

Reduced intensity conditioning with fludarabine, busulfan and anti-T lymphocyte globulin (FluBu3ATLG) for older patients with myeloid disease: determinants of outcome and the role of cytokine-based predictive modelling



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PLAGIARISM DECLARATION

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

I agree to deposit this thesis in the University's open access institutional repository or allow the Library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Dublin Library conditions and use and acknowledgement.

I consent to the examiner retaining a copy of the thesis beyond the examining period, should they so wish (EU GDPR May 2018). St. James' Hospital, Dublin is the data controller for all patient-related data. The processing of data undertaken as part of this study was done so with permission from said data controller following the submission of a Data Protection Impact Assessment (DPIA).

Full and informed consent was obtained from all research participants for use of personal and clinical data. The storage, processing and analysis of all patient blood samples was conducted with explicit full and informed consent. No animals were involved in this study.

This body of work is of my own creation, including data collection and analysis, recruitment of patients, oversight of sampling, laboratory analysis, thesis writing and dissemination. Oversight and guidance were provided by my supervisor, Dr. Nina Orfali. All clinical care was carried out under the auspices and in conjunction with the Department of Haematology at St. James' Hospital and laboratory processing with the Cryobiology and Stem Cell Facility at St. James' Hospital.

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Abbreviations

ADV: Adenovirus

AlloSCT: Allogeneic stem cell transplant

AML: Acute myeloid leukaemia

APC: Antigen presenting cell

ATG: Anti-thymocyte globulin

ATLG: Anti-T-lymphocyte globulin

BFUE: Burst-forming unit erythroid

BUCY: Busulfan and cyclophosphamide

CD: Cluster of differentiation

CFU-GM: Colony-forming unit – granulocyte macrophage

CIBMTR: Centre for International Blood and Marrow Transplant Research

CMML: Chronic myelomonocytic leukaemia

CMV: Cytomegalovirus

CNI: Calcineurin inhibitor

CPSS(Mol): CMML-specific Prognostic Scoring System (Molecular)

CR: Complete response

CRi: Complete response with incomplete haematological recovery

CSO: Central statistics office

CTL: Cytotoxic T-lymphocyte

DIPPS-Plus: Dynamic International Prognostic Scoring System - Plus

DLCO: Diffusion capacity for carbon monoxide (alveolar)

DLI: Donor lymphocyte infusion

DRI: Disease Risk Index

EASIX: Endothelial activation and stress index

EBAO: Ethidium bromide acrinide orange

EBMT: European Society for Blood and Marrow Transplantation

EBV: Epstein-Barr virus

ECOG: Eastern Cooperative Oncology Group

EF: Ejection fraction

ELISA: Enzyme-linked immunosorbent assay

ELN: European Leukaemia Network

FLAG(Ida): Fludarabine, cytosine-arabioside, granulocyte colony stimulating factor (idarubicin)

FluBu3: Fludarabine and busulfan (3 days)

FluBuATG: Fludarabine, busulfan and ATLG

GCSF: Granulocyte colony stimulating factor

GVHD: Graft-versus-host disease

GVL: Graft-versus-leukaemia

HCT-CI: Hematopoietic Cell Transplantation-specific Comorbidity Index

HHV6: Human herpes virus 6

HLA: Human leucocyte antigen

HR: Hazard ratio

HSV: Herpes simplex virus

ICC: International Consensus Classification

IFD: Invasive fungal disease

IFN: Interferon

IL: Interleukin

JAK/STAT: Janus kinase/signal transducer and activator of transcription

KPS: Karnofsky performance score

LDH: Lactate dehydrogenase

LIS: Laboratory Information System

MAC: Myeloablative conditioning

MAGIC: Mount Sinai Acute GVHD International Consortium

MAHA: Microangiopathic haemolytic anaemia

MAIT: Mucosa-associated invariant T-cell

MDS: Myelodysplastic syndrome

MF: Myelofibrosis

MHC: Major histocompatibility complex

MIPSS-70: Mutation-Enhanced International Prognostic Scoring System – 70

MLFS: Morphological leukaemia-free state

MPN: Myeloproliferative neoplasm

MRD: Minimal/measurable residual disease

MSD: Mesoscale Discovery

NCRI: National Cancer Registry of Ireland

NGS: Next-generation sequencing

NIMIS: National Integrated Medical Imaging System

NK: Natural killer

NMA: Non-myeloablative

NRM: Non-relapse mortality

OS: Overall survival

PBSC: Peripheral blood stem cells

PCR: Polymerase chain reaction

PI3K: Phosphoinositide 3-kinase

PIL: Patient information leaflet

PRES: Posterior reversible encephalopathy syndrome

PTCY: Post-transplant cyclophosphamide

PTD: Patial tandem duplication

PTLD: Post-transplant lymphoproliferative disorder

RAS/MAPK: Rat sarcoma virus/Mitogen-activated protein kinase

RIC: Reduced-intensity conditioning

RIPSS: Revised International Prognostic Scoring System

SAA: Severe aplastic anaemia

SOS: Sinusoidal obstructive syndrome

ST2: Suppression of tumorigenicity 2

STR: Short tandem repeats

TCI: Transplant conditioning intensity

TGF: Transforming growth factor

TNF: Tumour necrosis factor

UD: Unrelated donor

VAF: Variant allele frequency

WHO: World Health Organisation

Summary

Reduced intensity conditioning (RIC) allogeneic haematopoietic stem cell transplant (alloSCT) for older patients with myeloid disease is an area of evolving practice, with a range of treatment protocols varying by chemotherapy backbone, serotherapy, graft source and donor type. The National Adult Stem Cell Transplant Programme at St. James' Hospital in Dublin has provided alloSCT services to patients on the island of Ireland since 1984, and since the early 2000s has offered a combination of fludarabine, busulfan and rabbit anti-T lymphocyte globulin (ATG-Fresenius/Grafalon®) to eligible older patients with acute myeloid leukaemia, myelodysplastic syndrome, and myeloproliferative neoplasms. Demand for alloSCT is growing nationally and internationally and is expected to continue to do so based on population projections showing an aging and fitter population. Defining the optimal treatment approach for older patients requires a balance of acceptable long-term efficacy and a limited toxicity profile. Comparative prospective clinical trials of the various modalities are lacking and therefore longitudinal studies of consistent practice can help inform the current landscape.

We explore six principal questions in this study to identify potential areas for optimisation and to guide future prospective studies. The first is the objective assessment of biological frailty and impact of increasing chronological age on a range of transplant outcomes. The impetus for this investigative avenue is the year-on-year increase in transplant activity for older patients and rising median age at transplant. The historical age cut-off of 70 years for consideration of alloSCT at this institution was lifted shortly before commencement of this study and safety, efficacy and resource-related consequences of this change require exploration. The second objective is a review of optimal donor choice and the impact of the stem cell graft characteristics on outcomes. The landscape in donor choice has shifted in recent years, with increased safe use of haploidentical donors, with the attendant alternative post-transplant cyclophosphamide (PTCY)-based approach to conditioning. The position of ATG-based regimens are therefore under increased scrutiny, in addition to alternatives to the traditional busulfan alkylator backbone. The deterministic role of donor and recipient variable matching is similarly in evolution, particularly with new means to modulate CMV mismatch and the potential impact of donor age. The outcomes after transplant with graft cryopreservation, warranted during the COVID-19 pandemic, are now in more mature follow-up and explored in this patient group to determine if a preference for fresh product is necessary in modern practice.

The third area of interest is the role of ATG in balancing graft-versus-host disease (GVHD) and the graft-versus-leukaemia (GVL) effect. The speed and strength of immune reconstitution and the risk of post-transplant relapse underpins some of the controversy of ATG in RIC alloSCT. New challengers to its use have come in the form of PTCY, which has a firmly established role in haploidentical transplantation and has now supplanted ATG in the mismatched unrelated donor setting. It is not yet clear that PTCY will replace ATG in the HLA matched setting, as direct comparator trials are lacking. ATG may have further optimisation potential to limit interpatient variability, particularly with refinements in dose schedule and patient-specific dosing.

A lack of decision support and prognostic tools in allogeneic transplantation informed the fourth area of investigation: the role of the Endothelial Activation and Stress Index (EASIX) for sequential prediction of major transplant outcomes including survival and non-relapse

mortality (NRM). We sought to apply this score at sequential peri-transplant time-points and extend its utility by assessing the predictability of high-grade acute GVHD and relapse. The fifth domain examines the use of donor lymphocyte infusions (DLI) in three approaches: pre-emptive abrogation of relapse risk in those with mixed donor chimerism, prophylactic reduction of relapse risk in high-risk disease and augmentation of salvage strategies in post-transplant relapse. International use of DLI is variable and the balance of toxicity and effect is explored in this older patient group.

The final area of interest is the interplay of serum cytokines at sequential time-points through transplantation and their impact on clinical outcomes. Many of the complications of myeloid disease and the treatment thereof are driven by inflammatory signals and the cytokine secretome is known to modulate several transplant-related outcomes including GVHD, treatment mortality and relapse. 32 patients were recruited for prospective serum sampling and a 10-cytokine multiplex electrochemiluminescence panel was applied at the time-points of pre-transplant, day 0, 7, 14, 28, 60, 100 and 180. A combination of proinflammatory and anti-inflammatory cytokines were chosen, including IFN γ , IL1- β , IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A, IL-33 and TNF α .

143 sequentially treated patients between 2015 and 2021 were retrospectively reviewed, the results of which are documented in chapter 2. The additional clinical outcomes of the further 32 patients included in the cytokine analysis cohort were pooled to report on outcomes of 175 patients treated with a standardised protocol of fludarabine 180mg/m², busulfan 8mg/kg (IV) and ATLG 30-60mg/kg. The median overall survival had not been reached by median follow-up of 26.3 months and the 2-year overall survival was 72.35%. The cumulative incidence of non-relapse mortality was 12.13% at 2-years, and the incidence of relapse was 29.51% at 2-years. Acute GVHD was common at 60.34%, but moderate to severe disease was modest at 11.21% and chronic GVHD incidence was low at 17.3% by 2 years. Delayed immune reconstitution was notable, with a median CD4⁺ count of 113 x 10⁶/L by 6 months and frequent viral reactivation, although with low associated mortality.

Chronological age, including in a small group of patients over 70s, played no significant role in any major transplant outcome, although additional resources were required to fully assess and optimise the oldest patients before transplantation. The most significant impact by donor and recipient variable matching in this dataset was ABO matching, where inferior survival and increased relapse risk was seen in mismatched pairs. CMV mismatch saw decreasing significance following the introduction of routine letermovir prophylaxis. Donor age had no effect on serious transplant complications except for increased risk of acute GVHD in younger donors, which correlated closely with unrelated donor status. Interestingly, high T cell content in the donor graft including CD3⁺, CD4⁺, CD8⁺ and dual positive CD4⁺/CD8⁺ compartments and graft cryopreservation prior to infusion resulted in an increased relapse risk.

