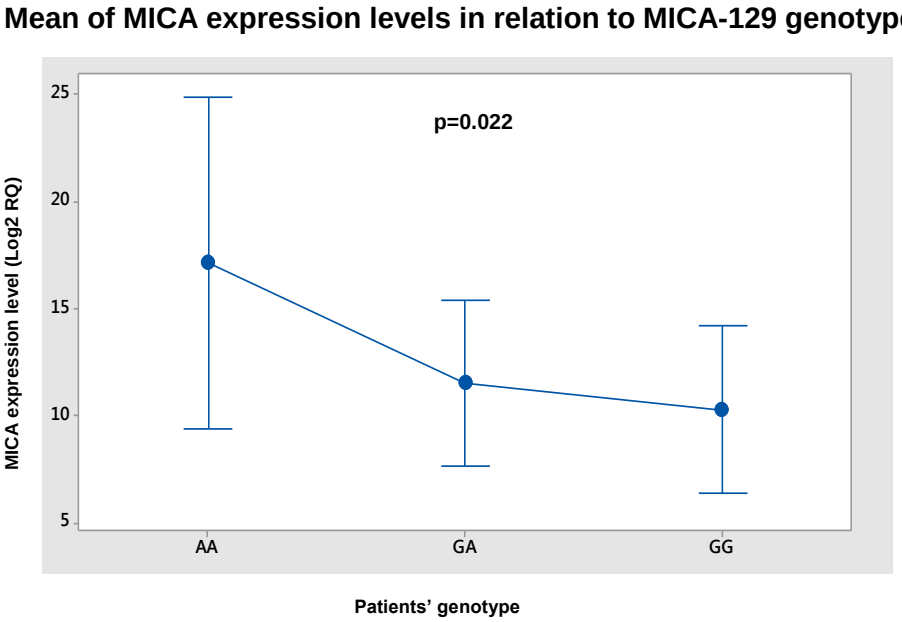


Figure 4.6. Variation of mean of MICA mRNA expression levels in relation to patients MICA-129 genotype. MICA mRNA expression levels in patients (blue dots) in comparison to the MICA-129 dimorphism showed that the mean level of MICA expression (followed by the red line) was higher in patients carrying the MICA-129 Met allele and was the highest in patients homozygous for this allele who carried the Met/Met genotype.

Comparison between the mean expression of MICA mRNA within different genetic groups showed a clear link between high MICA mRNA levels and the Met/Met (AA) genotype (Figure 4.7).



Variable	Mean	St Dev	SE Mean	95% CI
AA	17.12	16.33	5.16	(5.44, 28.80)
GA	11.51	10.79	1.71	(8.06, 14.96)
GG	10.28	12.75	2.04	(6.14, 14.41)

Figure 4.7 Comparison between the mean levels of MICA mRNA levels and MICA-129 genotype. Variation of the levels of MICA mRNA in the GI tract of patients post-HSCT showed that for patients carrying the Met/Met genotype (AA) the mean value of MICA mRNA level was the highest with 17.12 Log2 RQ. The mean of MICA mRNA level in patients carrying the Val/Met genotype (GA) was intermediate with 11.51 Log2 RQ. This mean was the lowest in the case of patients carrying the Val/Val genotype (GG). ANOVA analysis showed that the additive effect of the MICA-129Met was significantly associated with the increase in the levels of expression of MICA (p=0.022).

4.3.6 Immunofluorescence investigation of MICA protein expression in gastrointestinal tissue post HSCT

A series of optimisation experiments were conducted to select the optimal antibodies and concentrations for staining of MICA protein in gastrointestinal sections (optimisation process is described in Chapter 2, section 2.9.5).

A cohort of n=23 gastrointestinal tissue sections collected from the patients recruited at the Transplantation centre of Regensburg, were stained for MICA. These clinical GI sections were obtained from patients who underwent transplantation at Transplantation Centre, University Clinic of Regensburg, Germany and for whom the

MICA-129 genotype is known (data obtained from the genotyping study described in Chapter 3). Both clinical and histological grades of GvHD corresponding to the GI sections are illustrated in Table 4.3.

Table 4.3 Clinical characteristics of patient gastrointestinal tract sections considered for MICA immunofluorescence analysis.

Characteristics of GI sections		**N (%)**
Clinical GvHD grade*	Grade 0	12 (52.17%)
	Grade 1	3 (13.04%)
	Grade 2	4 (17.39%)
	Grade 3	2 (8.69%)
Histological GvHD grade	Grade 0	8 (34.78%)
	Grade 1	5 (21.73%)
	Grade 2	4 (17.39%)
	Grade 3	6 (26.08%)

*Clinical GvHD grades were not available for 2 GI biopsies

For the purpose of statistical analysis, the histological grades of GvHD were considered, as these grades are specific to the gut and thus more reflective of the investigation of MICA protein levels in the GI tract.

MICA expression was observed within the intestinal epithelial cells of the crypts. Confocal microscopy images revealed that MICA staining intensity, corresponding to its expression, was significantly higher in gastrointestinal sections graded 0-1, and MICA intensity gradually decreased in sections with high aGvHD grades (grade 3-4) ($p=0.002$) (Figure 4.8).

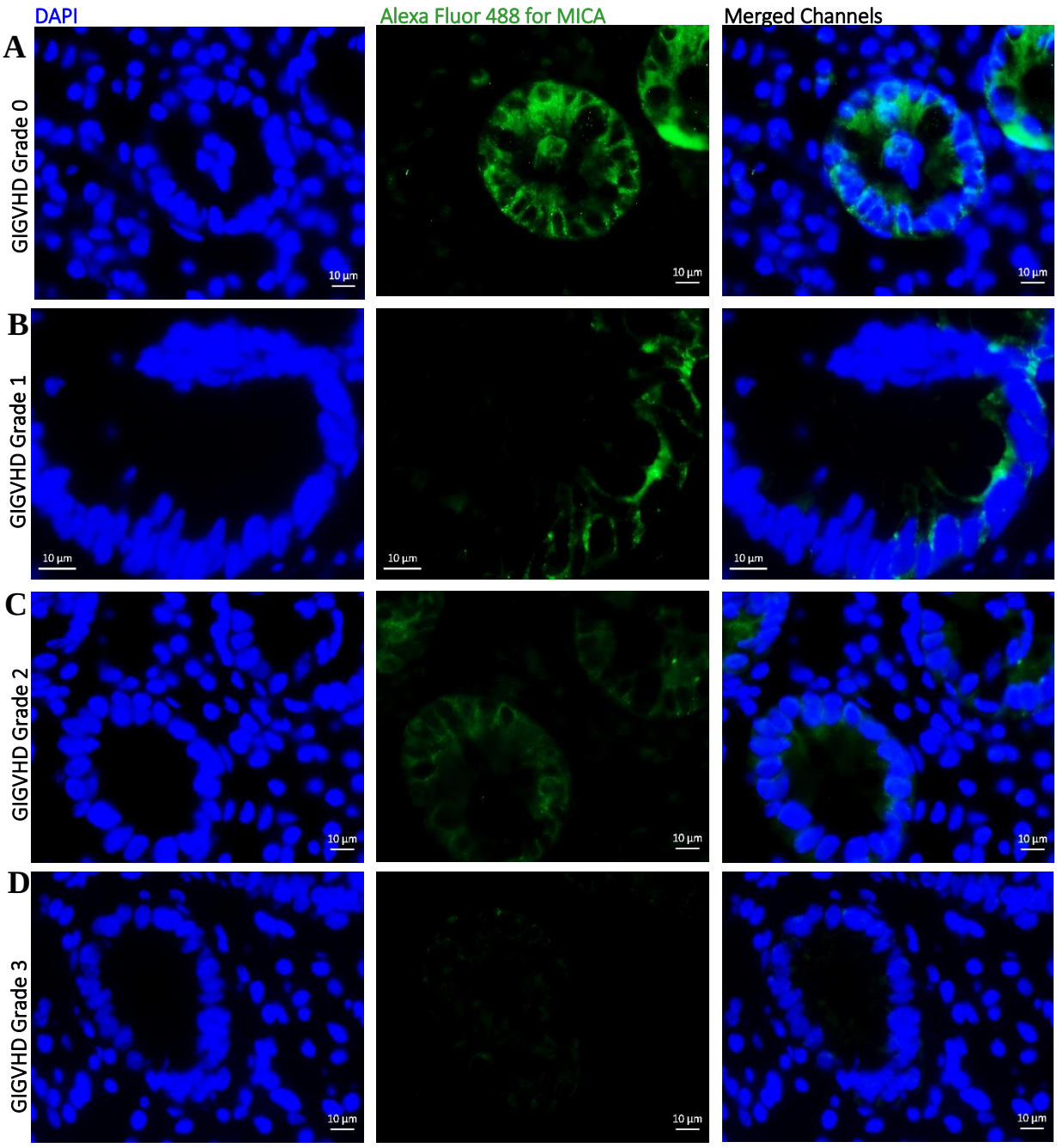


Figure 4.8 Differential expression of MICA in the GI tract in relation to GIGvHD grades. (A) MICA expression by intestinal endothelial cells in a grade 0 GIGvHD section showing the strongest intensity of Alexa Fluor 488 corresponding to MICA. (B) Grade 1 GIGvHD section with MICA expression less intense than grade 0, but relatively higher expression than grade 2 and grade 3 GIGvHD. (C) MICA expression in a grade 2 GIGvHD GI section. (D) Grade 3 GIGvHD GI section showing low expression of MICA.

During the imaging process (ZEN Lite Software, ZEISS Microscopy) a uniform exposure time was maintained for both DAPI and Alexa Fluor 488 (79.5 ms) for the purpose of statistical analysis via the quantification of MICA intensities.

ZEN images of MICA were exported into ImageJ, an image processing program, for the quantification of MICA intensity. Alexa Fluor 488 corresponding to MICA was measured against a uniform intensity of DAPI and against the background.

Statistical analysis using one way-ANOVA showed a significant decrease in MICA levels when comparing grade 0 GIGvHD to grade 3 GIGvHD (p=0.002) (Figure 4.9). This significance was maintained when comparing grade 0 to grade 1 GIGvHD (p=0.033) (Figure 4.9). The differential levels of MICA expression in gastrointestinal tissue are illustrated in Figure 4.9.

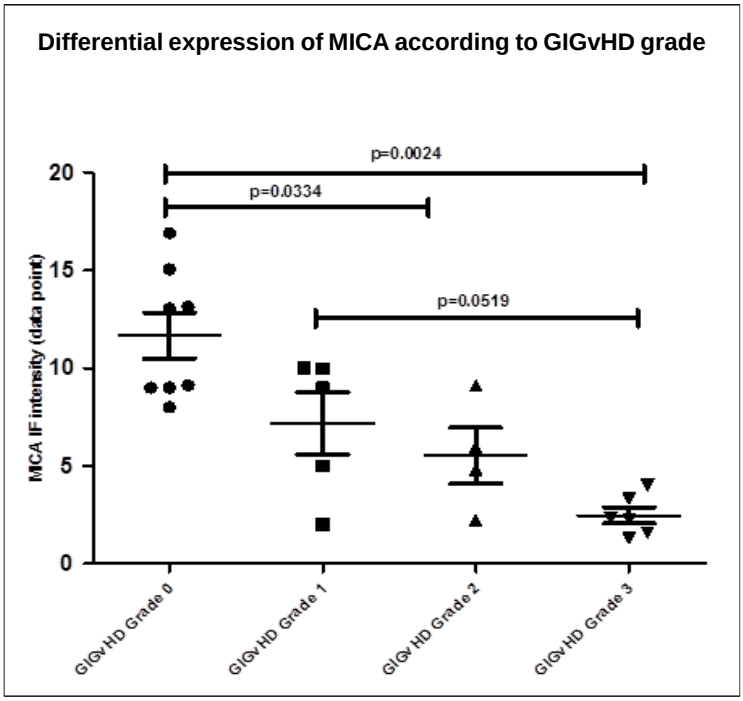


Figure 4.9 MICA immunofluorescence intensity in relation to GIGvHD grades. Statistical analysis using One-way ANOVA (GraphPad Prism v6.0) test showed that there was significant decrease in MICA protein level between GIGvHD grade 0 and grade 3 (p=0.002). Comparison between MICA protein levels in patients with grade 0 and grade 2 GIGvHD, showed that MICA protein levels were significantly higher in patients who had GIGvHD grade 0 (p=0.033). MICA protein levels were significantly higher in patients with grade 1 GIGHD in comparison with patients with grade 3 GIGvHD (p=0.051).

4.4 Assessment of soluble MICA levels in sera of patients pre and post-HSCT

4.4.1 Clinical characteristics of the study cohort

The study cohort comprised of n=129 serum samples collected from n= 55 patients recruited from the Newcastle Upon Tyne Transplantation Centre, The Newcastle Upon Tyne NHS Foundation Trust, Newcastle upon Tyne, United Kingdom. These samples were collected from patients pre-transplantation, and at day 14, day 28 and 3 months post-transplantation. All clinical characteristics of the patients are detailed in Table 4.4.

Table 4.4 Clinical characteristics for the patients recruited for sMICA level association with HSCT-outcome (n=55)

Clinical Characteristics		**N (%)**
Patients gender	Female	18 (32.72)
	Male	29 (52.72)
	Missing	8 (14.54)
Donors gender	Female	8 (14.54)
	Male	30 (54.54)
	Missing	17 (30.90)
Adult		55 (100)
Patients age range (years)		20-68
Donors age range (years)		19-55
Relationship	SIB	12 (21.81)
	MUD	26 (47.27)
	Missing	17 (30.9)
Disease	Acute Myeloid Leukaemia	9 (16.36)
	Myelodysplastic syndrome	5 (9.09)
	Non-Hodgkin's Lymphoma	5 (9.09)
	Acute Lymphoblastic Leukaemia	1 (1.18)
	Other	3 (5.45)
	Missing	32 (58.1)
Deceased		8 (14.54)
Relapse		7 (12.72)
aGvHD	Grade 0	13 (23.63)
	Grade 1	11 (20)
	Grade 2	10 (18.18)
	Grade 3	1 (1.18)
	Missing	20 (36.36)
TCD		16 (29.09)
RIC		26 (47.27)

Abbreviations: SIB: siblings, MUD: matched unrelated donors, GvHD: graft versus host disease, TCD: T cell depletion treatment, RIC: reduced intensity conditioning, HSCT: Haematopoietic stem cell transplantation, MICA: MHC class 1 chain related polypeptide A, Met: Methionine, Val: Valine

5.1.1 Serum MICA levels in patients pre and post-HSCT

Statistical analysis for the association between MICA serums levels and the incidence of aGvHD was performed using two samples independent t-test (GraphPad prism v6.0). For the association of sMICA levels with the OVS, NRM and relapse, a cut-off point of

sMICA level was defined and ranges above and below the cut-off value allowed for the transformation of continuous data (sMICA levels) into binary data.