We hypothesised that recipient lymphocyte count, in addition to weight at the time of ATLG infusion could determine the prolonged lymphotoxic effects of ATLG in some patients, however in this study we did not identify any variance in clinical outcomes based on these parameters. No significant differences were noted between the two dose schedules of ATLG, and further prospective *in vivo* pharmacokinetic studies coupled with dose-stratified, randomised clinical trials in older adults are needed to design an individualised approach, and thereby optimally balance GVHD prevention without compromising the GVL effect.

The EASIX score proved robust, and its known utility in predicting survival and non-relapse mortality were replicated in this treatment group, endorsing its use as an additional monitoring tool through transplantation. An increased score correlated with inferior survival pre-transplant, at the onset of GVHD and 1-year post-transplant, in addition to increased risk of non-relapse mortality when measured pre-transplant. Additional utility was identified in predicting moderate to severe GVHD pre-transplant and at day 0, and increased risk of relapse at 1-year post-transplant.

DLI use, guided by chimerism studies was an important relapse prevention tool in this cohort, showing significant reduction in relapse in those with mixed chimerism at day 90 or beyond. DLI was well tolerated, including in the elderly, with no high grade acute GVHD, extensive chronic GVHD or NRM events occurring post-infusion. A small number of patients received prophylactic DLIs with positive outcomes, although further study in this application is needed. Sequentially dose-escalated DLI improved survival in post-transplant relapse, most commonly in combination with azacitidine therapy.

Serum cytokine secretion patterns offered an insight into donor and recipient inflammatory processes, their interdependence and correlation with clinical outcomes. Secretion followed patterns, including early surges as seen in IL2, IL-10, IL-17A, mid-transplant peaks as seen with IL-4, IL-6 and IFN γ or more subtle changes as in IL-1 β , IL-12p70 and TNF α . At various time-points, cytokines correlated with subsequent outcome, including increased risk of death in those with early high levels of IFN γ , IL-1 β or IL-6, increased risk of high-grade acute GVHD with high IL-6 or IL-10 between day 28 and day 100, and reduced acute GVHD in those with higher IFN γ at day 14 and 180. Further site-specific associations between several cytokines and acute GVHD were evident, including early IL-17A expression in those who developed gastrointestinal GVHD and IL-2 secretion in cases of liver GVHD, which may have a role as predictive biomarkers. The pleiotropic effect of several cytokines was evident, with time-dependent association with outcomes, including opposing effects depending on the stage of transplant. These data offer insights into the temporal biology of inflammation and recipient and donor immune interactions. A smaller panel of analytes at the significant time-points defined in this study would expand upon these preliminary findings and strengthen clinical associations, potentially paving the way for the introduction of a targeted cytokine panel in routine clinical monitoring.

The results of this study highlight the competitive performance FluBu3ATLG, with positive safety and efficacy signals, including in carefully selected elderly patients. These data endorse a preference for fresh rather than cryopreserved grafts, but do not validate donor age as a significant determinant of outcome. Attempts to optimise ATLG dosing and schedule are similarly encouraged by these data, which identifies a favourable GVHD-related toxicity profile but with room for improvement on relapse-risk and the pace of immune reconstitution. The EASIX score is endorsed by this study as a practical adjunctive tool for serial monitoring and should be recorded at pre-transplant, day 0, at onset of GVHD and at 1-year post-transplant across all transplant settings. The importance of supplemental therapy with DLI guided by chimerism analysis and in those who experience relapse is encouraged by these data and has proven efficacy and an acceptable toxicity profile. Additional studies of prophylactic DLI use, particularly alongside the ongoing studies of post-transplant prophylactic maintenance treatments are eagerly awaited. Rational interventional studies guided by the data presented here are feasible and will help answer some of the outstanding questions in RIC alloSCT for older patients with myeloid disease.

Lay summary

Stem cell transplant from another person (allogeneic haematopoietic stem cell transplant) can be used to treat several blood cancers in older patients, including acute myeloid leukaemia, myelodysplastic syndromes and myelofibrosis. The options for treatment continue to grow, including varying chemotherapies and immunotherapies to prepare the patient to receive donor stem cells (conditioning). The type of donor and the similarities between the donor and the patient need to be considered. The National Adult Allogeneic Stem Cell Transplant Programme at St. James' Hospital Dublin has performed such transplants for several decades. Since the early 2000s, a programme of reduced intensity treatment, which older patients and those with significant medical conditions are more suited to, has been available, with a treatment regimen including two chemotherapeutic drugs (fludarabine and busulfan) and an immunotherapy derived from rabbit serum called anti-T lymphocyte globulin (ATLG), which reduces the complications of donor and recipient immune system interactions. As the population ages, more patients are diagnosed with these diseases and may need a transplant. The best approach for the oldest patients is not clear but any approach must balance the benefit of potential cure with the potential to do significant harm. There are no large clinical trials to help identify the best treatment approach and we therefore rely on studies of the outcomes of past patients.

We explored six principal questions in this study with a goal of optimising our current treatment strategy and potentially identify areas that can be studied in greater detail in the future. The first is the assessments of older patients including how to identify frailty and how increasing age might affect fitness for transplant. The average age of transplant patients rises each year and the growth in the older population means more patients are going to need transplants. Immediately prior to this study, patients over the age of 70 were considered for transplantation for the first time in Ireland, and the results from these patients must be studied to ensure this has proven safe and effective. The second question considers the choice of the most appropriate stem cell donor and the matching of various characteristics between the donor and recipient including blood type, history of exposure to certain viruses (CMV) and how the stem cells are collected.

The third question considers the role of ATLG, which reduces the risk of donor immune cells attacking the recipient (GVHD) but may increase the risk of the underlying disease relapsing if the ATLG stays in the recipient system for too long. The use of ATLG is not universal and some transplant centres use alternative drugs, and with ATLG there are many different doses and timings that can be used. New ways of dealing with GVHD by replacing ATLG with post-transplant cyclophosphamide (PTCY) have shown good outcomes in some types of transplants, but not all. By optimising dosing, there may be a continued role for ATLG.

Predicting outcomes for individual patients is difficult in transplantation and therefore new tools to help guide prognosis are needed. The Endothelial Activation and Stress Index (EASIX), which uses simple data from routine blood tests, has been used to help identify patients at risk of dying during transplant. We intend to assess this ability in this patient group and additionally test if it can predict other outcomes such as GVHD and relapse. A further area of interest is the use of donor mature immune cells (donor lymphocyte infusion) to reduce the risk of relapse or help treat those who have relapsed and improve their survival.

The last area of interest concerns monitoring for inflammation-related blood proteins (cytokines) at different time-points through transplant and how they affect outcomes such as GVHD, treatment-related death and relapse. 32 patients were recruited to have additional blood samples taken at several time-points during their transplant, from pre-transplant to the day of stem cell infusion and then at increasing time-points up to 6 months post-transplant. We examined 10 different cytokines.

We initially reviewed the cases of 143 patients from 2015 to 2021 and their results are reviewed in chapter 2. When the additional 32 patients are added, the outcomes of 175 patients are reported. At 2-years post-transplant 72.35% of patients were still alive. The risk of dying from complications of the transplant (non-relapse mortality) was 12.13% at 2-years. The risk of the underlying disease relapsing was 29.51% at 2-years. Acute GVHD was common, seen in 60.34% of patients but only 11.21% had moderate to severe disease. Only 17.3% of patients developed longer-term GVHD (chronic GVHD). Return of a strong immune system was slow in this group, with only modest immune cell numbers at 6 months post-transplant, which was associated with various infections, although deaths from these infections were rare.

Older age, including over 70, did not result in any major complications from transplant, however more time and resources were required to ensure these patients were fit enough to proceed with transplant. A difference between donor and recipient blood type was associated with an increased risk of death and relapse, but the risk from CMV mismatch was less important now that there is access to preventative anti-viral medicines (letermovir). High levels of immune cells (T cells) in the stem cell infusion and freezing of the stem cells before administration (cryopreservation) resulted in an increased risk of relapse.

We hypothesized that the level of immune cells in the patient at the time ATLG was given could determine how long ATLG continued to last in the blood, however we could not identify this link in our study. No difference was seen in those who received either dose of ATLG and further studies to look at the drug levels in the blood after infusion in addition to a trial of different ATLG doses are needed.

The EASIX score was used to predict death and treatment-related mortality as was seen in other studies. We also noted it could predict moderate to severe GVHD and relapse when measured at various time-points. Use of DLI was guided by a test called chimerism, which measures the proportion of blood cells after transplant that are now being made by the donor stem cells. In those who had evidence of their own (and potential disease-containing) bone marrow continuing to grow, using DLI helped reduced the risk of their original disease relapsing. DLI was well tolerated in all age groups with no significant high-grade complications after infusion.

The cytokine patterns seen through transplant gave an insight into the inflammation processes, how the cytokines interacted and how they affected clinical outcomes. Various patterns of cytokine levels were seen. High levels of some were associated with the risk of death, GVHD or relapse. Many cytokines were associated with opposite outcomes depending on the stage of the transplant they were measured at.

This study shows that this treatment regimen is effective and has good safety outcomes, including in the oldest patients. The EASIX score is a useful aid to transplant monitoring and should be recorded in the future. DLI is an important tool for ensuring the success of this type

of transplant and is well tolerated. Further study in larger numbers of those over the age of 70 are required, as are studies into the impact of the stem cell graft characteristics, the best dose of ATLG to use and how cytokine levels can be incorporated into transplant monitoring.

Ethics

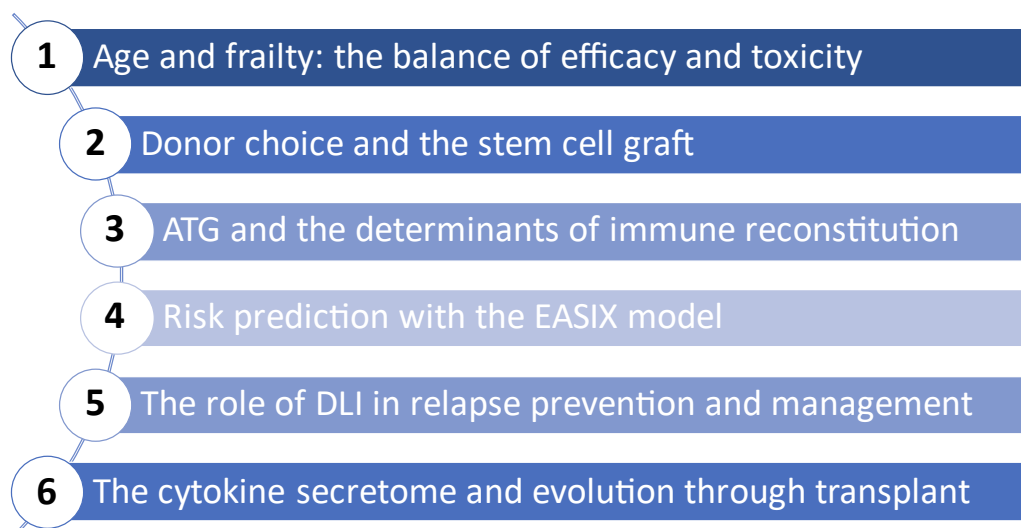
All patients in the retrospective component of this study provided express written consent at the time of transplantation for the use of data for research purposes. All patients enrolled in the prospective cytokine sampling phase of this study provided consent for biobanking of blood samples and the processing thereafter for research purposes. A copy of the biobanking patient information leaflet (PIL) and consent form is available in Appendix 1. Ethical approval for this study was granted by the Joint Research Ethics Committee (JREC) of St. James' Hospital and Tallaght University Hospital. A data protection assessment was completed in conjunction with this application and approved by the Data Protection Office at St. James' Hospital as compliant with GDPR regulations.

Project ID: 1800.

Funding

A research grant to the value of €12,000 was generously awarded by the Trinity/St. James' Cancer Institute (TSJCI) through competitive application for one of 10 Cancer Research Stimulus Awards (CREST). This project aligns with the TSJCI "Cancer Immunology" research theme. A report and oral presentation were provided to the TSJCI Patient Representative Group (PRG) as part of this award. All grant money awarded was used for the procurement of Mesoscale Diagnostic (MSD) U-Plex bespoke multiplex ELISA kits. The grant funds allowed for the purchase of 12 x 96-well plates and the accompanying reagents.

Scope of study; aims and hypotheses



Research strands

Strand 1: Age and frailty: the balance of efficacy and toxicity

Strand 1 will encompass both the retrospective and prospective arms of this study and will interrogate outcomes based on chronological age. Surrogates of biological frailty will be examined to determine if the FluBu3ATLG platform is safe and effective for elderly transplant candidates.

Strand 2: Donor choice and the stem cell graft

Strand 2 will examine the hierarchies of donor and recipient characteristic matching and their relative importance in the reduced intensity setting. The cellular content of the stem cell graft and its processing will be considered in the context of clinical outcomes.

Strand 3: ATG and the determinants of immune reconstitution

Strand 3 will assess the impact of ATLG on infectious complications, GVHD risk and the cumulative incidence of relapse. The relationship of recipient lymphocyte count and graft T cell content during ATLG exposure will be assessed to identify modifiers of ATLG persistence and delayed immune reconstitution.

Strand 4: Risk prediction with the EASIX model

Strand 4 will interrogate the predictive power of the EASIX model to predict a range of transplant outcomes including overall survival, NRM, moderate to severe acute GVHD and relapse at serial time-points through transplantation. This adjunctive risk model will be assessed alongside traditional scoring systems including the HCT-CI and the CIBMTR DRI.

Strand 5: The role of DLI in relapse prevention and management

Strand 5 will determine the effectiveness and toxicity profile of DLI use in older patients in 3 domains: pre-emptively for relapse prevention in mixed donor chimerism, prophylactically in a small number of patients with high-risk disease and therapeutically in patients with post-transplant relapse as part of a salvage strategy.

Strand 6: The cytokine secretome and evolution through transplant

Strand 6 will examine the pattern of cytokine secretion of 10 different proinflammatory and anti-inflammatory cytokines in a group of prospectively recruited patients with examination of the relative relationships of expression between cytokines over time and their potential association of clinical outcome. We will assess whether cytokine expression is predetermined by donor and recipient characteristics and transplant complications such as GVHD, or whether cytokine secretions play a deterministic and predictive role.

Phase 1 & 2 (Chapter 2 and 3)

- To assess performance of FluBu3ATLG RIC programme in modern era in a large national adult alloSCT centre and compare to international experience.
- To identify the impact of chronological age on key transplant outcomes including NRM, GVHD outcomes, relapse and survival.
- To interrogate the role of ATLG as an *in vivo* T cell depletion strategy in the context of emerging applications of PTCY by examining the determinants of immune reconstitution and the attendant risks of opportunistic infection and relapse.
- To determine if lymphocyte count at time of ATLG infusion correlates with ATLG exposure as seen in the paediatric setting with possible implications for individualized ATLG dosing schedules.
- To determine the impact of donor age, graft cryopreservation and graft T cell content on GVHD, NRM, relapse and survival in RIC alloSCT.
- To apply the EASIX risk prediction tool in a homogenous transplant population and assess its role in the prediction of GVHD and relapse.
- To identify trends in practice and the utility of DLI for relapse risk reduction and post-transplant salvage.

Phase 2 (Chapter 3)

- To compare outcomes between historical and contemporaneous groups of patients over a 7-year period and identify trends over time in patient population and transplant outcome.
- To establish a transplant-specific biobank repository for serial peripheral blood mononuclear cell (PBMC) and serum samples at key pre- and post-transplant timepoints.
- To chronologically profile the cytokine expression profiles of patients from pre-transplant to 6 months post-transplant using a 10-cytokine multiplex electrochemiluminescence modality.

- To clinically correlate cytokine levels with transplant outcomes including GVHD onset, NRM, relapse and survival.
- To correlate cytokine levels with clinical predictive models including EASIX.

Departmental Objectives

- To create a database for the comprehensive collection of transplant patient data that will facilitate retrospective data analysis, and which will be maintained to facilitate ongoing outcome analysis and the provision of clinical correlation for future translational work within the department.
- To formally schedule and standardize the measurement of post-transplant immune reconstitution within the transplant department.
- To establish and standardize the banking of post-transplant biospecimens for future translational research activity.
- To contribute to the formation of a formal cellular therapies collaborative research group between Trinity College Dublin, St. James' Hospital and the Irish Blood Transfusion Service.

Value of research

This study provides important insights into historical and contemporaneous practices and allows for comparison of the performance of this conditioning regimen against published comparators. This is an important endeavour as direct comparator trials are, as yet, unavailable, and the optimal treatment approach in this setting is not universally agreed. This study will allow our institution to critically appraise performance over time and offer accurate estimations to patients based on local data.

The findings of this study will help formulate targeted study at our institution in several domains, including ATLG dose optimisation, standardisation of DLI practice, audit of outcomes in elderly transplant candidates and informs translational study of biomarkers for risk stratification and prognostication.

Publications and presentations

1. Cryopreservation of allogeneic stem cells may be associated with increased early relapse compared with fresh product: a cohort study of Irish Patients. C Armstrong, H Foy-Stones, N Gardiner, P Hayden, E Conneally, CL Bacon, C Flynn, E Vandenberghe, P Browne, N Orfali. Poster presentation, EBMT 48th Annual Conference, Prague, Czech Republic (March 2022).
2. Donor lymphocyte infusions: a highly effective rescue remedy in reduced-intensity conditioning transplant. C Armstrong, R Finn, E Conneally, C Flynn, N Gardiner, H Foy-Stones, M Ni Chonghaile, G Lee, N Orfali. Poster presentation, Haematology Association of Ireland Annual Conference, Cork, Ireland (October 2022).
3. Post-transplant viral reactivations in recipients of anti-thymocyte globulin cause significant morbidity but limited mortality. C Armstrong, E Jones, B Crowley, E Conneally, C Flynn, N Gardiner, H Foy-Stones, M Ni Chonghaile, G Lee, N Orfali. Poster presentation, Haematology Association of Ireland Annual Conference, Cork, Ireland (October 2022).
4. The EASIX score predicts overall survival and late relapse in patients undergoing reduced intensity conditioned allogeneic stem cell transplantation for myeloid disease. C Armstrong, A Kelly, E Conneally, C Flynn, N Gardiner, H Foy-Stones, M Ni Chonghaile, G Lee, N Orfali. Poster presentation, Haematology Association of Ireland Annual Conference, Cork, Ireland (October 2022).
5. Allogeneic transplantation is safe and effective in older patients with myeloid disease: a 5-year review of national outcomes in patients conditioned with reduced-intensity fludarabine, busulfan and ATG. C Armstrong, E Conneally, C Flynn, N Gardiner, H Foy-Stones, M Ni Chonghaile, G Lee, N Orfali. Oral presentation, Haematology Association of Ireland Annual Conference, Cork, Ireland (October 2022).
6. The Easix score predicts overall survival and late relapse in patients undergoing reduced intensity conditioned allogeneic stem cell transplantation for myeloid disease. Chris Armstrong, Eibhlin Conneally, Catherine M Flynn, Nicola Gardiner, Hayley Foy-Stones, Aidan Kelly, Mairead Ni Chonghaile, Nina Orfali. Blood (December 2022) 140 (Supplement 1): 12900 – 12901.
7. Reduced intensity allogeneic transplantation combined with prophylactic/pre-emptive usage of donor lymphocyte infusion enables durable disease-free survival in older patients with myeloid disease. Chris Armstrong, Eibhlin Conneally, Catherine M Flynn, Nicola Gardiner, Hayley Foy-Stones, Mairead Ni Chonghaile, Nina Orfali. Poster presentation, Tandem Meetings, ASTCT and CIBMTR, Orlando, Florida, USA (February 2023).
8. Reduced intensity allogeneic transplantation combined with strategic donor lymphocyte infusion use offers an effective and tolerable curative therapy for older patients with myeloid disease. Chris Armstrong, Eibhlin Conneally, Catherine M Flynn, Nicola Gardiner, Hayley Foy-Stones, Mairead Ni Chonghaile, Nina Orfali. EBMT 49th Annual Conference, Paris, France (March 2023).
9. Predicting mortality and relapse in reduced intensity transplantation for myeloid disease: the EASIX score in practice. Chris Armstrong, Eibhlin Conneally, Catherine M Flynn, Nicola Gardiner, Hayley Foy-Stones, Mairead Ni Chonghaile, Nina Orfali. EBMT 49th Annual Conference, Paris, France (March 2023).

- 10.** The Combination of Fludarabine, Busulfan and Anti-T Lymphocyte Globulin is Well Tolerated and Offers Effective Reduced Intensity Conditioning Therapy for Patients with Myeloid Disease. C Armstrong, H Foy-Stones, N Gardiner, E Conneally, C Flynn, N Orfali. Poster presentation. EBMT 50th Annual Conference, Glasgow, United Kingdom (April 2024)
- 11.** Cytokine Secretion During Reduced Intensity Conditioning Haematopoietic Stem Cell Transplant for Myeloid Disease, An Opportunity for Outcome Prediction. Poster presentation. C Armstrong, H Foy-Stones, N Gardiner, D Doherty, A McElligott, F O'Connell, E Conneally, C Flynn, N Orfali. EBMT 50th Annual Conference, Glasgow, United Kingdom (April 2024)
- 12.** Transplant in the Elderly: FluBu3+ATLG is Safe and Effective in Myeloid Malignancies. Poster presentation. British Society of Haematology Annual Scientific Meeting, Liverpool, United Kingdom (April 2024).

Chapter 1

Introduction

Part 1: *Reduced intensity conditioning allogeneic stem cell transplantation for myeloid disease: a review of Irish and international practice.*

Reduced intensity conditioning and the history of transplant

The concept of allogeneic haematopoietic stem cell transplantation (alloSCT) as a curative option for haematological disorders has its origins in the late 1940s with recognition of radiation-induced bone marrow failure following the atomic bomb explosions that heralded the end of World War II. Early pre-clinical experimentation with rodents exposed to total body irradiation demonstrated the protective benefit of infusing allogeneic marrow cells and it was subsequently discovered that donor progenitor cells were capable of engraftment in the host bone marrow niche and thereafter producing the full gamut of physiological haematopoietic cells and sustaining life.