Analysis of the variation of sMICA levels in patient serum pre and post HSCT showed a mean value of 19.60pg /mL of sMICA pre-HSCT and mean value of 26.40pg/mL post-HSCT (Figure 5.1).

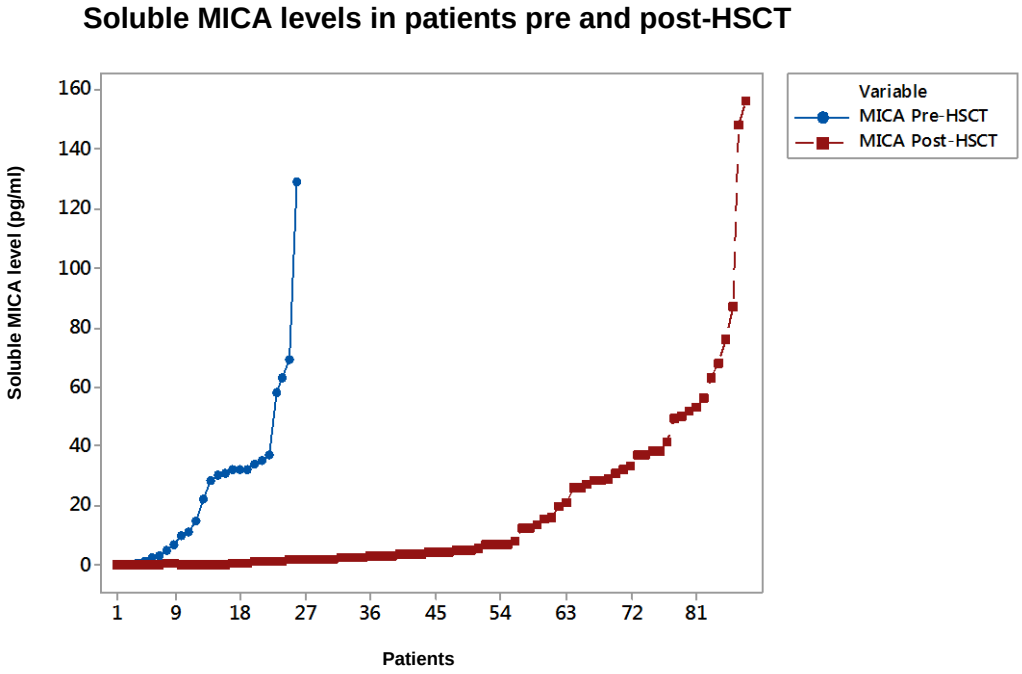


Figure 4.10 Variation of sMICA levels in serum pre and post-HSCT. sMICA expression showed a mean value of 19.60pg /mL of sMICA pre-HSCT and mean value of 26.40pg/mL post-HSCT. The highest levels of sMICA were of 156.00 pg/ml in patients post-HSCT and 126.13pg/ml in patients pre-HSCT.

Based on sMICA distribution profile post-transplant in patients (Figure 5.1), data was stratified according to a cut-off point of 12.3 pg/ml (Boukouaci *et al.*, 2009). This cut-off point was used to dichotomise expression to perform survival analysis statistical.

Survival analysis based on the cut-off value of 12.3 pg/ml of sMICA serum showed no significant association between the sMICA levels and OVS. There was no significant association between the levels of sMICA and relapse, NRM and cGvHD.

5.1.2 Association between sMICA levels and aGvHD

Levels of sMICA at pre-HSCT, day +14, day +28 and 3 months post HSCT were investigated for their association with the incidence of aGVHD. sMICA levels were significantly upregulated in the serum of patients with aGvHD at 3 months post-transplantation (p=0.012) (Figure 5.2). There was no significant association between

sMICA levels and the incidence of aGvHD pre-HSCT or post-HSCT at day 14 and day 28. No significance was also observed between sMICA levels and the severity of GvHD post-HSCT (grade 0 vs 1-3).

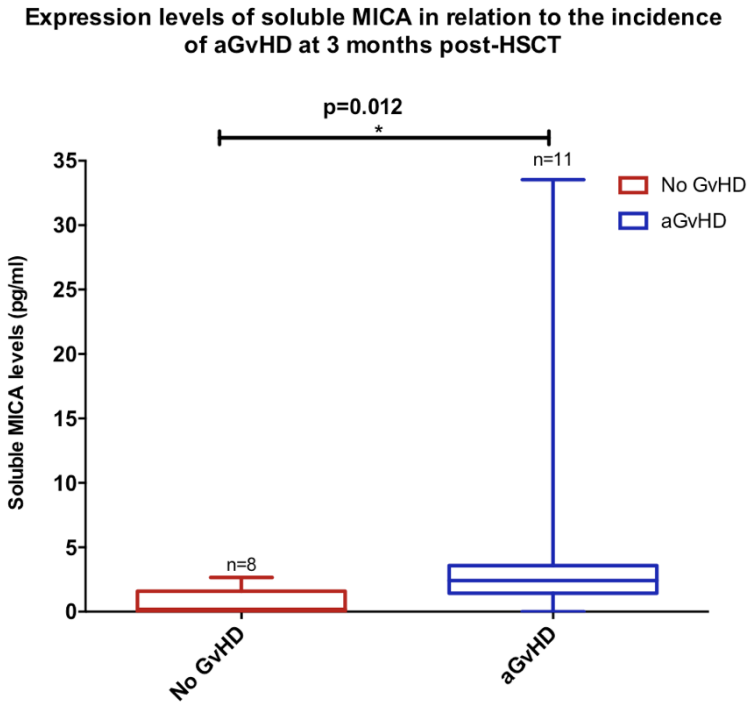


Figure 4.11 sMICA levels association with the aGvHD incidence at 3 months post-HSCT. Box plot presentation of sMICA levels in relation to the incidence of aGVHD. A t-test for unpaired samples showed that are sMICA levels were significantly upregulated in the serum of aGvHD patients at 3 months post-HSCT (p=0.012).

5.1.3 Association between MICA-129 dimorphism (rs10511792), MICA mRNA expression, sMICA levels and the outcomes of HSCT

In order to investigate associations between sMICA levels and MICA-129 genotype with the incidence of GvHD, patients data acquired from the MICA SNP genotyping study was correlated with the sMICA levels, this information was only available for n=23 patients (Table 4.5).

Table 4.5 Available MICA-129 genotypes for the patient within this study

MICA-129 genotype		N (%)
Patient's MICA-129 Genotype	Val/ Met [GA]	1 (4.34)
	Val/ Val [GG]	8 (34.78)
	Met/ Met [AA]	14 (60.86)

Plotting the levels of soluble MICA against the patients genotypes showed that high sMICA levels were attributed to the MICA-129 Val/Val genotype (p=0.406) (Figure 5.3).

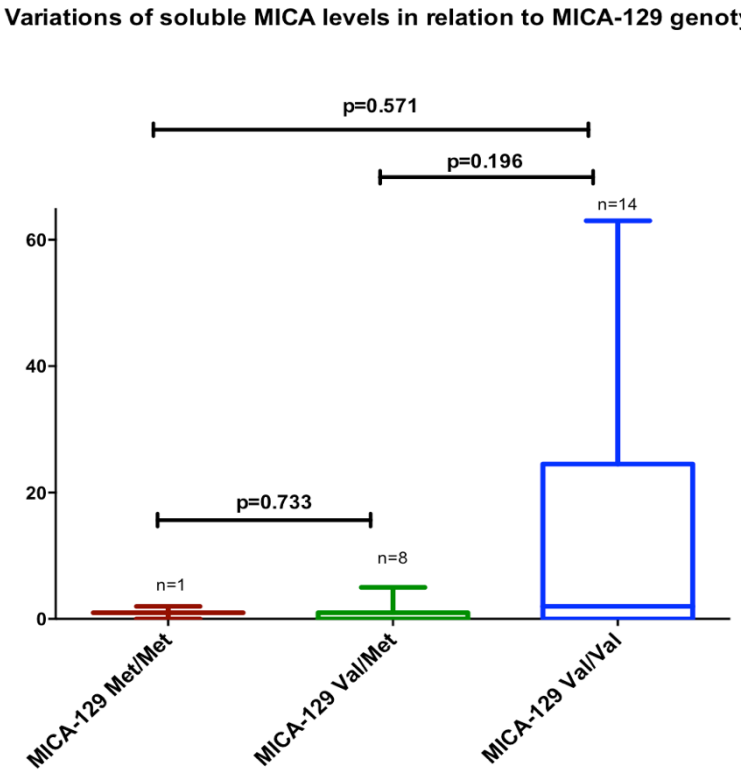


Figure 4.12 MICA-129 genotype in association with sMICA levels in serum of patients post HSCT. Variation of sMICA levels in association with the MICA-129 genotype in patients. Analysis were based on the additive genotype risk, (Met/Met, Val/Met and Val/Val), whereby additive effect of the minor allele, Met allele, increased the risk of aGvHD. No significant association was observed between the different MICA-129 genotypes and an increased level of sMICA..

4.5 Discussion

HSCT is the most potent curative therapy for many malignant and non-malignant disorders. A major complication of HSCT is GvHD, which is mediated by tissue damage resulting from the conditioning regimens before transplantation and the alloreaction of dual immune components (activated donor T-cells and recipient's APCs) (Ramadan and Paczesny, 2015). This tissue damage leads to the release of alarmins (which are of endogenous origin), together with the exogenous PAMPs, elicit similar responses of danger signals and represent the group of DAMPs. These DAMPs trigger the pathogen-recognition receptors that activate the innate immune system and subsequently the adaptive immune system. The immunopathology of aGVHD, triggered by tissue damage, induces secretion of proinflammatory cytokines, which is a critical step for the maturation and activation of host dendritic cells, and for initiation and amplification of donor-derived T-cell– mediated responses (Hill and Ferrara, 2000; Ferrara *et al.*, 2003). Tissue-specific expression of danger signals from injured host tissues might contribute to the pattern of clinical pathology. In this setting, MICA is induced upon cellular distress conditions such as DNA damage, malignant transformation, or intracellular infection (Groh *et al.*, 1999b; Groh *et al.*, 2001; Tieng *et al.*, 2002; Gasser *et al.*, 2005). MICA is recognized by the NKG2D activating receptor, which activates NK cells and costimulates effector T-cell subsets leading to cytotoxic lysis of the stressed target cells (Groh *et al.*, 1996b; Bauer *et al.*, 1999). Expression of the NKG2D ligands (NKG2D-L), such as MICA, is rare or absent on the cell surface of unstimulated normal human cells, although transcripts and intracellular proteins can be present in fibroblasts and epithelial or endothelial cells. Moreover, induction of MICA expression upon cellular distress has been mostly observed in epithelial cells of the GI tract, a pattern that fits with the tissue targets of aGVHD (Groh *et al.*, 1996a; Das *et al.*, 2001; Hue *et al.*, 2004).

The MICA-NKG2D system acts as an initial defence against infections and malignant transformation (Vivier et al., 2008). Once there is MICA-NKG2D binding on specific cells, DNAX-activating protein of 10kDa (DAP10) becomes phosphorylated, accompanied by the recruitment of phosphatidylinositol 3-Kinase (PI3K) or a growth factor-receptor-bound protein 2 (GRB2)-Vab1 complex for full activation of the NKG2D-expressing immune cells (Nausch and Cerwenka, 2008). The human NKG2D ligand MICA was initially described as a stress response molecule, as it could be induced on

cells by heat shock (Nausch and Cerwenka, 2008). It was subsequently discovered that expression of both human and murine NKG2D ligands can be induced upon infection of cells with a wide range of different viruses, including human cytomegalovirus, influenza A, hepatitis B, Epstein-Barr virus, and adenovirus (Groh *et al.*, 2001; Pappworth *et al.*, 2007; Vilarinho *et al.*, 2007; McSharry *et al.*, 2008). Constitutive expression of MICA in intestinal epithelial cells was first reported over ten years ago (Groh *et al.*, 1996b). The mammalian gastrointestinal tract harbours a dense and diverse microbial community which is composed primarily of bacteria but also includes fungi, archaea, and viruses; collectively, these are referred to as the intestinal microbiota (Lozupone *et al.*, 2012). Under such conditions, infection of epithelial cells with pathogenic *Escherichia coli* was described (Zhou *et al.*, 2007; Nausch and Cerwenka, 2008). Therefore, it is likely that NKG2D ligand expression levels in gastrointestinal epithelial cells are responsive to changes in the gut flora (Eagle *et al.*, 2009).

MICA gene expression was previously investigated in several diseases including celiac disease (Hue *et al.*, 2004) and laryngeal carcinoma (Wang *et al.*, 2016). Due to its polymorphic nature, it was assumed that MICA could be a novel transplantation antigen or alloantigen. Anti-MICA specific Ab were detected in sera of transplant recipients with different types of rejection episodes (Zwirner *et al.*, 2000b), these Ab were absent before the transplant, and they were effectors of complement mediated cytotoxicity (Zou *et al.*, 2002). In addition, renal and pancreatic allografts with acute or chronic rejection were shown to express MICA (Hankey *et al.*, 2002). Since ischemia-reperfusion injury induced to a solid organ induces a stress response in the graft that is associated with the hypoxia and activation of immune response genes (Strehlau *et al.*, 1997; Rauen and Groot, 2004), some cytokines and other proinflammatory mediators induced by the ischemia-reperfusion may also up-regulate the expression of MICA on the cell surface of endothelial and stromal cells of the grafted organ. Although this circuit of ischemia-reperfusion injury - proinflammatory cytokines – MICA expression may trigger graft rejection, studies to establish the relationship and timing of MICA expression, cellular infiltration and rejection are necessary to establish the specific role of MICA during graft rejection.