Throughout the 1950s and 60s, experimental work in both bone marrow failure patients and patients with leukaemia led to the discovery of many of the intricacies of stem cell transplantation including human leucocyte antigen (HLA) histocompatibility, graft-versus-host (GVHD) and graft-versus-leukaemia (GVL) effects, graft failure, transplant-related infections, and disease recurrence. From the late 1960s onwards advances in animal models led to refinement of a number of these issues, in particular histocompatibility and GVHD prophylaxis, with post-transplant methotrexate the first to show promise (2).

While many of the first transplants conducted in humans from this era were performed for inherited immune deficiencies, it was not long before patients with advanced haematological malignancies and severe aplastic anaemia were considered. Conditioning therapy pre-transplant for such patients initially centred on three agents: fractionated total body irradiation (TBI) and high doses of busulfan and cyclophosphamide (BuCy). These so-called myeloablative regimens were utilised to in part eradicate the host residual diseased bone marrow to allow growth and expansion of donor progenitors, but also to suppress the host immune system and prevent graft rejection. The issue of both acute and chronic GVHD led to the introduction of limited duration post-transplant calcineurin inhibitors, which remains a standard to this day. Many additional advances in supportive care occurred in tandem with transplant practice evolution, including therapeutic and prophylactic antimicrobials, organ function care and monitoring systems and advances in transfusion practice.

By the late 1970s and early 1980s, alloSCT had evolved from bench to bedside, with an international network of transplant centres emerging to offer this therapy to a wider population of patients. The first clinical transplant in Ireland was performed in St. James' Hospital in 1984 and over subsequent decades the centre has grown exponentially, with a national and cross-border catchment area. The 1980s also demonstrated the feasibility of using unrelated donor grafts as a viable alternative to sibling donors. By the late 1980s the discovery of the practicalities of using granulocyte colony stimulating factor (G-CSF) mobilised stem cells as an alternative to bone marrow grafts allowed for even further opportunities to offer transplant to suitable patients. During this era unrelated cord blood banks began to emerge, although the adoption of this technology in years since has been variable.

The intensity of conditioning remained a barrier for many older patients with diseases that were potentially curable by transplant, as evidence pointed to a significant increase in toxicity beyond the fourth decade of life. After this point the risk of organ toxicity from the conditioning agents and the intolerability of immune phenomenon post-transplant such as GVHD were progressively increased. As the median age of diagnosis of most haematological malignancies exceed this threshold, this resulted in transplant being a suitable treatment option for only a subset of patients with malignant disease. Similarly, younger patients with significant medical comorbidities were deemed ineligible.

In the late 1990s, Shimon Slavin and colleagues published the first *in vivo* results of a small set of patients who had conditioning with reduced intensity agents (fludarabine and anti-thymocyte globulin) with encouraging results (3). This was a critical step towards making transplant available to many older patients and those with co-morbidities. By accepting a higher risk of relapse in exchange for reduced treatment related mortality, reduced intensity conditioning (RIC) alloSCT has since proven effective in both disease control and tolerability, with many series showing reasonable outcomes compared to standard myeloablative (MAC) regimens (4, 5, 6, 7, 8, 9, 10, 11, 12, 13). These results have led to investigation of reducing intensity in younger patients, however it remains the case that for patients who are eligible for MAC alloSCT, published outcomes favour an intensive approach, most especially in patients who have detectable MRD prior to transplant. However, for most patients in RIC-eligible cohorts, a MAC approach is not a viable alternative, regardless of disease status pre-transplant (14, 15).

Advances in the understanding of the GVL effect are central to the success of the RIC approach, which demonstrates that conditioning therapy need not entirely ablate the host marrow to induce a therapeutic benefit (16, 17, 18). Indeed, the ability to directly induce such an effect by immunomodulation with donor mature lymphocyte infusions (DLI) has come to prominence in the RIC era. In addition to enhanced understanding of the GVL effect, RIC transplantation also offers insights into the relationship of GVHD and GVL (17, 18, 19, 20). As relapse risk is inherently increased, methods to harness this potent immunomodulatory effect were needed. Following on from work in 1980s with peripheral blood mobilisation of stem cells using G-CSF and apheresis methods, it became evident that there was an increased risk of GVHD with unmanipulated PBSC grafts due to the higher T-cell content compared to marrow grafts (21, 22). In some cases, particularly non-malignant disease such as sAA, this became the impetus for recommending bone marrow over PBSC to avoid GVHD where there was no GVL effect. In RIC transplants the increased risk of relapse is partially abrogated by favouring a PBSC source, leading to higher rates of GVHD and by correlation, an increased GVL effect in some. As most myeloid diseases are diagnosed later in life, this has led to a large proportion of modern transplants utilizing a RIC regimen, and therefore a PBSC graft.

In aggressive myeloid disease long-term survival is frequently poor in the absence of alloSCT (23). These diseases involve early bone marrow progenitors, and, most prominently in MPNs, involves the bone marrow stroma and microenvironment. Therefore, myeloablation is additionally effective in eradicating insidious disease and reduces the risk of post-transplant relapse. It is clear this is not a *sine qua non*, as in the RIC setting, despite non-myeloablative conditioning intensity, there is demonstrable therapeutic benefit to alloSCT and frequent achievement of long-term disease control and eradication even in advanced disease. This alludes to the centrality of the GVL effect at work deep within the bone marrow compartment (24, 25, 26, 27, 28, 29, 30, 31, 32).

A commonly used RIC regimen for myeloid disease in Europe comprises a combination of fludarabine, busulfan (FluBu) and ATG or ATLG, frequently with post-transplant methotrexate or mycophenolate mofetil (MMF) and calcineurin inhibitors (33, 34, 35, 36, 37, 38, 39, 40, 41, 42). With the exception of minor modifications, this combination has been a staple approach in RIC transplantation for over 20 years. Simple modifications of the total busulfan chemotherapy backbone can produce varying transplant intensities, which makes the combination attractively adaptable. However, to an extent this can cloud international comparison of case series where intensity definitions, particularly in the RIC domain, vary. The primary alternative agents in modern practice are melphalan and treosulfan and sometimes in conjunction with thiotepea. Although there are no randomized trials comparing the optimal alkylating agent, a number of retrospective studies allude to conflicting results both for melphalan and treosulfan (41, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58). A trade-off of reduced non-relapse mortality (NRM) in busulfan regimens appears to come at the expense of an increased relapse risk, leading to similar overall survival in many comparisons. However, preference of one approach over the other is increasingly a bespoke decision based on factors such as pre-transplant measurable residual disease (MRD) status, where increasing toxicity may abrogate the heightened relapse risk. While the Pre-MEASURE study suggests a more favourable outcome in those with MRD positivity at the time of conditioning using a MAC approach, in the older patient cohort where RIC is often the only choice, choosing between the varying RIC intensities may help reduce this risk (59).

The busulfan total dose varies in many studies, and although lowest and highest doses define the non-myeloablative (NMA) and myeloablative polar ends, a lack of firm consensus on the definition of reduced intensity obfuscates comparisons between series (60). Poorly standardised definitions of intensity such as “reduced toxicity” attempt to stratify this interface zone, however in the absence of consensus guidelines and randomised clinical trials, the clearest delineation is myeloablative vs. non-myeloablative, with the intervening doses lying on a spectrum. The NMA regimen FluBu2 commonly refers to a cumulative dose of 6.4mg/kg of busulfan, given over 2 days. The myeloablative FluBu4 regimen refers to a cumulative busulfan dose of 12.8mg/kg given over 4 days. The range of busulfan doses defined as RIC in published material ranges from 8mg/kg given over 3 days, to 9.6mg/kg, again over 3 days, and referred to as FluBu3. In Ireland, the standard protocol varies only by ATLG dose intensity between sibling and unrelated donor source and is modelled on the protocol employed in the EBMT prospective study of RIC alloSCT in primary and secondary myelofibrosis (61, 62, 63). The dose intensity used in Ireland is 8mg/kg total dose, given over 3 days, and lies in the RIC zone between NMA and MAC. When the components of the regimen are assessed using a modern European model for conditioning intensity characterisation, this amounts to a transplant conditioning intensity (TCI) score of 2.5, placing it in the lower end of the “intermediate” intensity category (64). For clarity, all references to FluBu3 in this study imply an 8mg/kg total dose unless otherwise specified and will be referred to as a RIC regimen, in contrast to the accepted MAC FluBu4 platform. The treatment protocol is provided in Appendix 2.

Outcomes in the published literature using a FluBu RIC approach vary by both the defined busulfan dose and patient cohort. Disease relapse is a larger contributor to mortality in RIC alloSCT as compared to MAC alloSCT across all disease subtypes and therefore reduction in NRM frequently does not translate to a survival advantage. Reported GVHD outcomes typically identify a 1 in 3 risk of grade II-IV GVHD and chronic GVHD in between 30 and 60% of patients. The median NRM risk across published studies is typically between 14 and 17%, but

frequently higher. Rates of relapse at 2 years have been reported to range from a modest 17% to almost 50%, with a median relapse risk of around 25-30% at 2 years across all settings. Relapse-free survival is again variable across studies and varies depending on the treatment population, ATG use and the follow-up duration (6, 26, 63, 65, 66, 67).

A recent attempt to expand transplant eligibility in myeloid disease came in the form of the augmented sequential RIC platform described in the FIGARO trial (68, 69). Initially promising as an attempt to address suboptimal disease control immediately pre-transplant, the results of the study showed that outcomes were similar regardless of MRD status pre-transplant, and that attainment of full donor T cell chimerism by 3 months had the strongest association with a successful outcome. In high-risk disease, where the time to development of a GVL effect may be surpassed by aggressive relapse or progression, it appears that the depth of remission induction is the most important determinant of whether disease quiescence can be maintained long enough for full donor engraftment and maturation to occur.

Age and transplant

The median age of diagnosis in AML, MDS and MPN is over 60 years of age and many patients are never considered alloSCT candidates, primarily due to frailty and life-time accrued comorbidity (70) (71, 72). There remains a large unmet need in this cohort of patients, where long-term disease-free survival rates, particularly in AML, are dismal in the absence of allogeneic transplantation (23). Higher risk disease is more common in elderly patients and therefore by disease characteristics alone there is an inherent demand for effective therapy in this age group (73). Over 60% of alloSCT recipients in Europe are over the age of 55 at the time of transplant, and trends in the oldest cohort over the age of 65 show increasing growth year on year, from a negligible proportion in the year 2000 to 6.7% by 2016 (74) (*Fig. 1 and 2*). Almost 60% of alloSCTs in Europe are performed for myeloid disease each year, which highlights the burden of disease, but also alludes to the fact that there is an even larger and underserved group of patients who, by virtue of the age, are considered ineligible for transplantation. It is important to note that objective evidence has shown that elderly patients stand to benefit from alloSCT and therefore, where feasible and tolerable, should be considered as candidates on a patient-by-patient basis (24, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86).

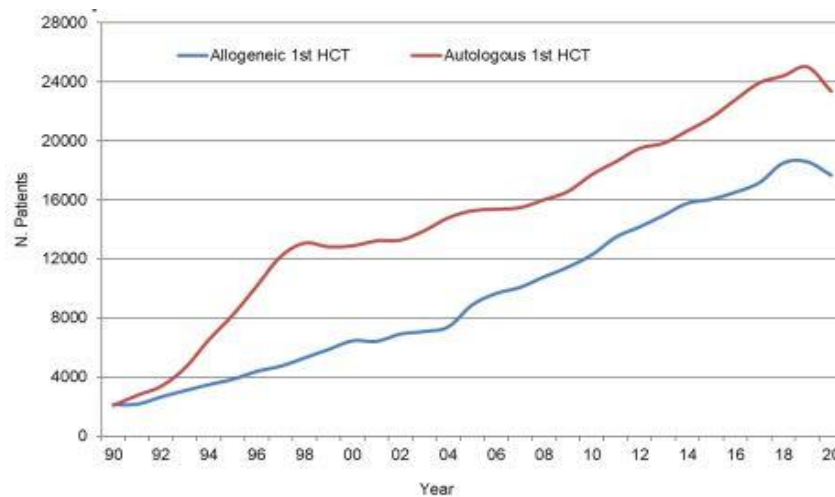


Figure 1: EBMT 2020 Transplant Activity: allogeneic and autologous activity. EBMT.

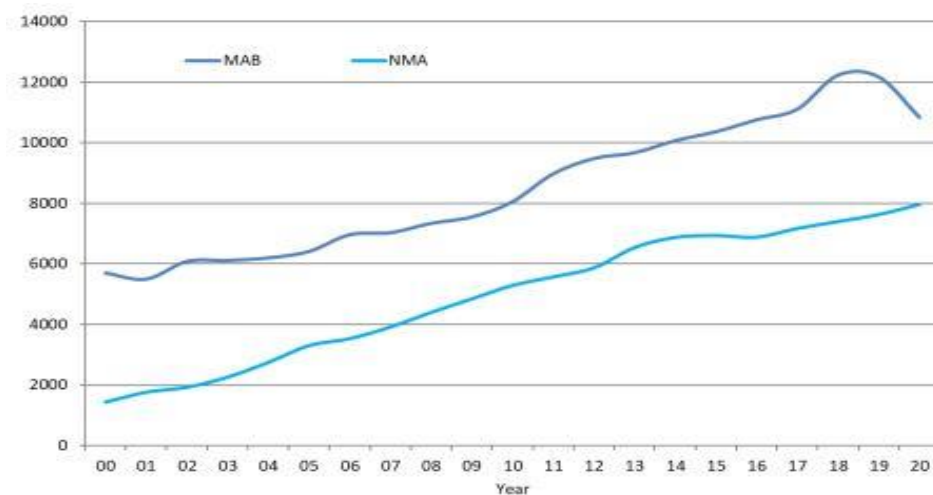


Figure 2: EBMT 2020 Transplant Activity: proportion of myeloablative and non-myeloablative transplants per year. EBMT.

While chronological age has historically determined candidacy, it has poor predictive power for outcomes post RIC transplantation (87). As the median age at transplant continues to climb, those over the age of 70 are increasingly considered for transplantation on an individual basis. With this comes the need for appropriate and specific tools for prognostication, and the resources for optimising prognosis pre-transplant (88, 89, 90, 91). Current predictive tools, such as the age-adjusted Hematopoietic Cell Transplant-specific Co-morbidity Index (HCT-CI), are useful, but are limited in sub-stratifying risk by age group and furthermore offer only a pre-transplant single snapshot, which does not account for the dynamic nature of transplantation in the elderly. While ongoing efforts to expand access for older patients attempt to address an unmet clinical need, the conflicting outcomes reported in older patients reflect the difficulties in accounting for population variation, selection bias, follow up and treatment algorithms (74, 92, 93, 94, 95). Similarly, the optimal conditioning regimen for those patients at the upper limits of age is not agreed (96).

The impact of immune regulation, T cell expression and maturation and organ function all play a role in successful recovery post-transplant. In addition to adequate disease control, objective measures of transplant success include adequate immune reconstitution to provide protection against common and uncommon microbes, without significant morbidity from GVHD. Age-related immunological phenomena such as clonal haematopoiesis, monoclonal gammopathy, thymic involution and T cell senescence are all relevant when considering transplant in the elderly, both from a recipient and donor point of view, where most sibling donors are over the age of 50 (97, 98, 99, 100, 101).

Geriatric assessment of candidates is desirable prior to, during and after transplant, however there are few standard validated assessment tools in this cohort, and a comprehensive multi-system assessment of frailty is time and resource intensive, although many adaptable and office-based adjuncts are currently under review (102, 103, 104, 105, 106, 107). Some assessments can be carried out by the transplant physician, such as frailty questionnaires and the timed up and go test, while others may require specific equipment such as grip-strength manometers and specialised interpretations by geriatric physicians and physiotherapists. Involvement of geriatric physicians with an interest in haemato-oncology and transplantation is desirable but is not always available. Critical to the utility of frailty assessments is the ability to capture evolution over time and following major transplant milestones and then providing sufficient resources to support and proactively prevent deterioration through targeted medical and psychosocial interventions (108). In addition to formal frailty assessments, there are several simple clinical risk tools such as the age-adjusted HCT-CI, Karnofsky performance score (KPS) and endothelial activation and stress index (EASIX), which capture elements of biological frailty and support decision making as part of a wider patient assessment.

The areas of highest risk for elderly patients during alloSCT are akin to those of younger patients, but are compounded by a background of reduced physiological reserves to overcome sequential, and sometimes concurrent, obstacles (109). Risks include direct toxicity from the conditioning therapy, early infection risk during neutropenia, viral and atypical infection during immunosuppression and the toxic effects of GVHD and its treatment. Steroid treatment, which is the mainstay of grade II and higher acute GVHD, exposes older patients to a range of troublesome side-effects which are more common in aging such as hypertension, hyperglycaemia, osteoporosis, cataracts, and neurocognitive disruption.

The routes by which patients with myeloid disease achieve disease control prior to transplant are continually being refined, with reduced toxicity induction regimens and targeted therapies. This means some older patients can achieve a meaningful remission without compromising organ function and remain transplant eligible, in comparison to historical counterparts, where lower-intensity induction treatments were less likely to lead to a sufficiently sustained remission. In AML, there have been a number of developments leading to effective reduced toxicity therapy, most prominently following the VIALE-A study, where azacytidine and venetoclax was shown to induce more durable remissions than azacytidine alone, broadening candidacy for RIC transplantation consolidation in select patients (110). In secondary AML and AML MRC, CPX-351 offers an alternative to standard induction and furthermore has cardioprotective benefits from the liposomal preparation (111, 112). Disease markers such as FLT3, IDH1, IDH2, CD47 and TP53 are currently being targeted in clinical practice or are under ongoing investigation, again leading to deeper remissions and widening windows for consideration of allogeneic transplantation (113, 114, 115, 116, 117, 118). Evolving practice in MDS mirrors many of the developments in AML and changes in disease

definition are likely to expand the access of patients with high risk MDS to AML-type therapy (119). In MPNs, newer generations of janus kinase (JAK) inhibitors are delivering both reasonable disease control pre-transplant and are also offering a path to salvage for relapsed disease post-ruxolitinib, and an additional window for transplantation in high-risk disease (120, 121, 122, 123). Unfortunately, the overlap syndrome chronic myelomonocytic leukaemia (CMML) remains a significant challenge and there have not been as many advances in induction therapies. Depending on where the individual disease lies on the spectrum of dysplasia and proliferation, differing strategies can be employed as a bridge to alloSCT, which remains the only curative treatment for this disease.

Demographic demand

Worldwide, advances in basic principles such as sanitation, nutrition and reduced smoking rates, mean people are living to ever older ages (124). Life expectancy at birth in many developed countries is now in the mid-ninth decade of life. Population projections predict a world population surpassing 10 billion by the year 2100 with an accompanying increase in disease prevalence and demand for transplantation. In line with this growth, cancer registries report a trend towards an increased disease incidence of myeloid leukaemia over the past 3 decades, although enhanced reporting strategies are likely to account for some of this apparent growth (125, 126) (*Fig. 3*). Incremental medical practice advances mean many patients are aging with less significant co-morbid diseases and with increased fitness (127). Indeed, a new metric introduced by the WHO now attempts to account this interplay of health and age in the form of the health life expectancy (HALE).

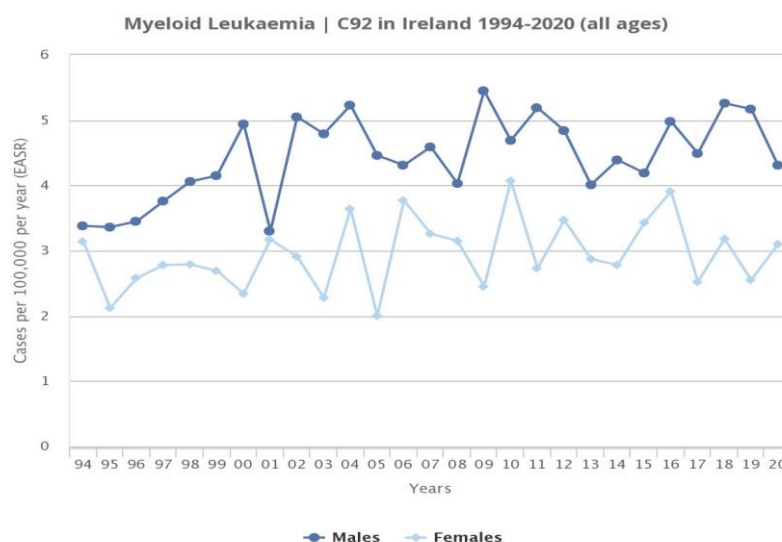


Figure 3: Myeloid leukaemia incidence in the Irish population, 1994 – 2019. NCRI.

Demographic predictive modelling in most developed nations now show a consistent shift in the average age of citizens, with falling birth and death rates. While Ireland has until recent times had a relatively young population with high birth rates, it too is moving towards an aging profile, and with it, the potential demand for reduced-intensity transplants is also growing, both due to the increased incidence of myeloid disease in older patients and expanding eligibility for transplant in this cohort (*Fig. 4 A-E*).