In the present study, it was revealed that high levels of MICA mRNA in the GI tract are observed in patients with no apoptosis and no active GIGvHD. Such outcomes may be attributed to the fact that within the gastrointestinal tract, NKG2D is chronically exposed

to its ligand, MICA (Danier *et al.*, 2011). Under chronic exposure, infected cells react by shedding MICA from the cell surface and this leads to downregulation of NKG2D and thus, less cytotoxic immune responses (Danier *et al.*, 2011). Expression of MICA in intestinal epithelium is thought to be stress-induced rather than constitutive (Groh *et al.*, 1998). Stress-induced expression of MICA and its recognition by specific T cells was previously shown to serve as an immune surveillance mechanism for the detection of damaged, infected, or transformed intestinal epithelial cells or to stimulate T cell secretion of growth factors for the maintenance of epithelial homeostasis, as originally proposed for murine intraepithelial T cells (Witherden *et al.*, 2014). Thus, elevated MICA mRNA levels in the case of low GIGvHD grades could be the first response of the GI tract to the development of GIGvHD. When GIGvHD grades become higher, MICA molecules becomes more abundant and thus, the NKG2D receptor becomes chronically exposed to its ligand which leads to its downregulation.

Since there is considerable genetic variation in the MICA gene, allelic variants of MICA substantially differ in their binding affinity for NKG2D, which could have significant effects on the modulation of T cell responses. Association analysis between the levels of MICA protein expression and the MICA-129 genotype within the cohort of the present study linked the high levels of MICA protein expression to the MICA-129 Met allele. However, the majority of patients were carrier of the MICA-129 Val allele. This finding is consistent with previous investigations reporting that the weak binder, MICA-129 Val, binds to NKG2D with low affinity and thus, leads to a weak immune response (Isernhagen *et al.*, 2015b). This might explain the observation of less apoptosis and less active GIGvHD in the patient cohort. In a study by Iserngagen et al investigating the effects of MICA expression on target cell killing by apoptosis, it was demonstrated that MICA intensity had a negative influence on killing of target cells expressing the MICA-129Met variant (Isernhagen *et al*., 2015b). In contrast, killing increased with the expression intensity of the MICA-129 Val allele (Isernhagen *et al*., 2015a). This supports the finding of no apoptosis and less active GIGvHD at high MICA expression levels.

In order to statistically correlate the levels of MICA expression with GIGvHD grades, MICA gene expression investigation in the GI tract, was followed by immunofluorescence investigation of its protein levels in GI sections collected from patients post HSCT. Staining of GI sections from GvHD patients who had undergone biopsies for diagnostic purposes showed that MICA was expressed in intestinal

epithelial cells as originally described (Groh *et al.*, 1996b). The finding of the present study has confirmed previous investigations and showed that, indeed, high MICA protein levels in GI sections correspond to tissue diagnosed with low grades of GIGvHD. These high intensities of MICA protein in the GI tract are the product of the translation of the MICA-129 Met/Met genotype. Previous studies have shown that the MICA-129 Met variant is a stronger trigger of NK cell cytotoxicity but the MICA-129 Val isoform out performs at high MICA expression intensities on infected cells. Binding of the MICA-129 Met isoform to the NKG2D receptor at high levels causes down regulation of the MICA-NKGD system and thus, less target cell killing and less cytotoxicity (Isernhagen *et al.*, 2015a). Investigation of MICA protein levels in the GI tract was previously performed by Groh et al., who's aim was to generate specific monoclonal antibodies for MICA to be used in immunochemical experiments with normal and transfected mutant cell lines, as well as for immunohistology (Groh *et al.*, 1996b). Their investigation showed that there was almost exclusive expression of MICA in gastrointestinal epithelium combined with transcriptional regulation of the MICA gene by a promoter heat shock element, implying that this MHC class I molecule functioned as a ligand for a subset of T cells in the intestinal intraepithelial lymphocyte compartment (Groh *et al.*, 1996b). Further studies investigated the MICA protein levels in the GI tract during celiac disease (Hue *et al.*, 2004) and showed that MICA is strongly expressed at the epithelial cell surface in these patients (Hüe *et al*., 2003).

In the SNP study conducted in the study cohort of PBMC samples collected from patients and donors who underwent allo-HSCT (Chapter 3. Impact of SNPs on HSCT outcome), it was demonstrated that in blood, patients carrying the MICA-129 Met allele were characterised with low OVS post-HSCT and also with an increased risk of relapse, while the MICA-129 Val allele was significantly associated with an increased risk of aGvHD. Although MICA expression is thought to be limited to enterocytes (Groh *et al.*, 1996b; Janeway *et al.*, 2005), the reality is that MICA expression is yet to be investigated and assessed in different tissue. Hence, there is still a remaining question about which tissues or organs express MICA and how does that influences its consequent surface expression and shedding.

It has been almost 15 years since the identification of MICA as a member of the MIC gene family (Bahram and Spies, 1996), but there is rare transcriptional analysis of the MICA gene published to date (Schrambach *et al.*, 2007).

Scharmabach et al., showed that MICA transcripts were found in virtually every organ examined, with the notable exception of the central nervous system (Schrambach *et al.*, 2007). NKG2D behaves as a guard used by CD8 T cells and NK lymphocytes to detect cells that have upregulated ligands such as MICA as a result of cellular insults (Diefenbach and Raulet, 2003). Thus, MICA-NKG2D binding results in T cell activation and proliferation and the presence of the strong binder was associated with incidence of the disease. MICA was previously reported to be associated with squamous carcinoma (Chen *et al.*, 2015a), BK polyomavirus reactivation and associated nephropathy after kidney transplantation (Tonnerre *et al.*, 2016), breast cancer (Bargostavan *et al.*, 2016), lung cancer (Okita *et al.*, 2016), abscess formation (Martinez-Chamorro *et al.*, 2016), acute and recurrent pericarditis (Markel *et al.*, 2016) and hepatocellular Carcinoma (Li *et al.*, 2016).

The association of the MICA-129 dimorphism with NKG2D binding, NK cell and T cell activation and proliferation, aGvHD, relapse and OVS, implies that the abundance of this isoform comes with more GvL effect. Differential mechanisms of regulation of MICA may allow for the segregation of GVHD and GVL and provide the foundation to specifically modify different responses by targeting distinct pathways of T cell-mediated pathways. Differences in the findings between MICA gene expression in blood and the GI tract can, in some way, be associated with the differences amongst the study cohorts as samples were collected from 6 transplantation centres including Newcastle, Paris, Prague, Vienna, Regensburg and Munich. Different approaches of disease diagnosis, HSCT protocols, conditioning regimens and GvHD prophylaxis can majorly affect the analysis and outcomes.

MICA is reported to produce sMICA through alternative splicing, proteolytic shedding or exosome secretion (Salih *et al.*, 2002). Shedding of MICA has been well investigated, and raised levels of sMICA have been associated with various malignancies, such as Crohn's disease and ulcerative colitis (Glas *et al.*, 2001), type I diabetes (Sanjeevi *et al.*, 2002), rheumatoid arthritis (Martinez *et al.*, 2001), primary sclerosis cholangitis (Norris *et al.*, 2001), systemic lupus erythematosus (Gambelunghe *et al.*, 2005), psoriasis (Gonzalez *et al.*, 2001), psoriatic arthritis (Gonzalez *et al.*, 2002), Addison's disease (Gambelunghe *et al.*, 1999), Behcet disease (Mizuki *et al.*, 1999), and familial Mediterranean fever (Touitou *et al.*, 2001). In the transplantation setting, the presence of sMICA in sera taken from patients post cardiac transplantation was shown to be

associated with a lower incidence of rejection (Fernández-Sánchez *et al.*, 2013). Through functional studies, the authors also reported that sMICA molecules downregulated NKG2D surface expression which led to a functional impairment of cell-mediated cytolysis (Fernández-Sánchez *et al.*, 2013). In renal transplant patients, specific antibodies against MICA were detected in the serum of patients collected at different time points after organ rejection (Zwirner *et al.*, 2000a). This outcome was later confirmed by Mizutani et al., where they showed that MICA-antibodies were produced more frequently in rejected renal transplant patients (Mizutani *et al.*, 2006).

The present investigation of sMICA level variation in sera of HSCT patients pre and post-transplantation showed that sMICA levels were higher post-HSCT than pre-HSCT in these patients. Such an outcome may be expected as MICA is a stress-induced molecule and the transplantation procedure, treatments, prophylaxis and development of GvHD are conditions under which patients are prone to cellular stress and inflammation. This increase in the levels of sMICA post HSCT was also observed by Boukouaci et al., who demonstrated that high levels of MICA post-HSCT were associated with the incidence of cGvHD (Boukouaci *et al.*, 2009).

The present study showed that high levels of MICA at 3 months post-transplantation were significantly associated with the incidence aGvHD. This was expected, as the previous finding in Chapter 3 (Impact of SNPs on HSCT outcome) showed that high levels of MICA-129 Met were associated with the incidence of relapse and a low overall survival. Shedding of MICA was shown to decrease the amount of cell surface MICA (Tomuleasa *et al.*, 2015) and sMICA has been demonstrated previously to downregulate NKG2D on NK and T cells, thus subverting the NKG2D-mediated immune surveillance (Groh *et al.*, 2002b).

Correlation between sMICA levels and the MICA-129 genotype showed that majority of the patients were homozygous for the Val variant which is the weak binder for NKG2D. At higher intensities, the MICA-126 Val variant was previously shown to induce the cytotoxic activity of T lymphocytes and killing by the NK cells (Isernhagen *et al.*, 2015a). Thus, the observation made in this study associating high levels of sMICA in serum, and the MICA-129 Val/Val genotype, with the incidence of aGvHD are in agreement with Isernhagen et al. This finding is also in coherence with the balance of action previously described between both the MICA-129 Met and the MICA-129 Val allele whereby the MICA-129 Val variant, if expressed at high intensities,

triggered increased degranulation whereas at very high intensities, the MICA-129 Met even decreased target cell killing (Isernhagen *et al.*, 2015a).

In conclusion, this study is novel as it explored MICA gene expression and MICA protein levels in the GI tract and correlated both with the MICA-129 dimorphism. This study showed that in the GI tract of GvHD patients, high levels of MICA mRNA were associated with less apoptosis and thus, less GIGvHD and showed that high MICA protein levels in GI tissue were correlated with low GIGvHD grades. This study has also highlighted the importance of sMICA levels as an indicator of the incidence of aGvHD post HSCT and results are in concordance with previous findings in relation to MICA-129 dimorphism. Thus, serum levels of MICA showed potential to be considered as non-invasive biomarker for aGvHD.

MICA has been shown to play a role in very different aspects of immune response, such as transplant rejection, immune response against viruses and intracellular bacteria, inflammation, homeostasis of epithelia, mother-foetus tolerance and immune response against tumours. It is likely that clinical testing for the presence of anti-MICA alloantibodies might be implemented to avoid early rejections. Simultaneously, molecular typing strategies to genotype MICA (Leelayuwat *et al.*, 1994; Fodil *et al.*, 1996; Petersdorf *et al.*, 1999; Stephens, 2001; Stephens, 2002; Collins, 2004) may avoid the transplantation of MICA-mismatched grafts and lead to an improved graft survival (Zwirner *et al.*, 2006).

Chapter 5. Investigation of the impact of significant immune response-related genes and their corresponding proteins on HSCT outcome

5.1. Introduction

Despite HSCT being the only potentially curative treatment for many malignant and non-malignant haematological diseases, the overall survival rate after transplantation is still only 40% to 60% (Dickinson *et al.*, 2004). This is due to severe post transplantation complications, including GvHD, relapse and infection (Robin *et al.*, 2007; Mohty and Mohty, 2011).

Matching for HLA genes is essential to reduce the risk of graft rejection and GvHD (Flomenberg *et al.*, 2004). However, non-HLA genes also impact on transplant outcome and aGvHD can be lethal even in patients receiving transplants from HLA-identical matched siblings donors (MSD) (Dickinson and Norden, 2015b). As MSD are currently available for only one third of patients, transplantation using HLA-MUD is more common than the use of cord blood or mismatched related donors. Several studies comparing MUD to MSD transplants showed that there was a 2.44 times higher risk of grade II to IV aGVHD in 8/8 matched MUD compared to MSD transplants (Arora *et al.*, 2009) and the incidence of grade II to IV aGVHD was still higher in 10/10 matched MUD compared to MSD transplants (Yakoub-Agha *et al.*, 2006).

The higher risk of GvHD after MUD compared to MSD transplants could be due to a higher degree of similarity in non-HLA genes for siblings, who share 50% of their genome with respective recipients (Novota *et al.*, 2011a). Accordingly, there is still a need for the identification of genes that contribute significantly to the risk of developing acute GvHD. These genes, or gene markers, may be used to assess the risk of developing GvHD, for the diagnosis of GvHD, for monitoring treatment of GvHD and for screening for immunomodulating substances which may be useful in the treatment of GvHD.

The use of novel gene markers as a method of predicting GvHD risk was developed under a patent (application number PCT/EP2011/072804) by Prof Ralf Dressel, Prof Anne Dickinsona Prof Bent Rolstadt (partners in the CellEurope Training Network) and Lutz (Dressel *et al*., 2011). The inventors identified rat and human MHC and NKC genes but also non-MHC and non-NKC genes that are regulated during GvHR in skin explant assays and could therefore serve as biomarkers for GvHD (Zinöcker *et al.*, 2012).

The method comprises determining the prognostic transcript of one or more genes selected from the group of genes consisting of *UBD, C2, LST1, AIF1l, C1QTNF7, CEACAM4, MME, IGFBP5, TAP1, CTGF, ANP32A, HCLSl, HTRA1, LGALS7, PTGER2, PTPN7, TGM2, TREM2 and CARD11, PIK3AP1, PSTPIP1, MSR1*; or their corresponding cDNAs, or their expression products. In an exploratory experiment, the inventors analysed the expression of 169 genes with human homologues, including the respective MHC and NKC region genes, identified in the rat in human skin explant samples (Dressel *et al*., 2011).

From this investigation, a selected list of genes, which were identified as significantly associated with GvHD (*C1QTNF7, LGALS7, ANP32A, HTRA1, PIK3AP1, PSTPIPI, MSR1* and *CXCL9*) were considered in this study for further validation by qPCR, as the rest of the genes were investigated elsewhere in a separate study. Gene expression levels of this list was compared to their protein levels in samples taken from patients and donors post-HSCT.

For the purpose of investigation of the listed genes, a parallel investigation to identify a suitable endogenous control for normalisation of qRT-PCR data was conducted (for further explanation, Chapter 2, section 2.6.4.3). Whilst there are several studies that have used RT-qPCR to analyse levels of immune response-related gene expression in GvHD, there remains a lack of data when it comes to evaluating the use of suitable reference genes specific to tissues of interest mostly affected by GvHD, pre and post-HSCT. The MIQE Guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) describe that qPCR studies should include the use of an endogenous and stably expressed reference gene, selected appropriately for the tissues or cell types used in the study (Bustin *et al.*, 2009).

5.2. Study aims

The aim of this study was to determine the expression levels of candidate RNA transcripts in blood samples (PAXgeneTM Blood RNA System) obtained from transplant patients, comparing expression of these genes with a corresponding baseline value of uniformly expressed endogenous genes (*GAPDH, B2M, ACTB, EIF4A2, ATP5B and 18s*, genes annotation and functions are explained in Chapter 2, section 2.6.4.3) in patient and donor samples obtained from the Transplantation Centre of Newcastle, United Kingdom.

Six candidate housekeeping genes were tested in this study in order to identify a suitable reference gene stably expressed in aGvHD patients. These were chosen due to their existing use as common reference genes in RT-qPCR; *GAPDH, B2M, ACTB, EIF4A2, ATP5B* and *18S* (Chapter 2, section 2.6.4.3). Their expression was analysed in a carefully selected cohort of GvHD patients, with samples collected from different tissues across different GvHD grades, and varying time points before and after allo-HSCT.

5.3. Results

Clinical information for the study cohort

Gene expression analysis of the target genes (*C1QTNF7, LGALS7, ANP32A, HTRA1, PIK3AP1, PSTPIP1, MSR1* and *CXCL9*) was conducted in n=400 samples collected from n=186 patients (peripheral blood collected in PAXgene Blood Tubes) of patients collected at different time points pre and post-HSCT (including pre-HSCT, day -7, day +14, day +28 and day +100). For some of the patients, not all time points were available All clinical characteristics for the patients are shown in Table 6.1.

Table 5.1 Clinical characteristics for the patient cohort (n=186)

Clinical Characteristics		**N (%)**
Patient gender	Female	71 (38.2)
	Male	115 (61.8)
Patient age range (years)		20-72
F to M ratio		15.6
Adult		186 (100)
Relationship	MUD	123 (66.1)
	SIB	73 (39.2)
Alive		109 (58.6)
Disease	Acute lymphoblastic leukaemia	22 (11.82)
	Acute myeloid leukaemia	60 (32.25)
	Hodgkin lymphoma	12 (6.45)
	Non-Hodgkin lymphoma	36 (19.35)
	Myelodysplastic syndrome	23 (10.21)
	Others	33 (17.4)
Relapse		48 (25.80)
aGvHD grade	Grade 0	74 (42.04)
	Grade 1	51 (28.97)
	Grade 2	49 (27.84)
	Grade 3	4(2.27)
TCD		96 (51.61)
RIC		135 (72.6)

Abbreviations: F to M; Female to male, MUD: Matched unrelated donors, SIB: siblings, matched related donors, TCD: T cell depletion treatment, RIC: Reduced intensity conditioning

5.3.1. Identification of a suitable reference gene for qRT-PCR (This work was performed by an undergraduate student, Matthew Mankarious, under my supervision)

To obtain optimal expression data for the list of genes of interest, geNormPLUS Kit and software (PrimerDesign, UK) was utilised to determine the most suitable reference gene to be used during qPCR and quantification. *GAPDH, B2M, ACTB, EIF4A2,*

ATP5B and *18s* (for further information regarding gene annotations and functions see Chapter 2, section 2.6.4.3) were tested for their suitability in this study.

A total of 11 peripheral blood samples were used for this study. Samples were collected from patients at different time points including pre-HSCT, day 14, day 28, 3 months, 9 months and 12 months post-HSCT.

Ct values for each qPCR reaction were recorded. Samples returning a Ct value over 40 were not considered as standardised by the MIQE guidelines (Bustin *et al.*, 2009) and thus, excluded from the analysis. A mean Ct value was recorded for each candidate reference gene along with standard error of the mean (SEM) and coefficient of variance (CV) (Table 6.5).

Table 5.2 Endogenous control expression. For each of the genes considered, the standard error mean, the Ct mean, the coefficient of variation and the standard deviation are shown.

Gene Name	Ct Mean	SEM	CV
GAPDH	29.87	0.45	4.77
B2M	27.46	0.69	7.94
ACTB	28.02	0.56	6.29
EIF4A2	32.50	0.55	5.36
ATP5B	33.07	0.67	6.39
18S	18.34	0.46	8.00

Ct: Threshold cycle, SEM: Standard error mean, CV: Coefficient of variance

An ideal reference gene should have ΔCt values close to zero with low SEM (Nguewa *et al.*, 2008). To identify the most suitable gene for normalization in whole blood, mean Ct values for each candidate gene were assessed in relation to SEM (Figure 6.3) and Ct values representative of the expression of candidate genes in the different samples were also examined (Figure 6.4).

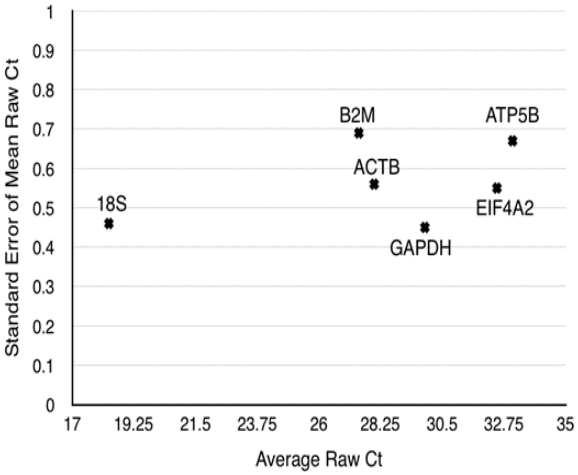


Figure 5.1 Average expression stability for the reference genes. Mean C_t values for the amplification of each gene plotted against the standard error of mean (SEM).

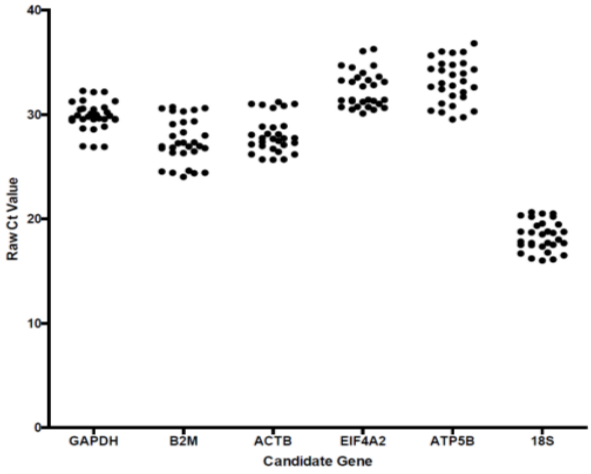


Figure 5.2 Raw Ct values shown in all peripheral blood samples. Each PBMC sample has 3 individual points for each of the 3 repeats.

Mean Ct values ranged from 33.07 in *ATP5B*, to 18.34 in 18S, representing the lowest and the highest levels of expression in patients' blood samples, respectively. Using SEM as a representation of variability of each gene, *GAPDH* was indicated as the most stably expressed candidate gene across all samples with SEM=0.45, closely followed by 18s with SEM=0.46, and the least stable genes was B2M with SEM=0.69 (Table 6.5).

Expression stability of the six candidate genes was assessed in respect to the time point of the sample collection (Figure 6.5 A) and the occurrence of GvHD (Figure 6.5 B). GAPDH had stable expression across the time points studied and during both states of no GvHD and GvHD (any grade). Ranking genes based on their SEM showed that the most suitable gene for the study of gene expression in blood samples was GAPDH (SEM=0.45).

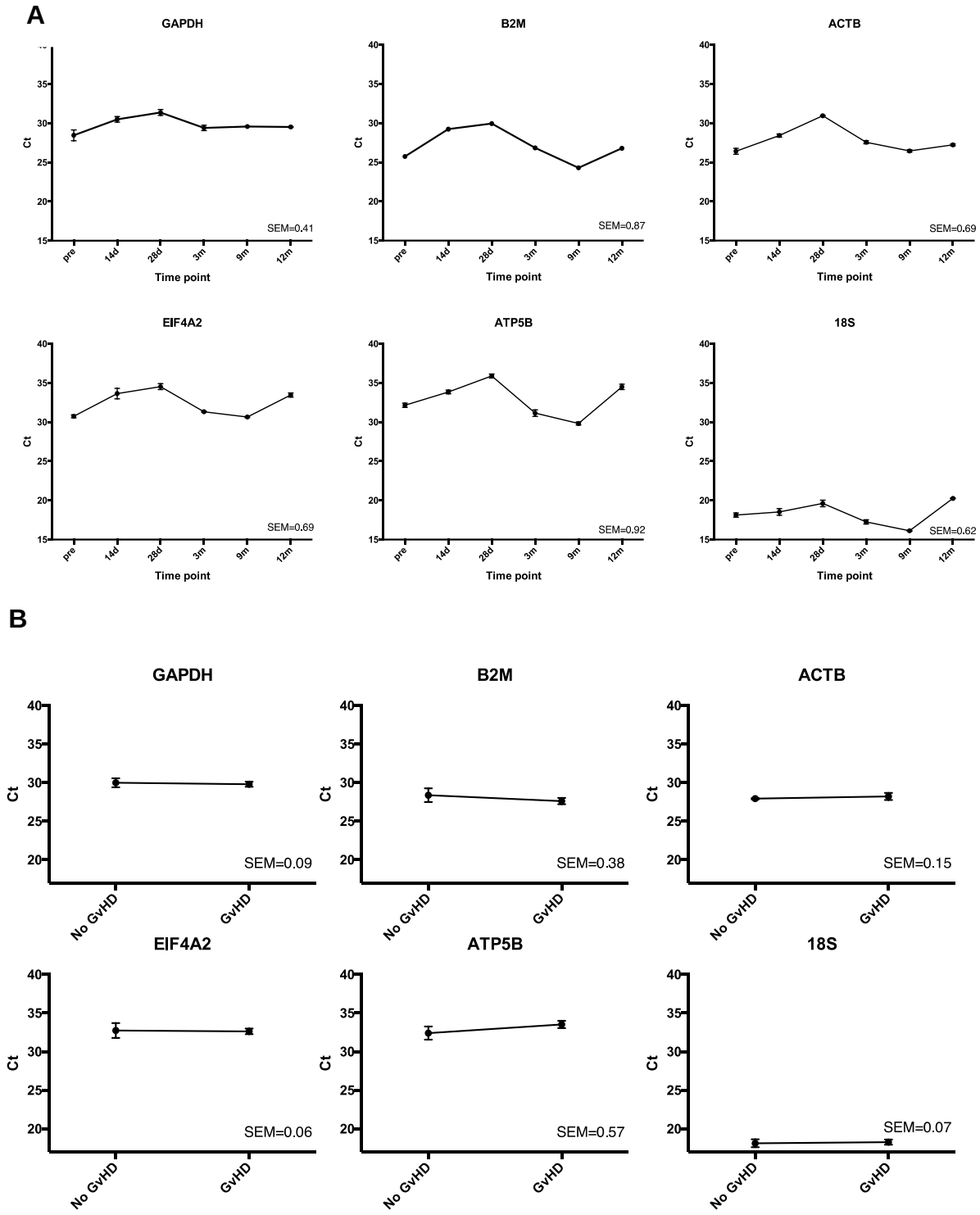


Figure 5.3 Mean C_t values of candidate gene amplification in blood samples in respect to time point and the incidence of GvHD. (A) Ct values were compared between time points relative to allo-HSCT. Error bars indicate SEM for Ct average for time point, and SEM values are indicated for the mean of the gene across all time points. (B) All blood samples with GvHD are grade 1. Error bars indicate SEM for Ct average for GvHD group, and SEM values are indicated for the mean of the gene between the GvHD groups.

5.3.2. Gene expression studies

For the quantification of candidate gene expression by Taqman RT-qPCR, GAPDH, was utilised for the normalization process.

An example of the raw data representing the mean Ct values obtained from the Taqman RT-qPCR for the genes of interest is represented in Table 6.6.

Table 5.3 Raw data representing the Ct values obtained for the genes of interest after Taqman qPCR

	Mean Ct values						
Time point	**GAPDH**	**PIK3AP1**	**PSTPIP1**	**HTRA1**	**ANP32A**	**MSR1**	**CXCL9**
Pre-HSCT	28.251	32.773	31.311	34.857	29.603	33.507	32.467
Day -7	28.462	32.783	33.098	33.039	30.638	33.490	32.467
Day +14	28.461	32.568	32.969	34.435	29.660	34.133	33.161
Day +28	28.351	31.924	31.070	33.063	29.280	35.512	32.478
Day +100	28.763	29.602	29.080	34.108	32.446	35.572	32.835

RT-qPCR outcome analysis showed that in peripheral blood samples collected from patients pre and post-HSCT from patients recruited from the Transplantation centre of Newcastle, no significant association was observed between the expression of *HTRA1, PIK3AP1 or PSTPIPI* and the incidence of aGvHD at the different time points in this study. *C1QTNF7 and LGALS7* were not expressed in any of the samples.

Expression of *ANP32A* was significantly downregulated in patients who developed aGvHD compared to patients who did not develop aGvHD at day 14 post-HSCT (p=0.01654) (Figure 6.6 A). *MSR1* and *CXCL9* was not associated with aGvHD when the analysis was performed for each time point separately. Overall levels of *MSR1* were significantly down regulated in the case of patients who developed aGvHD (p=0.04293) (Figure 6.6 B). In contrast, *CXCL9* overall levels were significantly upregulated in the presence of aGvHD (p=0.009) (Figure 6.6 C).