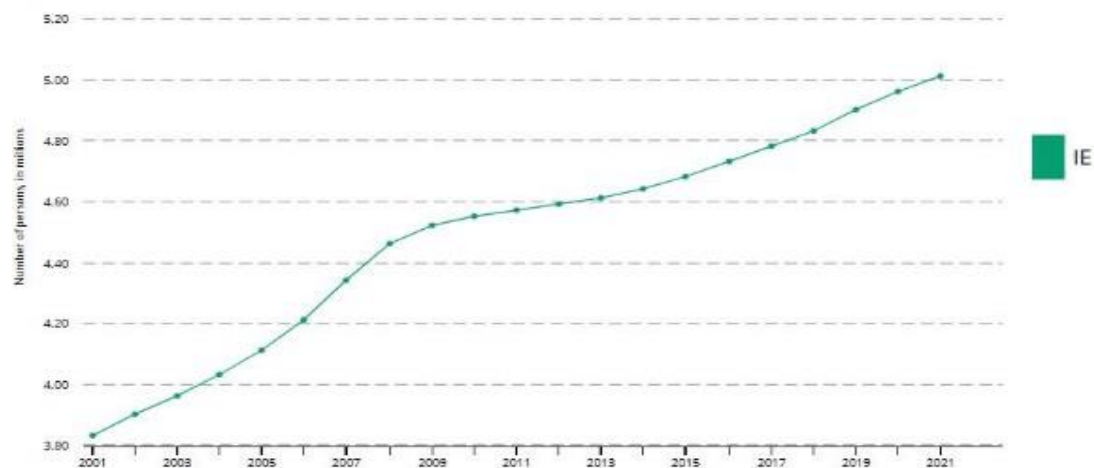
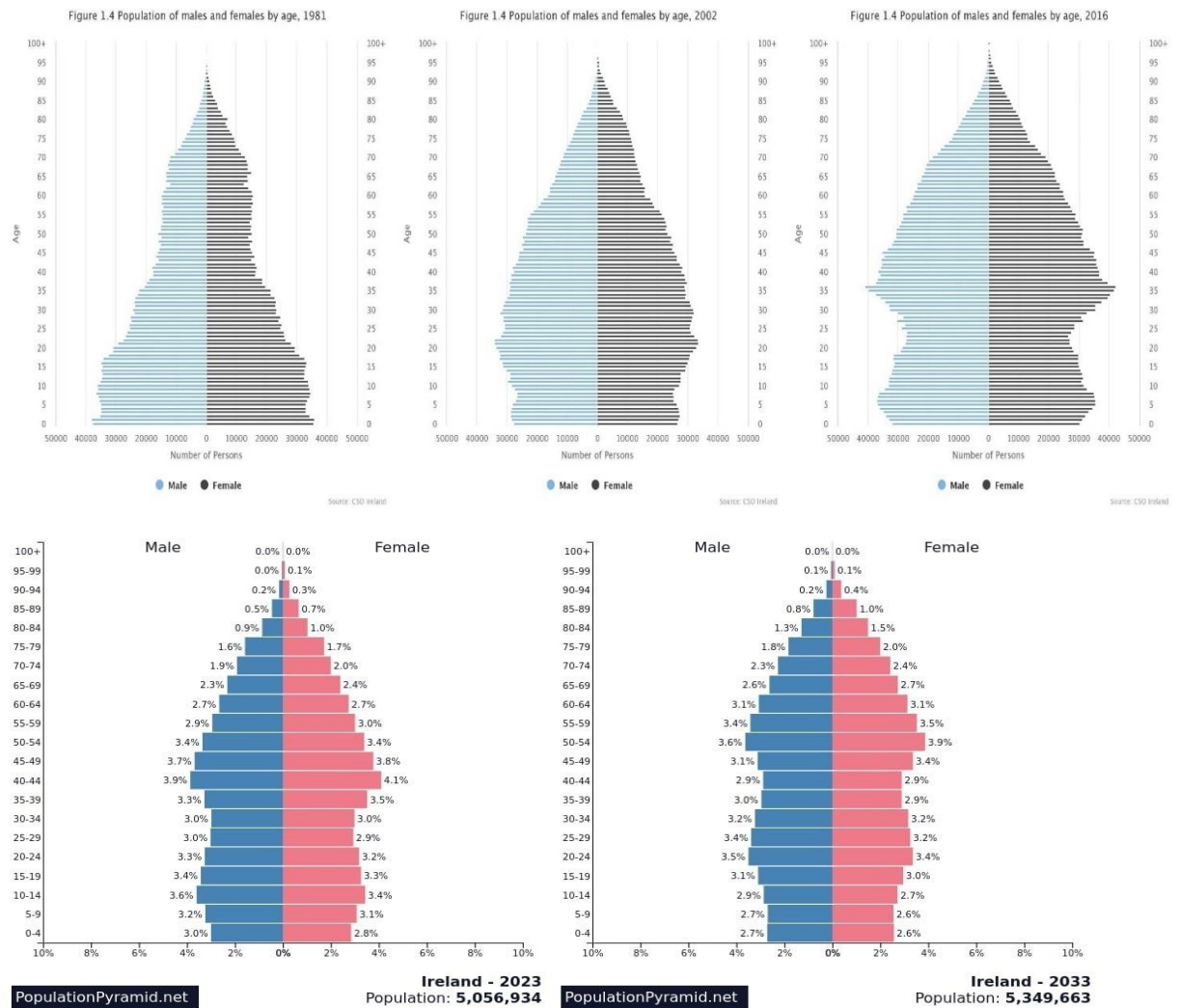


Figure 4. A: Ireland population pyramid 1981 census. B: Ireland population pyramid 2002 census. C: Ireland population pyramid 2016 census. D: Ireland projected population pyramid 2023. E: Ireland projected population pyramid 2033. Source: Central Statistics Office of Ireland & PopulationPyramid.net. F: Population growth, linear graph 2001-2021. Source: Eurostat.

The Centre for International Blood and Marrow Transplant Research (CIBMTR) and European Society for Blood and Marrow Transplantation (EBMT) registry activity reports highlight growing activity in the oldest cohort of patients over the age of 65 years old. In fact, where transplant activity has reached a stable level in most age groups, it continues to rise in the elderly, from a non-existent base in the early 2000s, to now accounting for almost a quarter of transplants for AML (128, 129, 130). As the eligibility criteria for transplant expand and the population at risk grows, issues of resource allocation, medical justice and equity are becoming more pertinent (131). It is therefore important to have a comprehensive and defined understanding of the role of transplant in older patients, in terms of patient selection, assessment and knowledge of both historical and contemporaneous outcomes in this cohort.

Diseases and classification changes

AlloSCT in myeloid disease is an established treatment paradigm for many patients as consolidative therapy for higher risk disease and as part of salvage strategies in relapsed disease. It has shown efficacy in all the primary myeloid disease families, including AML, MDS and MPN (132, 133, 134, 135, 136, 137, 138, 139).

Acute Myeloid Leukaemia

Patients with AML represent the largest individual cohort of allogeneic transplant recipients, although there is considerable heterogeneity in disease profiles and multiple indications for transplantation within the disease. With an older median age of diagnosis, by default, many patients undergoing consolidative alloSCT for AML are ineligible for myeloablative conditioning and are considered for RIC alloSCT. Disease-specific indications for alloSCT are in continual evolution, driven by refinements in diagnostic criteria, prognostication guidelines and by the impact of targeted therapies. AML has seen the largest changes in recent years both in terms of diagnostic criteria, clinically available diagnostic tests, prognostication tools and individualized therapies.

Recent changes to the classification of AML have seen some divergences in opinion from the World Health Organisation (WHO) and the International Consensus Classification (ICC) (140, 141). Many of the changes from previous editions, and between sources are categorical, and do not materially affect the role of alloSCT in these disease subtypes. Importantly however, the overlap between AML and MDS is now defined in different terms between the WHO and the ICC. The ICC recommendation of classifying MDS with blasts over 10% as MDS/AML will help allow more patients access AML-orientated clinical trials and acknowledges the biology of high risk MDS as a dynamic entity in transformation to overt AML. The WHO classification retains a blast cut-off of 20% for most cases of AML in keeping with previous classifications (142). Ultimately this will result in additional care being required interpreting comparative studies, both prospectively and retrospectively, to ensure similar populations are being studied.

Further subclassification of disease by molecular profile is driven by ongoing advances in clinical access to important molecular testing, which at the time of previous editions of

classification were not as widely available outside of research areas. Leukaemic exome gene sequencing has expanded significantly beyond single gene mutation testing for *FLT3* and *NPM1*, with many diagnostic panels now incorporating over 40 of the most implicated mutations in myeloid disease (143). Additional markers for germline predisposition are also available and help guide risk assessment, family screening and treatment choices. Combinations of diagnostic modalities such as conventional cytogenetics, chromosomal microarray, FISH, whole genome sequencing, single gene PCR testing, RNA sequencing of the leukaemic transcriptome and DNA-based NGS panels can accurately define and prognosticate individual patients at increasingly sophisticated levels. Retrospective reviews are ever more nuanced given major changes in diagnostic tools and even within a series timeframe the evolution of contemporaneous practice must be considered. This lends strength to the need for frequent and iterative large-scale, multi-centre, randomized controlled trials to lend sufficient power to observations.

New technologies are particularly important in guiding prognosis, with guidelines from the European Leukaemia Network (ELN) in both 2017 and 2022 increasingly utilising molecular diagnostics to stratify risk (144, 145). The most recent revision now incorporates a number of MDS-related molecular lesions in the adverse-risk category, in addition to the down-scaling of the adverse impact of *FLT3* mutations to intermediate risk, which in the previous guideline were adverse in the absence of a concomitant *NPM1* mutation or with high allelic ratio. This particular change is likely reflective of the synergistic benefit of *FLT3* inhibitors, primarily midostaurin, in addition to standard therapy. Undoubtedly in retrospect some transplant decisions may have been made differently with new information, and therefore caution is needed to consider risk in the context of the contemporaneous guidance and available therapies at the time of transplantation.

Further recent developments in next-generation sequencing, digital droplet technology and highly sensitive flow cytometric methods have also changed the landscape in treatment pathways for AML (146, 147, 148). Historically alloSCT in favourable risk disease was reserved for second complete remission (CR2). With the advent of serial MRD analysis during and post-induction and consolidation, impending relapse on a molecular level is identified prior to overt haematological relapse. In those proceeding to alloSCT, this allows for patients to progress to transplant in better overall condition and frequently requiring less or indeed no salvage therapy.

Current indications for alloSCT in AML are adverse risk disease in CR1, intermediate risk disease in CR1 following assessment of risk and benefit, any disease risk in CR2 and for favourable risk disease, MRD positivity predictive of relapse in those who have already achieved CR1 (149). The 2022 ELN risk classification is summarized in *Table 1*. Importantly in the RIC alloSCT domain, many of the high-risk features outlined in the adverse risk group occur more commonly in older patients. High-risk clinical features such as secondary AML due to antecedent MDS or MPN and therapy-related disease are also overrepresented in the elderly.

Table 1: ELN 2022 Risk classification of AML (145).