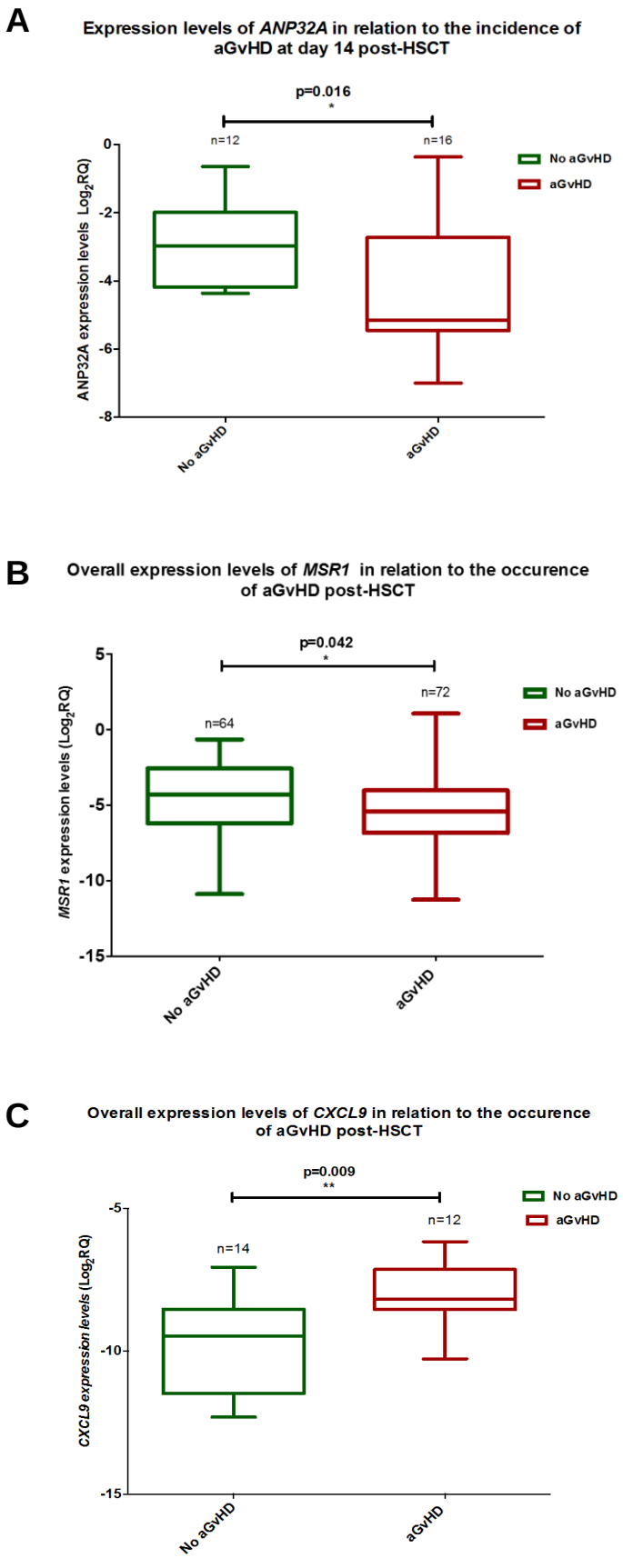


Figure 5.4 Significantly dysregulated gene expression levels in association with the occurrence of aGvHD. (A) Boxplot presentation of *ANP32A* gene expression levels were significantly downregulated in patients developing aGvHD at day 14 post-HSCT, p=0.016. (B) Boxplot presentation of overall expression levels of *MSR1* were significantly down-regulated in patients developing aGvHD, p=0.042. (C) Boxplot presentation of *CXCL9* overall levels of expression were significantly upregulated in patients without aGvHD, p=0.009.

5.3.3. Protein level investigation of significantly dysregulated genes pre and post-HSCT

A protein Biochip Array (Evidence Investigator®, RANDOX) was utilised for measuring soluble LGALS7, MSR1 and CXCL9 levels in n=129 serum samples from allo-HSCT patients collected at pre-transplantation. Day-7, day+14, day+28 and 3 months post transplantation samples were included in this study.

Since the technology provided by Randox did not include C1QTNF7, HTRA1, PSTPIP1, PIK3AP1 or ANP32A on the protein Biochip, serum levels of these proteins were investigated with a different approach using the Cusabio™ ELISA kit n=80 (as two kits only were available for this investigation.

Statistical analysis showed that there was no significant association between the levels of expression of C1QTNF7, HTRA1, PSTPIP1, PIK3AP1, ANP32A, MSR1 or CXCL9 protein in sera of patients at all time points and the incidence of aGvHD post-HSCT. However, LGALS7 protein levels were shown to be upregulated in patients developing aGVHD at day 28 post transplantation (p=0.021) (Figure 6.7).

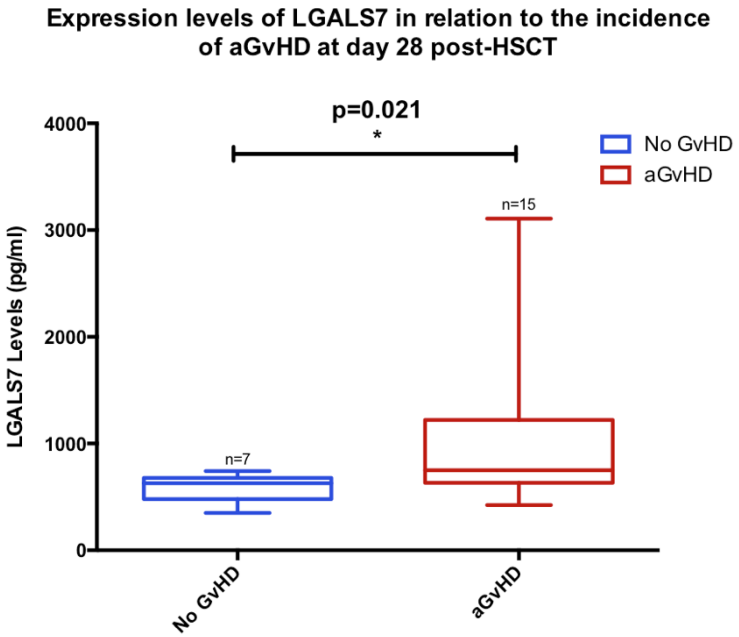


Figure 5.5. Soluble LGALS7 levels in relation to the occurrence of GvHD. Boxplot presentation of LGALS7 protein levels showing upregulation of in patients developing aGvHD at day 28 post HSCT, p=0.021. The p value was calculated using the independent samples t-test.

5.3.4. Messenger RNA expression and protein level correlation and their impact on the outcomes of HSCT

Summarised in Table 6.6 are the results of both the gene expression study in PBMCs and the corresponding protein level analysis in serum. Statistical analysis showed that there was no significant association between the levels of *LGALS7* gene expression in blood and the occurrence of GvHD. In serum, however, the levels of LGALS7 protein was significantly upregulated in patients with GvHD (Grade 2-4) at day 28 post-HSCT (p=0.0211) (Table 6.6).

For *MSR1* in peripheral blood samples, gene expression was significantly down-regulated in aGVHD patients (p=0.042), while there was no significant association between the levels of soluble MSR1 protein and the occurrence of GvHD (Table 6.6).

The gene expression of CXCL9 was up-regulated in the peripheral blood of aGvHD patients (p=0.009) but there was no significant association between the protein levels of CXCL9 and the occurrence of aGvHD post-HSCT.

Similar results were obtained for ANP32A. Gene expression was downregulated in aGvHD patients at day 14 post-HSCT (p=0.016), but no significant association was observed between the protein levels of ANP32A and the occurrence of aGvHD (Table 6.6).

Table 5.4 Comparison between the outcome for the gene expression studies in PBMCs and protein level analysis in serum.

Gene ID	Gene Expression in PBMCs	p value	Protein Expression in Serum	p value
LGALS7	X		↑	p=0.021
MSR1	↓	p=0.042	X	
PSTPIP1	X		X	
CXCL9	↑	p=0.009	X	
HTRA1	X		X	
C1QTNF7	X		X	
ANP32A	↓	p=0.016	X	
PIK3AP1	X		X	

X: no significant association between the gene expression levels/protein expression levels and the incidence of aGvhD
↑: Expression of the gene/protein was upregulated in patients developing aGVHD post-HSCT
↓: Expression of the gene/protein was downregulated in patients developing aGVHD post-HSCT

6.6. Discussion

Diverse cell populations, such as T cells, APCs, NK and T cells have been reported to play immunoregulatory roles in aGvHD (Morris and Hill, 2007). However, the molecular mechanism underlying the behaviour of these inflammatory cell populations in aGvHD is not yet fully determined. Recently, several analyses of gene expression profiling of GvHD target tissues including liver (Ichiba *et al.*, 2003), skin (Sugerman *et al.*, 2004), gastrointestinal tract (Snover, 1990) and peripheral blood (Alam *et al.*, 2012), in both murine and human GvHD have been reported. These studies provided comprehensive information supporting the molecular mechanism of acute GVHD. In order to gain a better understanding of acute GvHD, a comparison of the gene expression profiles of non-HLA genes: *C1QTNF7, LGALS7, ANP32A, HTRA1, PIK3AP1, PSTPIP1, MSR1* and *CXCL9,* in peripheral blood was conducted in the present study and the levels of their corresponding proteins in the serum of patients pre and post-HSCT was investigated. These genes were identified as part of a patent developed by Prof Anne Dickinson, Prof Ralf Dressel, Prof Bent Rolstadt and Lutz Walter (MHC genes and risk

of graft versus host disease: https://patentscope.wipo.int/search/en/ WO2012080359), where the inventors identified not only rat and human MHC and NKC genes, but also non-MHC and non-NKC genes that are dysregulated during GvHD.

In order to perform optimal normalization of gene expression patterns by RT-PCR in blood samples collected pre and post HSCT, a set of 6 candidate reference genes, *GAPDH, B2M, ACTB, EIF4A2, ATP5B* and *18S,* were tested for their stable expression in peripheral blood in relation to the different time points considered in this study (day 7 pre-HSCT and day 14, day 28, 3 months and 9 months post-HSCT) and in relation to the incidence of GvHD. *GAPDH* was chosen as the reference gene of choice for normalization and determination of gene expression levels of the target genes, as this gave the lowest variation across samples.

In this study cohort, log transformed expression levels of the target genes showed that there was no significant association between gene expression and protein levels of HTRA1, PIK3AP1 or PSTPIP1 and the incidence of aGvHD. In blood, *C1QTNF7* was not expressed. However, LGALS7 showed significant association between its soluble levels and the incidence of aGvHD, whereby protein levels were upregulated at day 28 in patients who developed aGvHD.

LGALS7 is implicated in modulating cell-cell and cell-matrix interactions and has previously been reported to be specifically expressed in keratinocytes and found mainly in stratified squamous epithelium, so it's surprising that there a very low expression of this gene in PAXgene blood (Uhlen *et al.*, 2005; Ponten *et al.*, 2008; Uhlen *et al.*, 2010; Uhlen *et al.*, 2015). Soluble LGALS7 levels however, were found to be significantly upregulated in aGvHD patients at an early day-post transplantation (day 28). Soluble LGALS7 are pro-apoptotic proteins that function intracellularly, and were reported to be responsive to stress stimuli, such as cytokines (http://www.genecards.org/). As cytokine production is a major step during the pathophysiology of GvHD, induction of the shedding of the stress-induced LGALS7 proteins caused an increase of the protein levels of LGALS7 in the serum of aGvHD patients, thus justifying the observation made in this study where protein levels of LGALS7 were upregulated in the serum of patient post-HSCT. LGALS7 could also be shed in the blood as a result of GvHD in the skin, thus giving rise to elevated levels of soluble LGALS7 in serum of GvHD patients.

For HTRA1, gene expression was reported in the lymph nodes, liver, colon, ovaries and kidneys as well as the cytoplasm of cells of different tissues (http://www.proteinatlas.org/). However, there is no information available for *HTRA1* gene expression in the blood (Uhlen *et al.*, 2005; Ponten *et al.*, 2008; Uhlen *et al.*, 2010; Uhlen *et al.*, 2015) and thus, the observation made in this study showing no significant association between the gene and protein levels of HTRA1 and the incidence of aGvHD is in agreement with the current data.

PIK3AP1 was previously reported to be a B-cell specific protein adapter, which is known to activate the PI3K-AKT signalling pathway (Pimienta *et al.*, 2015). PIK3AP1 contributs to B cell development by linking B cell receptor (BCR), and alternatively, linking TLR signalling, a process preventing excessive inflammatory cytokine production (Uhlen *et al.*, 2005; Ponten *et al.*, 2008; Uhlen *et al.*, 2010; Uhlen *et al.*, 2015). Recent data revealed an important role for B cells in the pathogenesis of GvHD, whereby increased B cell activation and survival were reported by Allen *et al.,* to be triggered in patients with GvHD (Allen *et al.*, 2012). Despite PIK3AP1 paying a major role in B cell activation and inflammatory cytokine production, its gene expression pattern and protein levels shown in this study were not associated with the incidence of aGvHD. This outcome could be due to the timing of samples, as only early times point (day14, day 28 and day 100 post-HSCT) were considered in this study.

PSTPIP1 was reported to be involved in the regulation of endocytosis and cell migration in neutrophils (www.genecards.org). Schwab L et al., have previously illustrated the role of neutrophils in GvHD, showing that recipient neutrophil granulocytes impact the severity of GvHD through their activation and production of reactive oxygen species (ROS) in the GI tract. This ,in turn, enhanced the pro-inflammatory environment and accelerated the pathogenesis of GvHD (Schwab *et al.*, 2014). In this study, no association between the gene expression levels of *PSTPIP1* and the occurrence of GvHD was noted. This observation could be due to the low number of aGvHD patients with high grade GvHD (grade 3, n=5) recruited for this study.

There was no expression of C1QTNF7 mRNA in PBMCs and its serum protein levels showed no significant association with the incidence of aGvHD. Information collected from Genecards and the Human Protein Atlas regarding C1QTNF7 was scarce; no expression was reported in PBMCs and low levels of C1QTNF7 were reported in the extracellular compartment (www.genecards.org; www.prtoeinatlas.org). These levels

maybe have been too low at the time points investigated to show any association with GvHD incidence.

Statistical analysis showed a decrease of MSR1 and ANP32A gene expression levels in blood samples from aGvHD patients. Chen Y et al, suggested in a recent study investigating tumour suppressor function of *MSR1* in leukaemia stem cells of chronic myeloid leukaemia (CML), that *MSR1* supresses the proliferation of leukaemia stem cells (LSCs), and that *MSR1* deletion causes acceleration of CML development (Chen *et al.*, 2011). MSR1 was reported to be interacting with *ANP32A* (Warde-Farley *et al.*, 2010). ANP32A plays many roles in cells including apoptosis, necroptosis, transcriptional regulation, mRNA export and cell cycle control (Rainer et al., 2013). Rainer B et al. highlighted that a there was a restricted expression of ANP32A to poorly differentiated tumours and haematopoietic stem cells (Rainer *et al*., 2013). Such specific characteristics of ANP32A in HSCs are reflected in the finding of the current study where in aGvHD patients, expression levels of *ANP32A* were downregulated. Studies have previously shown that ANP32A expression is regulated by miR-21, which is responsible for tumour growth (Schramedei *et al.*, 2011). In aGvHD rat skin explant model however, high levels of ANP32A gene expression were associated with aGvHD (Novota *et al.*, 2011b).

Both *MSR1* and *ANP32A* interact with *CXCL9,* and in the present analysis there was an upregulation of *CXCL9* levels in patients developing aGvHD. Several have studies investigated the role of chemokines in aGvHD. Ji LH. et al, reported that CXCL9 levels increased one week before aGvHD was diagnosed and significantly correlated with the severity of aGvHD (Ji *et al.*, 2006). Expression of IFN-γ has been associated with a GvHD, even though the pathogenetic importance is controversial (Yang et al., 2005). Interferon-γ is a potent inducer for the expression of the CXCR3 ligands CXCL9, CXCL10 and CXCL11 (I-TAC) (Farber et al., 1997; Cole et al., 1998). Recent studies have shown by gene expression profiling that pro-inflammatory chemokines are upregulated in different target organs of GvHD (Ichiba et al., 2003; New et al., 2002). In addition, the influence of chemokines on the recruitment of activated CD4+ T cells into the skin of GvHD patients has been elucidated (Piper et al., 2007). CXCL9 promoted aGvHD at high expression levels while interacting with ANP32A to promote proliferation and differentiation of HSCs and MSR1 to decrease the proliferation of leukemic cells (Piper KP et al., 2007). Soluble levels of CXCL9 were also investigated by Kitko et al, and they showed that plasma levels of CXCL9 were elevated at the onset

of cGvHD, but they did not observe this in patients with cGvHD for more than 3 months (Kitko *et al.*, 2014).

In summary, this study demonstrates differential expression of a panel of non-HLA, immune response-related genes (C1QTNF7 with *MSR1, CXCL9* with *CXCL11* and *CXCL10*, and *LGALS7*) and their respective proteins in acute GvHD. Further validation of these finding in a larger cohort is needed to confirm the results of this study.

Chapter 6. MicroRNA profiling in GI biopsies of patients with aGvHD

6.1 Introduction

Since their discovery, miRNAs have become established as crucial regulators of gene expression in plants and animals (Lee *et al.*, 1993; Wightman *et al.*, 1993; Reinhart *et al.*, 2000; Alvarez-Garcia and Miska, 2005; Wienholds *et al.*, 2005). In most organisms there is a limited number of miRNAs compared to the number of mRNAs and proteins. As an example, the human genome is believed to encode ~1000 miRNAs (Friedlander *et al.*, 2014), whereas the number of mRNAs is typically estimated at 30,000 (Strachan and Read, 1999). However, one miRNA may regulate hundreds of mRNAs and, as a result, may have a substantial effect on gene expression networks. MicroRNAs are believed to target more that 60% of mammalian and human transcripts (Friedman *et al.*, 2009) and thus, their expression patterns can be rich in biological information.

MiRNA expression profiling studies have helped identify miRNAs that are involved in the regulation of various processes, including organism development and the establishment and maintenance of tissue differentiation (Alvarez-Garcia and Miska, 2005; Wienholds *et al.*, 2005). MiRNAs have also been investigated as molecules for the reprogramming of cell fate in stem cell applications, as well as being applied as biomarkers for identifying the tissue differentiation state of cancers of unknown tissue origin (Lu *et al.*, 2005; Rosenfeld *et al.*, 2008). MiRNAs have been shown to be well preserved in a range of specimen types including blood plasma or serum, urine and formalin fixed tissue, and are also measurable with a much greater sensitivity than proteins (Ma *et al.*, 2009; Blondal *et al.*, 2013). Accordingly, miRNA profiling has become of interest to investigators working in diverse research areas of biology and medicine and there is considerable interest in the development of miRNAs as biomarkers for diverse molecular diagnostics applications, including cancer (Lu *et al.*, 2005; Rosenfeld *et al.*, 2008; Boeri *et al.*, 2011), cardiovascular and autoimmune diseases (Tili *et al.*, 2008) and forensics (Courts and Madea, 2011).

MiRNA profiling applications are growing. Comparing miRNA profiles between the different stages of disease development can facilitate identification of miRNAs involved in developmental transitions or cell differentiation (Alvarez-Garcia and Miska, 2005; Wienholds *et al.*, 2005). MiRNA expression patterns can be cell-type-specific and specific miRNAs can function to buffer developmental transitions and/or to maintain differentiation states. Many studies investigating gene expression patterns (Yao *et al.*,

2007; Bizuayehu *et al.*, 2012) are not well characterised at the genomic or transcriptomic levels and therefore, miRNA profiling is a powerful approach to identify novel miRNAs in this setting. Consequently, this could lead to the investigation of miRNA–mRNA and miRNA–protein interactions using techniques such as crosslinking immunoprecipitation (CLIP) (Ule *et al.*, 2003; Ule *et al.*, 2005). In the case of RNA sequences that are bound by protein of interest (for example the argonaut protein), they can be detected by high-throughput sequencing (HITS) in a combined approach known as HITS-CLIP or photoactivatable-ribonucleoside-enhanced CLIP (PAR-CLIP), in which photoreactive analogues are also incorporated (Hafner *et al.*, 2010). Along with interaction identification, miRNA profiling can be analysed with other large scale genomic data sets to identify specific miRNAs in the context of gene regulatory networks. This can be performed using *in silico* tools such as MAGIA (Sales *et al.*, 2010) and mirConnX (Huang *et al.*, 2011), as well as with databases of miRNA expression pattern in human disease, such as miR2disease (Jiang *et al.*, 2009), miConnXaldo interface with the miR-Ontology Database (Laganà *et al.*, 2009) , which is a compendium of miRNA-phenotype associations in humans.

Although miRNA singatures have been investigated in the setting of HSCT (Serody, 2015), there is currently no detailed information in the litterature about miRNA profiling in the GI tract of GvHD patients. Althought GvHD may affect any organ, intestinal GvHD is particularly important because of its frequency, severity and impact on the general condition of the patient (Takatsuka *et al.*, 2003). It has been shown that the gastrointestinal tract plays a major role in the amplification of systemic disease because gastrointestinal damage increases the translocation of endotoxins, which promotes further inflammation and additional gastrointestinal damage (Takatsuka *et al.*, 2003).

6.2 Study aim

This study performed miRNA profiling in GI biopsies of GvHD patients, recruited at the Transplantation Centre of Regensburg post HSCT, in order to identify significantly dysregulated miRs in the GI tract of patients with aGvHD. This was achieved using the nCounter miRNA Expression Assay (Nanostring technologies) which screened for ~800 miRNAs in total RNA samples extracted from GI biopsies. In order to validate the finding of the profiling investigation, significantly dysregulated miRNAs were then

assessed in a separate cohort of samples using qRT-PCR. As miRs target more than 60% of the human genome (Friedman *et al.*, 2009), target prediction was carried out for the identified miRNAs using publically available databases including TargetScanHuman, Tarbase, miRPath, miRBase, miRWalk, TargetMiner, PicTar, miRBaseTracker and the IPA web-delivered application.

6.3 Results

6.3.1 Clinical characteristics of the study cohort

For this study, n=13 GI biopsies collected from patients post-HSCT were initially considered. There were 2 samples flagged for poor quality control by the nSolver Analysis software (Nanostring Technologies), as the positive controls detected in these samples did not return any results. Thus, only n=11 samples were taken into consideration for bioinformatics analysis. Clinical characteristics for the study cohort are shown in Table 6.1.

The nCounter® miRNA Assay was utilised for the screening (Nanostring Technologies) (chemistry of the technology is further explained in further in Chapter 2, section 2.8.1).

Table 6.1. Clinical characteristics of the study cohort (n=11)

Clinical Characteristics		N (%)
Patients gender	Female	3 (27.3)
	Male	8 (72.7)
Donors gender	Female	2 (18.2)
	Male	9 (81.8)
Adult		11 (100)
F to M		18.18
Patients age range (years)		24 - 63
Donors age range (years)		29-59
Relationship	SIB	3 (27.27)
	MUD	8 (72.72)
Deceased		4 (36.4)
Relapse		3 (27.3)
aGvHD	Grade 0	3 (27.3)
	Grade 1	3 (27.3)
	Grade 2	1 (9.1)
	Grade 3	3 (27.3)
	Grade 4	1 (9.1)
TCD		8 (72.7)
RIC		10 (90.9)

Abbreviations: F to M; Female to male, MUD: Matched unrelated donors, SIB: siblings, matched related donors, TCD: T cell depletion treatment, RIC: Reduced intensity conditioning

6.3.2 Bioinformatic analysis for investigation of miRs profiles associated with aGvHD

Raw microRNA profiling data was exported as RCC files and loaded on the nSolver Analysis software (Nanostring Technologies) (details about the software and the analysis process is further explained in Chapter 2, section 2.8.2).

Data analysis was set to compare miRs signatures in relation to the incidence and to the severity of GvHD. Normalised data along with the fold change data were imported into R (The R Project for Statistical Computing, v3.3.0), where p values were calculated using the independent samples t-test, data was visualized as volcano plots, dendograms and heat-maps (all scripts and analysis pipelines were developed by Kile Green, Human DC lab, Haematological Sciences, Institute of Cellular Medicine, Faculty of Medical Silences, Newcastle University, UK). Stratification of the data was

first based on the incidence of aGvHD, comparing no aGvHD (grade 0) to presence of aGvHD (grade 1-4).

Based on the incidence of aGvHD (grade 0 vs 1-4), data analysis showed that there was a significant variation in the levels of 4 miRs (Table 6.2).

Table 6.2 Significant miRs based on the incidence of aGvHD (grade 0 vs 1-4). MiRs in red had an increased expression in aGvHD (1-4), while those in green had a decreased expression in aGvHD compared to no aGvHD (0).

aGvHD (grade 1-4) vs no aGvHD (grade 0)		
MicroRNA ID	**Fold change**	**p value**
hsa-miR-1247-5p	2.05	0.029
hsa-miR-297	1.88	0.041
hsa-miR-34a-5p	-1.54	0.023
hsa-miR-455-3p	-1.26	0.024

Data visualisation was performed using volcano plots on R to show miRs with a significant variation in their expression levels in patients with no aGvHD and those with GvHD (Figure 6.1).

Volcano plots were constructed by plotting the negative log of the p-value on the y-axis, which results in miRs with low p-values (highly significant) appearing toward the top of the plot (Li, 2012; Li *et al.*, 2014). The x-axis is the the fold change between the two categories of data, based on the grades of GvHD considered for the analysis. The fold-change is used so that changes in both directions appear equidistant from the centre (Li, 2012; Li *et al.*, 2014). Plotting miRs in this way resulted in two regions of interest in the plot: those miRs that were found towards the top of the plot that were far to either the left- or the right-hand side. These represented values that displayed large fold changes (hence being left- or right- of centre) as well as high statistical significance (hence being toward the top).

Visualisation of the data showed a significant increase in the levels of expression of miR-1247-5p (p=0.029), miR-297 (p=0.041) and decrease of miR-34a (p=0.023) and miR-455-3p (p=0.024) in patients with aGvHD (grade 1-4) compared to no aGvhD (grade 0) (Figure 6.1).

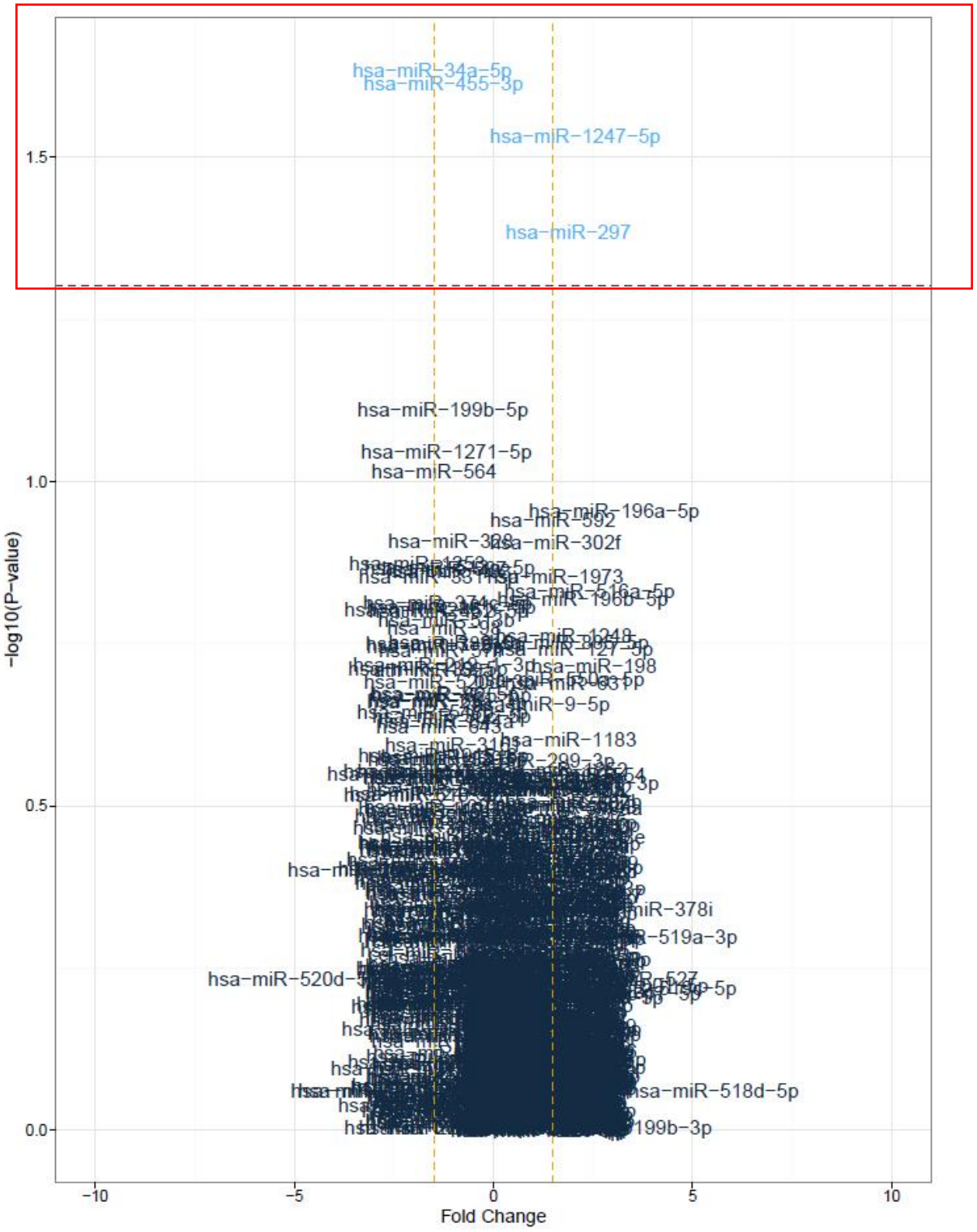


Figure 6.1 Volcano plot comparing miRs signature between no aGvHD (0) and the presence of aGvHD (1-4). A limit of significant variation in the levels of expression of miRs is marked by the red dotted line, while a fold change of 1 in the miR expression levels are marked by the dotted orange line (representing a fold change of 1 and -1 on each side).miRs with a significant variation in their expression levels are showed in light blue in the red box. MiR-1247-5p (p=0.029) and miR-297 (p=0.041) had increased expression and miR-34a (p=0.023) and miR-455-3p (p=0.024) were had decreased expression in aGvHD (1-4) compared to no aGvHD (0).