<i>Risk category</i>	<i>Genetic lesion</i>
<i>Favourable</i>	- t(8;21)(q22;q22.1)/<i>RUNX1::RUNX1T1</i> - inv(16)(p13.1q22) or t(16;16)(p13.1;q22)/ <i>CBFB::MYH11</i> - Mutated <i>NPM1</i> without <i>FLT3</i>-ITD - bZIP in-frame mutated <i>CEBPA</i>
<i>Intermediate</i>	- Mutated <i>NPM1</i> with <i>FLT3</i>-ITD - Wild-type <i>NPM1</i> with <i>FLT3</i>-ITD (without adverse-risk genetic lesions) - t(9;11)(p21.3;q23.3)/<i>MLLT3::KMT2A</i> - Cytogenetic and/or molecular abnormalities not classified as favourable or adverse
<i>Adverse</i>	- t(6;9)(p23.3;q34.1)/<i>DEK::NUP214</i> - t(v;11q23.3)/<i>KMT2A</i>-rearranged - t(9;22)(q34.1;q11.2)/<i>BCR::ABL1</i> - t(8;16)(p11.2;p13.3)/<i>KAT6A::CREBBP</i> - inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)/ <i>GATA2, MECOM(EVI1)</i> - t(3q26.2;q21.3)/<i>MECOM(EVI1)</i>-rearranged -5 or del(5q); -7; -17/abn(17p) - Complex karyotype, monosomal karyotype - Mutated <i>ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1, and/or ZRSR2</i> - Mutated <i>TP53</i>

Myelodysplastic Syndromes

MDS disease classification has also seen revision in recent years, with significant changes in nomenclature between the 2008 and 2016 WHO classification system, and further changes again in 2022 (71). Similar caveats to that of AML apply when comparing contemporaneous with historical groups of patients with MDS, with attention required to the classification schema used. MRD based decision-making has not been as widely used in MDS, where the core genetic lesions amenable to high sensitivity molecular monitoring are often AML-specific or AML-defining. Some gene fusion transcripts are measurable and highly sensitive multi-colour flow cytometry methods for disease-associated immunophenotypes can be used, but standardisation across laboratories is variable.

Risk stratification for MDS in practice continues to use the Revised International Prognostic Scoring System (IPSS-R) (150) and indications for alloSCT are primarily based on this system, where it is recommended for intermediate-2, high and very high-risk disease. Additionally, alloSCT is considered on a case-by-case basis in lower risk patients with additional risk factors or significant disease-related morbidity such as chronic transfusion dependence. Diagnostics have improved in MDS in tandem with those seen in AML, with many of the clinical available NGS panels having shared utility for defining many myeloid diseases. Access to these tests forms the basis of the increasingly used IPSS-Molecular (IPSS-M), which adds further clarity to individual patients in this heterogeneous disease family and is now supplanting the IPSS-R in the pre-transplant counselling session and clinical trials (151).

Myeloproliferative Neoplasms

Diagnostic criteria for MPNs have seen comparably less change in recent revisions of myeloid disease classification compared to MDS and AML. Myelofibrosis constitutes the largest indication for alloSCT in the MPN field. Diagnostic criteria and clinical features show a somewhat more homogenous profile than MDS and AML, but attempts to sub-classify and prognosticate the disease are ongoing (72). The primary disease indications for consideration of allograft are intermediate-2 and high risk primary and secondary myelofibrosis by the Dynamic International Prognostic Score Plus (DIPPS-Plus), and in rarer non-myelofibrosis MPNs, on a case-by-case basis. In myelofibrosis, additional prognostic information is gleaned from molecular analysis, which underpins the increasingly used Mutation Enhanced International Prognostic Score System for Transplant (MIPSS-70/v2), where mutations in *ASXL1*, *EZH2*, *SRSF2*, *IDH1* and *IDH2* help identify a higher risk group of patients who may benefit from alloSCT (152). In secondary myelofibrosis the MYSEC is seeing increased use as a validated risk score and the Myelofibrosis Transplant Scoring System (MTSS) helps assess risk at the decision to transplant in both primary and secondary disease (153, 154). Splenic debulking has recently been highlighted as an important pre-transplant consideration, however the optimal disease modification modality to bridge to alloSCT remains JAK inhibition, and indeed disease eradication is not necessary pre-transplant, allowing for a number of therapeutic pathways to transplant (155, 156, 157).

Chronic myelomonocytic leukaemia

CMML diagnostic criteria remain unchanged in recent revisions of myeloid disease classification, however the disease remains at the difficult liminal area between dysplasia and proliferation, with a marked heterogenous clinical profile within the patient cohort. Proliferative CMML and CMML-2 or lower risk CMML with additional risk factors are the currently accepted disease-based indications to consider alloSCT. Risk stratification has evolved with increasing molecular data and has now evolved from the CMML prognostic scoring system (CPSS) to the CPSS-Molecular (CPSS-M) with the addition of *ASXL1*, *NRAS*, *RUNX1* and *SETPB1* adding prognostic stratification (158, 159, 160). Unfortunately there remains no optimal treatment for CMML, with only modest responses reported to hypomethylating agents (161). Those who do achieve a disease response and are deemed eligible for alloSCT are frequently transplanted with RIC regimens, which remains the only curative option for this disease.

Assessment of risk

The pre-transplant consultation involves an assessment of disease biology, medical comorbidity, psychological reserve, physical fitness and incorporates a significant degree of patient education. There are both objective and subjective elements to this review, from quantifiable organ function assessments, to building rapport and probing patient engagement with the process. In many institutions, a limited amount of time is available to complete this review and frequently, the pre-transplant course is delivered at other centres. An important

element of the feedback to patients involves providing as accurate and bespoke as possible a risk assessment for the procedure. Such tools are limited, and no single one can truly capture the intricacies and dynamic nature of alloSCT. A significant limitation is the timing of their application, where a single snapshot in time may not account for the evolution and interplay of variables.

The CIBMTR Disease Risk Index (DRI) assignment tool is a validated system that can broadly categorise transplant patients into low, intermediate, and high-risk categories based on disease characteristics alone, with utility in grouping retrospective mixed-risk groups of patients. The score estimates survival based on disease stage and for AML and MDS, cytogenetic risk group. Although limited to broad estimations its application is simple and uses universally available data (162).

Two widely used assessments of general function include the Karnofsky Performance Score (KPS) and the Eastern Cooperative Oncology Group (ECOG) score. Although relatively crude in terms of patient fitness, they have shown decades of utility across a wide variety of haemato-oncological settings (163, 164). In the alloSCT setting, these scores can help group patients into broad cohorts, which aids with retrospective analysis. Additionally, as a numeric result, changes over time can be calculated to assess the effect of therapy at sequential time-points. A minimum pre-transplant KPS of 60 and a maximal ECOG of 2 is generally required to proceed to alloSCT.

The EBMT risk score incorporates additional factors including patient age, disease status, time-to-transplant, donor type and gender, to offer a more individualised risk assessment for NRM and OS. The score has been adapted and modified subsequently and is derived from a comprehensive dataset of over 50,000 patients (165). A more comprehensive validated risk assessment tool is the HCT-CI and the age-adjusted HCT-CI (built upon the Charlson Comorbidity Index), which are both designed to incorporate the historical co-morbidities of the patient pre-transplant and the objective measurements of organ function (166, 167, 168, 169). The score has been validated to accurately stratify patient outcome based on increasing score, which helps to plot an individual patient's risk and provide an objective estimate of the likelihood of NRM and overall survival. Primary limitations of the score include the static nature of the assessment, which does not account for clinical variability under extraneous stressors. For example, a patient with fragile renal function, which may decompensate readily during episodes of sepsis or inflammation, may demonstrate normal function between episodes and be unremarkable at the pre-transplant assessment. The score is also limited in sub-stratifying outcome for cohorts involving only older patients, as in the RIC alloSCT setting, most patients will score 1 point on the age-adjusted HCT-CI for age above 40. While this may be useful when comparing outcomes between younger and older patients, it is less discerning when reviewing groups of older patients in isolation. The age-adjusted HCT-CI has additional utility however in predicting outcome on a conditioning intensity basis, including non-myeloablative, reduced intensity and myeloablative and is widely recorded internationally as standard in transplant counselling clinics.

New risk predictive models are needed to inform the transplant decision-making process, in particular tools to identify those at high risk of severe GVHD and early relapse (170). Increasingly, artificial intelligence is driving pattern recognition and outperforming historical models, which is leading the way to exciting comprehensive personalised decision support in the transplant domain (171, 172). Accuracy of these assessments is enhanced by longitudinal and sequential timepoint sampling, which can identify trends over time and correlate

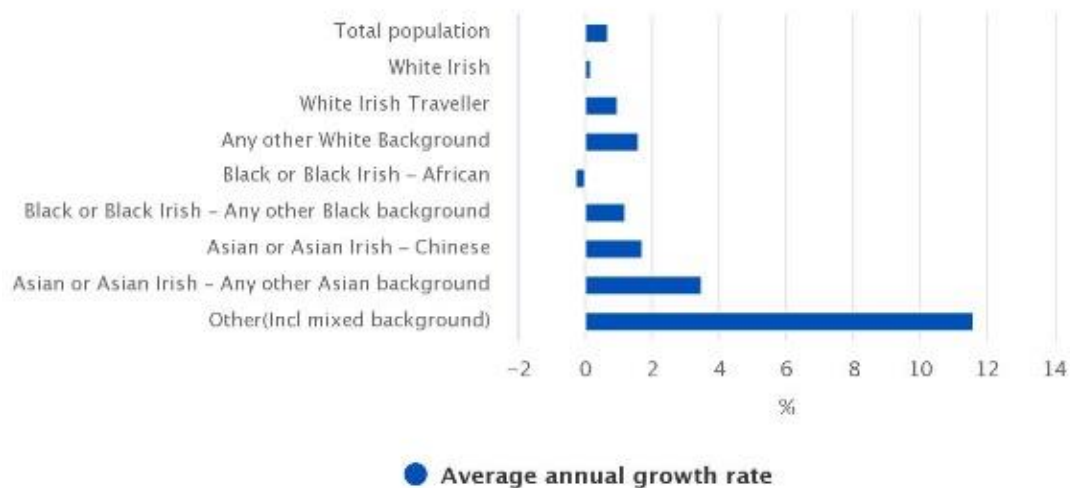
appropriately. Access to these tools is not routinely available however and is likely to require investment in information technologies to incorporate into daily practice. In busy modern practice, tools need to be accessible, reproducible, time-efficient and low-cost. The Endothelial Activation and Stress Index (EASIX) score passes these tests, and is gaining increasing awareness and application, particularly in transplant medicine.

Graft content and preparation

Donor considerations

Donor selection hierarchies are an important part of transplant preparation. The individual weighting of the variables including donor age, sex, fitness, CMV status, ABO matching, HLA matching, weight and stem cell source vary depending on the given context (173, 174, 175, 176, 177). In practice, after HLA matching, many of the additional desirable variables are secondary to donor availability. The current EBMT-recommended hierarchy of variables after a matched sibling donor is donor age, CMV status, sex matching and then ABO matching in a 10/10 matched unrelated donor, and donor age, donor specific antibodies and centre experience in mismatched and haploidentical donors (178). Many patients do not have a fully HLA matched donor option and decisions regarding mismatch and haploidentical transplantation are required (179, 180, 181, 182). The current alternative donor platform in those without a full HLA match in the National Adult Stem Cell Transplant Programme in Ireland is a 1-antigen permissive mismatch, either related or unrelated. Sibling donors are an important source of suitable stem cells in many transplant settings as they can typically be assessed and prepared for donation in shorter timeframes than unrelated donors. Siblings of those undergoing RIC are primarily older than typical unrelated donors and therefore additional care and attention is required to screen for age-related donor medical concerns that may be a risk to the donor or recipient. Co-morbidity prevalence increases with age, as do the incidence of occult abnormalities on screening donor tests and seropositivity for CMV. The rapidly diversifying ethnic makeup of Ireland will have future implications for the RIC programme for older patients, as the population HLA allele pool becomes more heterogeneous and finding unrelated donors becomes more complex (*Fig. 5*). This may drive consideration for alternative approaches to donor selection and the increased use of haploidentical donors. Increasing haploidentical activity will therefore dictate the choice of conditioning regimen for some patients.