6.3.3 Validation form hsa-miR-34a-5p

6.3.3.1 Clinical characteristics of the validation cohort

The validation study was carried out for miR-34a, as the litterateur showed a strong link between this miR and HSCT outcome and it was shown there that this miR was associated with severe GvHD however, interestingly, in our cohort this miR's expression increased in patients with no GvHD. Validation was performed in a cohort of n=20 GI cDNA samples. All clinical characteristics of the patients from whom gastrointestinal biopsies were obtained are shown in Table 6.3.

Table 6.3 Clinical characteristics for the miRNA validation cohort (n=20)

Clinical Characteristics		**N (%)**
Patients gender	Female	11 (55)
	Male	9 (45)
Donors gender	Female	8 (40)
	Male	12 (60)
Adult		20 (100)
F to M9		(10)
Patients age range (years)		17 - 71
Donors age range (years)		15 - 56
Relationship	SIB	4 (20)
	MUD	16 (80)
Deceased		5 (25)
Relapse		4 (20)
aGvHD	Grade 0	6 (25)
	Grade 1	3 (20)
	Grade 2	8 (40)
	Grade 3	2 (10)
	Grade 4	1 (5)
TCD		13 (65)
RIC		11 (55)

Abbreviations: F to M; Female to male, MUD: Matched unrelated donors, SIB: siblings, matched related donors, TCD: T cell depletion treatment, RIC: Reduced intensity conditioning

Comparison between the study cohort (n=11) and the validation cohort (n=20) comprised of GI samples collected form patients recruited at the transplantation centre of Regensburg, Germany is shown in Table 7.9. Data showed that both cohorts were comparable and no significant difference was observed (Table 6.4).

Table 6.4. Comparison between the study and the validation cohort

		Study cohort (n=11)	Validation cohort (n=20)	p val*
		n (%)	n (%)	
Gender	Female Patients	3 (27.3)	11 (55)	0.180
	Male patients	8 (72.7)	9 (45)	
	Female donors	2 (18.2)	8 (40)	0.714
	Male donors	9 (81.8)	12 (60)	
Age (years)	Patients	24 - 63	17 - 71	0.060
	Donors	29 - 59	15 - 56	0.762
Relationship	Matched Unrelated donors	3 (27.3)	4 (20)	0.865
	Siblings	8 (72.7)	16 (80)	
T cell depletion		8 (72.7)	11 (55)	0.601
Relapse		3 (27.3)	4 (20)	0.973
Reduced intensity conditionning		10 (90.9)	11 (55)	0.350
Acute GvHD	Grade 0	3 (27.3)	6 (25)	0.518
	Grade 1	3 (27.3)	3 (20)	
	Grade 2	1 (9.1)	8 (40)	
	Grade 3	3 (27.3)	2 (10)	
	Grade 4	1 (9.1)	1 (5)	

*For continuous data an independent samples t-test was performed while a Chi-squared test was performed in the case of categorical data.

Investigation of the expression levels of the hsa-miR-34a-5p was performed using Taqman qRT-PCR and data analysis was carried out using GraphPad Prism v6.0 (see Chapter 2 section 2.6.2 and section 2.11).

Two endogenous controls including U6 and HY3 were used alongside the miRs during Taqman qRT-PCR for normalisation. An example of row data data showing the mean Ct values obtained from the Taqman qRT-PCR is depicted in Table 6.5.

Table 6.5 Example of Row Ct values obtained for the Taqman qPCR for miR-34a

Sample ID	Ct Values for endogenous controls		Mean Ct values for hsa-miR3-4a
	U6	HY3	
1	19.458	19.325	16.005
2	16.911	19.501	17.074
3	21.287	19.102	16.008
4	22.096	21.747	17.116
5	20.208	19.360	20.489
6	15.318	18.896	17.828
7	19.101	20.420	20.279
8	18.650	18.642	24.343
9	20.332	19.526	22.821
10	18.170	19.346	18.840

6.3.4 Variation of the expression levels of miR-34a-5p

Data analysis for the investigation of expression levels of miR-34a-5p in relation to the incidence of aGvHD in the validation cohort showed that miR-34a-5p expression was significantly increased in patients with no aGvHD (p=0.007) (Figure 6.3). This outcome is in agreement with the findings observed in the study cohort, where miR-34a-5p expression was shown to be decreased in patients with aGvHD compared to patients with no aGvHD (p=0.014).

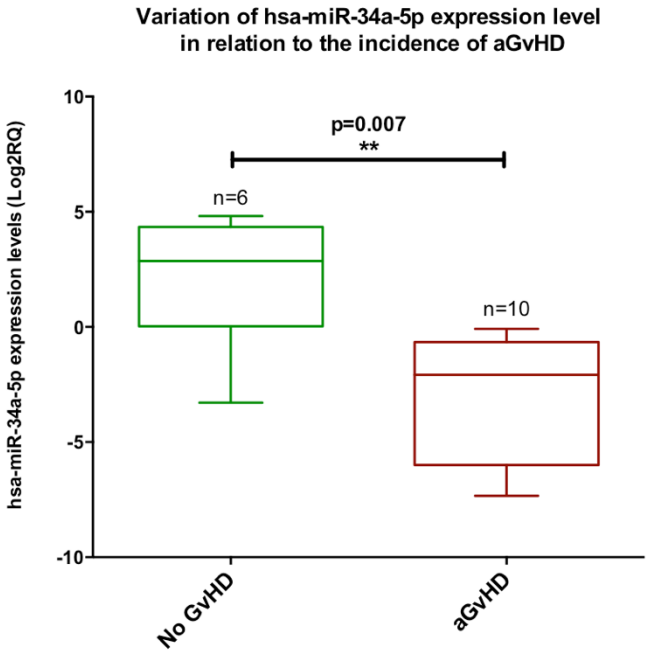


Figure 6.2 Variation of the expression levels of miR-34a-5p according to GvHD incidence. (A) Boxplot presentation of the variation of miR-34a-5p expression in relation to the incidence of aGvHD. MiR-34a-5p levels were significantly upregulated in the case of no aGvHD (p=0.007).

6.4 Discussion

Diagnosis of GvHD is usually performed clinically but despite this, an anatomopathological confirmation is often performed (Tomuleasa *et al.*, 2015). Several targets have been identified recently as promising GvHD biomarkers, however, no internationally recognised consensus has yet been established (Schultz *et al.*, 2006; Ye *et al.*, 2012). These targets include trappin-2, peptidase inhibitor-3 (PI3) and skin-derived anti-leukoproteinase, which were reported as elevated in dermatological GvHD (Paczesny *et al.*, 2013; Vander Lugt *et al.*, 2013). Patients with GI GvHD have shown increased levels of regenerating islet-derived 3alpha protein (REG3α) (Ferrara *et al.*, 2011; Levine *et al.*, 2012; Harris *et al.*, 2013) and serum markers have also been used as promising biomarkers including IL-2 receptor α (IL2Rα), hepatocyte growth factor (HGF) and IL-8 (Harris *et al.*, 2012; Goldberg and Giralt, 2013; Paczesny, 2013b; Sjoqvist and Snarski, 2013; Sung and Chao, 2013).

Recent advances in research identified miRs as potential biomarkers and it might be hypothesised that these short non-coding RNAs can also be used for the differential diagnosis of aGvHD. MiRs have been reported as important regulators of immune cells, including T cells (Ranganathan *et al.*, 2012) and recent studies have implicated miRs in the pathogenesis of aGvHD (Xiao *et al.*, 2013; Xie *et al.*, 2014). MiRs are also dysregulated in a variety of autoimmune diseases including systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis (Stanczyk *et al.*, 2008; Xiao *et al.*, 2014; Yan *et al.*, 2014; Zan *et al.*, 2014; Zhu *et al.*, 2014).

A better understanding of the regulation of human genes by miRs has an enormous potential. The identification of miR expression patterns in diseases and the understanding of their accurate involvement in pathogenesis process allows not only for the development of new potential molecular diagnostic markers but also for the development of new gene therapy strategies.

In this study, miR profiling was conducted in total RNA samples collected from the GI tract of GvHD patients. Damage in the GI tract is one of the main characterization of aGvHD in HSCT, during which donor T cells that are transferred along with the allo-graft execute an immunological attack on target recipient organs and tissues including the GI tract (Chen *et al.*, 2015b). It has been previously proposed that at the initial stages of aGvHD both TBI and high-intensive chemotherapy, as part of the conditioning regimen can reduce or eliminate the tumour load and thus cause sufficient immunosuppression to prevent graft rejection (Chen *et al.*, 2015b). However this treatment can also stimulate host tissues to secrete inflammatory cytokines which directly affect the epithelial cells of the GI tract allowing for the translocation of intestinal microbes and their products such as LPS into the systemic circulation (Chen *et al.*, 2015b).

The results of this microRNA profiling study identified miRs that were significantly altered in patients according to the incidence. MiRs that were shown to be significantly downregulated in patients with aGvHD (grade 1-4) compared to no aGvHD were miR-34a-5p and miR-455-3p. miR-34a was considered for further validation using qPCR. This miRs was chosen based on their association in the literature with various diseases and molecular mechanisms related to aGvHD.

It was revealed in this study that miR-34a-5p levels were significantly downregulated post-HSCT in patients with aGvHD (grade -14) (p=0.014) compared to patients with no aGvHD (0) which was confirmed in a separate validation study (p=0.0075). Depending on the cellular conditions and context (He *et al.*, 2007), over expression of miR-34a has been shown to induce cell cycle arrest (Sun *et al.*, 2008), senescence (Tazawa *et al.*, 2007) or apoptosis (Yamakuchi *et al.*, 2008). MiR-34a is upregulated by p53 in the case of DNA damage (Chang *et al.*, 2007; He *et al.*, 2007; Raver-Shapira *et al.*, 2007), but miR-34a can also be transcriptionally activated independently of p53 (Chang *et al.*, 2007; Christoffersen *et al.*, 2010). The miR-34a locus was reported to be deleted in neuroblastoma, breast, thyroid and cervical cancer (Welch *et al.*, 2007; Bagchi and Mills, 2008). In the case of colorectal, pancreatic, mammary, ovarian and renal cell carcinomas cancers, miR-34a expression was reported to be epigenetically reduced by hypermethylation (Vogt *et al.*, 2011). In mice, miR-34a administration inhibited tumour outgrowth (Tazawa *et al.*, 2007). In the present study, miR-34a expression levels were downregulated in the case of aGvHD (1-4) compared to no aGvHD (0) in GI samples taken from patients post-HSCT. This finding is not consistent with the observation made by Wang et al, where upregulated levels of miR-34a were shown to be associated with the incidence of aGvHD (Wang *et al.*, 2013). However, other investigations revealed consistent outcomes with the present study; In a recent study by Yanfei Ma et al, in papillary thyroid carcinoma patients, miR-34a overexpression promoted cell proliferation, stimulated colony formation, and inhibited apoptosis (Ma *et al.*, 2013). In the same study, the author reported that miR-34a overexpression led to activation of receptor tyrosine kinase class RET and downstream PI3K/Akt/Bad pathway (Ma *et al.*, 2013). Silencing of Akt in papillary thyroid carcinoma cells inhibited cell proliferation, reduced colony formation, and induced apoptosis, in accordance with the fundamental role of Akt in papillary thyroid carcinoma tumorigenesis (Ma *et al.*, 2013). Importantly, silencing of Akt reversed miR-34a overexpression-induced cell growth and inhibition of apoptosis (Ma *et al.*, 2013). This is in agreement with the observation made in this study where over expression of miR- miR-34a is capable of regulating hundreds of genes (Lal *et al.*, 2011) and its functions in GvHD development may depend on the type prophylaxis, the clinical and histological stage of GvHD in the GI tract and the sample size and type considered for this study.

In conclusion, both the miRNA profiling and the validation studies conducted here have found several miRs, specifically miR-34a, to be dysregulated in GI tract of aGvHD

patients. Further functional studies assessing the molecular mechanisms within which this miRs is involved may help identify novel biomarkers for the diagnosis of aGvHD and also present potential therapeutic targets.

The regulation of miRNAs may be the next innovation in pharmaceutical research. Although studies exploring the impact of miRs on HSCT outcome remain limited in number, recent investigations have demonstrated a tremendous potential for these molecules as diagnostic, prognostic and therapeutic markers. The clinical application of these findings is critical for better management and treatment of aGvHD. Although several basic questions regarding the biological principles of human miRs remain to be answered, the flux of data and research about these small non coding molecules have triggered the biotechnology community to start exploring the possibilities of miRs as therapeutic entities. From a scientific point of view, multiple miRs appear to be important potential therapeutic targets and chemistries exist that can inhibit miRs in a safe manner. The explosion of scientific research on miR biology is not surprising and has resulted in a considerable amount of innovation in this area as reflected by significant increase in the number of patent application increasing over the last 10 years (Van *et al.*, 2012).

To advance miRs in the clinic, a definite process of research and development involving optimization of suitable drug candidates and performing pharmacokinetics (PK), pharmacodynamics, and absorption, distribution, metabolism, and excretion studies are required. There is currently great excitement surrounding miRs as potent therapeutic entities.

Chapter 7. Concluding remarks and future directions

7.1 Novel findings and future avenues

This study investigated rs2910164 and rs2431697 in miR-146a, rs3027989 in IRAK1 and rs1051792 in MICA for their association with HSCT outcome. Results showed that the C variant in rs2910164 miR-146a was associated with increased NRM in patients post-HSCT in a discovery cohort, which was then validated in an independent validation cohort. For rs2431697 miR-146a, the presence of the T allele was associated with a trend towards an increased NRM in patients post HSCT. In the case of rs3027898 in IRAK1, which is a validated target of miR-146a (Chatzikyriakidou *et al.*, 2010), this study revealed that the C allele was associated with a decreased risk of relapse in patients which was more apparent when patients were homozygous for the C allele. This was also confirmed in the validation cohort. An increased risk of NRM was also observed when patients carried the C allele of rs3027989 in IRAK1 in the study cohort only. For rs1051792 in MICA, this study showed that the MICA-129 Met variant was significantly associated with low OVS post-HSCT, which was more apparent in the group of patients receiving non-TCD treatment. This result was also confirmed in a separate validation cohort. This study also revealed that the presence of the MICA-129 Met allele in patients was significantly associated with an increased risk of relapse, which was again confirmed in the validation cohort. It was also revealed that the presence of the MICA-129 Val variant in patients was significantly associated with an increased risk of developing aGVHD post-HSCT. It is therefore clear the impact of the SNPs in miR-146a, IRAK1 and MICA on HSCT outcome. The data reported demonstrates that there is increasing evidence to indicate that non-HLA polymorphisms have a major influence on HSCT outcome (Paczesny et al., 2013). Genotyping patients prior to HSCT for rs2910164, rs2431697 in miR-146a, rs3027989 in IRAK1 and rs1051792 in MICA may help to avoid high risk genotypes and thus can be translated into donor selection, therefore benefiting patients in need of a lifesaving transplant.

Future work will aim at investigating the involvement of rs2910164 and rs2431697 in miR-146a, rs3027989 in IRAK1 and rs1051792 in MICA mechanistically in HSCT. *In vitro* functional analysis through culture work, further analysis of the precise mechanism through which these SNPs intervene in HSCT is necessary in order to build evidence to support or refute the findings of this study.

This study also investigated the gene expression patterns of various immune response-related genes for their possible association with the incidence of GvHD post HSCT. The MHC class I chain related gene A (MICA) expression pattern was the main focus of this study and was first investigated in the GI tract of patients. It was demonstrated by the initial analysis that high doses of steroids supressed the expression of this gene. Stratification of the data based on the steroid doses showed that high MICA expression levels were associated with no apoptosis in the GI tract of patients post-HSCT and thus no active GIGvHD. A comparison between the mean expression levels of MICA mRNA within different genetic groups (dominant (GG), additive (GA) and recessive (AA)) showed a clear association between high MICA mRNA levels and the Met/Met genotype. Immunofluorescence investigation of MICA protein expression in the GI tract of patients post-HSCT showed that MICA was expressed within the intestinal epithelial cells of the crypts. There was a significant decrease in MICA protein levels when comparing grade 0 GIGvHD to grade 3 GIGvHD. This significance was maintained when comparing grade 0 to grade 1 GIGvHD. Levels of soluble MICA in sera collected from patients at pre-HSCT, day +14, day +28 and 3 months post HSCT were investigated for their association with the incidence of aGVHD and it was demonstrated that sMICA levels were significantly upregulated in the serum of patients with aGvHD at 3 months post-transplantation. Observations made in this work regarding the involvement of MICA genotype, MICA gene expression and MICA protein levels in the GI tract on aGvHD have strengthened the observation that MICA plays a prominent role in the immune response. Molecular typing strategies for MICA genotype simultaneously with the clinical testing for the presence of MICA antibodies can be implemented in the clinic to aid in the donor selection process and thus help avoid the transplantation of MICA-mismatched grafts and lead to an improved survival. It may also be used to monitor GvHD and adapt treatments based on each patients conditions.

Future work will aim to validate the finding of this study concerning MICA gene expression in an independent validation cohort and will explore which specific cells and by which specific means soluble MICA molecules are acting in the GI tract and at which time point post-HSCT they start having an influence on the development of GIGvHD.

This study investigated the gene expression patterns of immune response-related genes including *C1QTNF7, LGALS7, ANP32A, HTRA1, PIK3AP1, PSTPIP1, MSR1* and *CXCL9,* in order to gain a better understanding of aGvHD pathophysiology. A

comparison of the gene expression profiles of these genes in peripheral blood with the levels of their corresponding proteins in serum of patients pre and post-HSCT was conducted.

The study showed that there was a significant downregulation in the gene expression levels of *MSR1* and *ANP32A* in aGvHD patients post-HSCT while a significant upregulation in the levels of *CXCL9* was observed in aGvHD patients. Investigation of the association between the levels of proteins and the incidence of aGvHD showed that there was a significant association between upregulated of LGALS7 protein levels and aGvHD (p=0.021).

Findings of this study prove that despite advances in HLA matching, GvHD remains the main significant complication of HSCT and that investigation of the non-HLA genetic background of this complication is crucial for a better understanding of the pathophysiology of aGvHD. Various other studies are investigating the expression pattern of gene encoding cytokines, chemokines, costimulatory molecules, DNA replication, protein folding and drug metabolism and this is key for the development of personalised medicine.

Future work will aim to validate the impact of these genes and proteins on the incidence of aGvHD is a separate validation cohort. Potential interactions between the variation of the gene expression and the protein levels will also be investigated. Further functional *in vitro* analysis are required to understand the molecular mechanism through which these genes act in HSCT settings.

The final aim of this study was to perform miRNA profiling in GI samples taken from GvHD patients for the discovery of miRNAs associated with aGvHD in the GI tract of patient after HSCT. Scanning for potential targets of the identified miRs was also performed using publically available databases. This study identified a set of microRNAs that were differentially expressed according to aGvHD incidence. MiR-34a was validated later on in a separate validation cohort.

Although several studies have investigated the impact of miRs in the setting of HSCT, the present study is the first to perform miR profiling in the GI tract of aGvHD patients. The GI tract is of a particular importance because of the major role it plays in the amplification of systemic disease as GI damage amplifies the translocation of endotoxins and thus, promotes further inflammation and additional GI damage.

The identified miR was reported to play crucial roles in the process of apoptosis, T cell activation and cytokine production, which are all key features of the pathophysiology

of aGvHD. The finding made here can be the first step toward the transition of these identified miRs from the profiling study, into the clinic as diagnostic and therapeutic markers.

Further validation of the outcomes of this study is required in a larger cohort. This study had investigated the basic level of the involvement of these miRs in the development of GvHD, future work will also take a step closer to the cellular level and take a closer look at the expression of these miRs in specific subsets of cells within the GI tract of aGvHD patients. This study will be necessary, as localisation of the expression of these miR is important in order to develop and discover new therapeutics.

7.2 Limitations

One of the limitations of this study was the occasionally missing clinical data for some of the patient cohorts included in the investigations. This is due to the fact that samples were collected from different centres and thus, the data retrieval processes were different in each country and different levels of authorisation were required to be obtained prior to clinical data management. Missing clinical data was also, in some cases, due to the inconsistencies in completion of data collection from complex datasets.

Clinical data and patient selection processes are of crucial importance for all research due to the rarity of some types of samples such as GI biopsies from GvHD patients. This, at times, limited the number of samples included in parts of this study and made the selection process complicated.

Multicentre studies require a standardised HSCT procedures and GvHD prophylaxis but most importantly standardised clinical data storage standards. One of the limitations of this study was the availability of standardized clinical data from patients and their entry onto local and international databases. More than 6 months were needed for clinical data clarification, standardisation and entry onto the database for patients from Germany for example. Thus, for optimum accuracy of research in the HSCT field, more adequate methods of clinical data management are required. In Multicentre studies, a larger sample size does not necessarily result in larger study sizes, but rather in increasing the number of variants conferring small effects and thus, possibly affecting the outcome of statistical analysis.

As data was collected from different transplantation centres, the heterogeneity of disease types, along with the protocol variations can affect the therapeutic outcome of HSCT thus the need for more sophisticated statistical models and algorithms that take into consideration all these variables during data analysis.

For biomarkers in the field of HSCT, the main barrier that must be overcome is the validation of biomarkers in different types of allo-HSCT settings including conditioning intensity, donor source, TCD treatment and while taking into consideration various clinical factors. This can be achieved via the creation of statistical models incorporating various clinical factors. This can then be used for the calculation of risk factors for GvHD post-HSCT for use in the clinic.

Appendix

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

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AJ/AJ/MS

10 September 2015

Professor Matthew Collin,
Newcastle University,
Institute of Cellular Medicine,
Leech Building,
Framlington Place,
Newcastle upon Tyne.
NE2 4HH

Dear Professor Collin,

Trust R&D Project:	**6980**
Title of Project:	**Improving haematopoeitic stem cell outcome through studies of alloreactivity, immune reconstitution, biomarkers and novel therapies**
Principal Investigator	**Professor Collin**
Number of patients:	**300**
Funder:	**NUTH**
Sponsor:	**NUTH**
REC number:	**14/NE/1136**
IRAS Project Code:	**129780**
First participant to be recruited by:	**10 October 2015**

After completing the necessary risk and site assessments for the above research project, The Newcastle upon Tyne Hospitals NHS Foundation Trust grants NHS Management Permission for this research to take place at this Trust dependent upon:

(i) you, as Principal Investigator, agreeing to comply with the Department of Health's Research Governance Framework for Health and Social Care, and confirming your understanding of the responsibilities and duties of Principal Investigators by signing the Investigator Responsibilities Document. A copy of this document will be kept on file within the Joint Research Office.

(ii) you, as Principal Investigator, ensuring compliance of the project with all other legislation and guidelines including Caldicott Guardian approvals and compliance with the Data Protection Act 1998, Health and Safety at Work Act 1974, any requirements of the MHRA (*eg* CTA, EudraCT registration), and any other relevant UK/European guidelines or legislation (*eg* reporting of suspected adverse incidents).

(iii) where applicable, you, as Principal Investigator, should also adhere to the GMC supplementary guidance *Good practice in research* and *Consent to research* which sets out the good practice principles that doctors are expected to understand and follow if they are involved in research – see http://www.gmc-uk.org/guidance/ethical_guidance/5991.asp

The NIHR requires NHS organisations to recruit patients to CLRN Portfolio studies within 30 days from the date of this letter. The 30 day deadline for recruiting the first patient is therefore 10 October 2015.

UNIVERSITY OF NEWCASTLE

Haematological Sciences,
Institute of Cellular Medicine
The Medical School, William Leech Building
Newcastle upon Tyne NE2 4HH

CONSENT FORM - PATIENT

Title of Project:

Improving haematopoietic stem cell transplantation outcome

Please agree/disagree

1. I confirm that I have read and understand the information sheets for the above studies and have had the opportunity to ask questions. YES/NO

2. I give permission for DNA samples and tissue obtained during the study to be stored and used for future research subject to approval by a research ethics committee and we do not intend to come back to you for further consent but ethical opinion will be sought from the Local Ethics Committee for any future research. YES/NO

3. I give permission for my samples and anonymised data to be used in other related studies (given ethical approval) and shared between researchers within the EU. I have read the information sheets concerned with these other related projects. YES/NO

4. I understand that my participation is voluntary and that I am free to withdraw at any time and have my samples discarded, without giving any reason, without my medical care or legal rights being affected. YES/NO

5. I understand that sections of any of my medical notes may be looked at by responsible individuals conducting the research or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. YES/NO

6. I agree with any potential commercialisation of this research and I agree that my samples can be used in collaboration with a commercial company to further the research. YES/NO

7. I understand that my cells may be “immortalised”/or replicated in culture by specific viruses or molecules. YES/NO

8. I understand that my cells may be used in the development of “humanised mouse’ models. YES/NO

9. I give my permission for photographs to be taken of skin rashes. YES/NO

10. I agree to take part in the above study. YES/NO

PLEASE TURN OVER

_____	_____	_____
Name of Patient		Date Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher		Date
	Signature	

1 for patient; 1 for researcher; 1 to be kept with hospital notes

UNIVERSITY OF NEWCASTLE

Haematological Sciences,
Institute of Cellular Medicine
The Medical School, William Leech Building
Newcastle upon Tyne NE2 4HH

CONSENT FORM - DONOR

Title of Project:

Improving haematopoietic stem cell transplantation outcome

Please agree/disagree

1. I confirm that I have read and understand the information sheets for the above studies and have had the opportunity to ask questions. YES/NO

2. I give permission for DNA samples and tissue obtained during the study to be stored and used for future research subject to approval by a research ethics committee and we do not intend to come back to you for further consent but ethical opinion will be sought from the Local Ethics Committee for any future research. YES/NO

3. I give permission for my serum samples to be screened for antiviral activity so that my cells can be used for antiviral therapy for patient use. YES/NO

4. I give permission for my samples and anonymised data to be used in other related studies (given ethical approval) and shared between researchers within the EU. I have read the information sheets concerned with these other related projects. YES/NO

5. I understand that my participation is voluntary and that I am free to withdraw at any time and have my samples discarded, without giving any reason, without my medical care or legal rights being affected. YES/NO

6. I understand that sections of any of my medical notes may be looked at by responsible individuals conducting the research or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. YES/NO

7. I agree with any potential commercialisation of this research and I agree that my samples can be used in collaboration with a commercial company to further the research YES/NO

8. I understand that my cells may be “immortalised”/or replicated in culture by specific viruses or molecules. YES/NO

9. I understand that my cells may be used in the development of “humanised mouse’ models. YES/NO

10. I agree to take part in the above study. YES/NO

PLEASE TURN OVER

_____	_____	_____
Name of Patient		Date Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Signature	Date

1 for patient; 1 for researcher; 1 to be kept with hospital notes

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