Figure 3.1 Average annual growth rates of each ethnic or cultural group, 2011–2016



Source: CSO Ireland
Highcharts.com

Figure 5: Demographic changes in Ireland 2011 – 2016, ethnicity. CSO.

Evidence supporting the potential therapeutic benefit of stem cell grafts procured from younger unrelated donors as compared to older sibling donors is particularly pertinent in this setting, where related donors are typically between the ages of 50 and 65 (183, 184, 185). In Ireland historically large families have provided reasonable access to sibling donors, however this demographic phenomenon is evolving, with a peak fertility rate of 4.07 in 1964 now standing at 1.63 in 2020, indicating an impending demand for alternative donors that will mirror the trends seen in the wider European context (186, 187) (Fig. 6). The position of donor age in the donor characteristic hierarchy could inform choice between older siblings, haploidentical and unrelated donors. This has considerable implications for unrelated registries and for transplant centres, where use of a sibling donors can result in less transplant delay and donor service costs. Additionally, reducing the upper age cut-off for older sibling donors while increasing the age limits for recipient candidacy narrows the pool of readily accessible donors. It is important therefore to corroborate the data showing benefit from younger donors in local datasets.

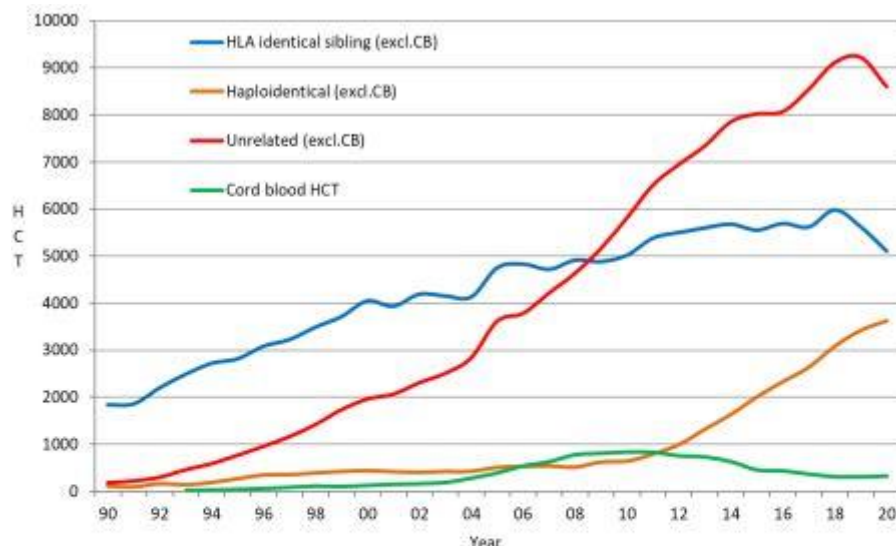


Figure 4: Change in the numbers of MAB versus NMA allogeneic HCT since 2020

Figure 6: EBMT Activity 2020, donor source trends. EBMT.

Stem cell source and processing

Peripheral blood stem cell (PBSC) grafts are the preferred stem cell source in the RIC setting (188). The reduction in direct anti-leukaemic effect from conditioning chemotherapy compared to MAC transplantation is balanced with a direct GVL effect from increased infused mature donor T cells and an increased risk of acute and chronic GVHD with the attendant simultaneous GVL effect (21, 189, 190). Peripheral harvest is less invasive for donors and reduces the need for exposure to general anaesthesia, hospital bed stays and operating theatre times. This increases eligibility of older donors, as they may meet the requirements for peripheral donation, but the risk of general anaesthesia may be prohibitive.

Graft cryopreservation came into sharp focus during the COVID-19 pandemic where, due to national and international logistical restraints, many alloSCTs were performed with cryopreserved stem cell grafts. Graft cryopreservation is established as a viable method of graft procurement and storage, and in the setting of short donor availability windows and transplant delays it is an important tool for maintaining the transplant candidacy of some patients (191, 192). However, many institutions, including those in Ireland, have a policy of first preferencing fresh product where available. During the most restrictive phase of the COVID pandemic, most unrelated donations were cryopreserved due to international travel and courier restrictions. Although wholesale practice change occurred in a short period of time, retrospective reviews of this process indicate broadly acceptable technical outcomes for engraftment and cell viability in keeping with historical data on the effect of freezing and thawing stem cells and the rate of subsequent engraftment (193). However, there are signals that there may be deleterious long-term effects on outcome when compared to fresh grafts, including an increased risk of relapse, NRM and a reduced rate of full donor chimerism

attainment, which underpins the continued widespread preference for fresh grafts in the post-pandemic world (194, 195, 196, 197, 198, 199).

Graft T cell content

Ex vivo T cell depletion and CD34⁺ selection is an alternative strategy to standard T cell replete peripheral blood stem cell grafts but is not universally available and has not demonstrated superiority in RIC alloSCT (200, 201, 202). In the absence of *ex vivo* graft manipulation, T cell content can vary significantly by donor. T cells may have a direct and indirect regulatory role in haematopoietic stem cell survival and function and influence successful engraftment (203). Naive donor T cells are responsible for the early instigation of alloreactive processes leading to acute GVHD and prompts the use of *in vivo* T cell depletion and GVHD prophylaxis with calcineurin inhibitors and methotrexate. Both donor and recipient T cells represent the ligand for ATG/ATLG, which initially selects recipient T cells during pre-transplant conditioning and, due to its long half-life, also targets graft T cells after infusion. The degree of T cell inhibition and persistence of ATG/ATLG will depend on both the host and recipient T cell population, where increased absolute lymphocyte numbers are felt to clear unbound ATG/ATLG faster (204). There are undoubtedly a myriad of factors determining ATG/ATLG exposure and persistence given its multiple cell surface targets, volume of distribution and non-linear relationship between dose and peak plasma levels (205). Variability in graft T cell content may contribute to clinical outcome as stem cell infusion in most RIC regimens occurs within the window of maximal serum concentration (i.e. serotherapy in the days immediately prior to stem cell infusion). The impact of CD4⁺, CD8⁺ and CD4⁺/CD8⁺ T cell subtypes in the donor graft on clinical outcomes such as GVHD, relapse, immune reconstitution and survival has been studied but definitive correlation is clouded by both the numerous additional variables that occur between infusion and these later clinical outcomes, and the categorical variables of the patient, disease and donor profile (206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216).

The EASIX model

The EASIX model has seen increasing use in several domains including alloSCT, chimeric antigen receptor T-cell (CAR-T) therapy and inflammatory syndromes (217, 218, 219, 220, 221, 222, 223, 224). In transplantation, it has demonstrated the ability to predict survival and transplant specific toxicity at serial timepoints (225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239). This simple tool allows for real-time and retrospective estimations of outcome using universally available biomarkers and may have a role in capturing the dynamic evolution of risk to the patient through the transplant process (Fig. 7) (240). The score has been examined in both RIC and myeloid domains and reproducibly predicts outcomes (238, 241, 242, 243).

The EASIX model shows promise in predicting key transplant-related outcomes, including overall survival, NRM and sinusoidal obstructive syndrome (SOS) (244, 245). The score has been validated at 3 time-points for survival: pre-transplant, at onset of GVHD and at 1-year post-transplant. SOS can be predicted using the day 0 time-point. In the pre-transplant setting, the score helps identify those patients at risk of all-cause death and non-relapse mortality by capturing those patients with low platelets, high serum creatine and raised LDH. Thrombocytopenia and a raised LDH are frequently hallmarks of disease activity, and at a pre-

transplant time-point may reflect higher risk patients at risk of early relapse and death, but also those who come to transplant with incomplete haematological recovery or in aplasia with the attendant risks of infection and bleeding. The day 0 time-point in the reduced-intensity setting is less discerning for survival as most patients experience significant thrombocytopenia following *in vivo* ATG/ATLG-based serotherapy. The proxy nature of thrombocytopenia, renal dysfunction and raised LDH likely also reflects an increased severity of inflammation and endothelial dysfunction at the onset of GVHD and therefore an increased risk of severe GVHD and NRM. At 1-year post-transplant the incidence of NRM and new onset GVHD is decreased but the correlation with survival may be due to late relapse manifestations including thrombocytopenia and raised LDH, frequent biomarkers of disease activity in myeloid disease. Late NRM in those with chronic severe GVHD and poor graft function beyond this time are also potentially captured by higher scores at 1-year.

The utility of the score in stratifying risk by age is unproven and is therefore of interest in RIC cohorts, as prognostication and risk-assessment tools are lacking, particularly those that can be used dynamically. Its role in predicting additional transplant outcomes such as relapse and risk of GVHD onset is similarly unknown but worthy of exploration.

$$\frac{\text{Lactate dehydrogenase (U/ml)} \times \text{Creatinine (mg/dL)}}{\text{Platelets} \times 10^9/\text{L}}$$

Figure 7: EASIX model.

Anti-thymocyte globulin and immune reconstitution vs. PTCY

ATG/ATLG is one of the most common and established *in vivo* T cell depleting therapies. In combination with various reduced intensity chemotherapy agents, it has been incorporated from the early days of RIC transplantation. *In vivo* T cell depletion reduces severe acute and chronic GVHD but does not improve overall survival, leukaemia-free survival or NRM and has been considered controversial in the RIC setting due to the potential increase in relapse (246, 247, 248, 249). ATG/ATLG is a non-specific, polyclonal and purified mixture of animal anti-human antibodies with potent activity against human T cells and is harvested after inoculation with sensitizing human antigens. The two animal incubation hosts are leporine and equine, the latter of which is typically reserved for the primary treatment of severe aplastic anaemia. Rabbit anti-human antibodies are stimulated by two methods to create both commercially available products: Thymoglobulin® (Sanofi-Genzyme) and Grafalon® (Neovii) (250, 251, 252). Thymoglobulin® uses human thymic tissue as antigenic material and is commonly used in the United States. Grafalon® (previously ATG-Fresenius) uses human T-lymphoblasts from the Jurkat cell line and is frequently used in European centres (253). These products are not biosimilar and due to differences in their manufacture contain differing antibody titres and profiles and therefore differing pharmacodynamic and pharmacokinetic profiles. Due to this difference in manufacture, Grafalon® is also referred to as anti-T lymphocyte globulin (ATLG).

The intended clinical effect however is broadly comparable and comparative studies show similar clinical outcomes in terms of survival, GVHD, relapse, NRM and immune reconstitution (254). A metanalysis alludes to a differential superiority in GVHD outcomes with ATG-Grafalon, equivalence in NRM but worse OS as compared to Thymoglobulin® (255).

Adaptations to the dose and schedule have occurred since early RIC alloSCT practice, and alternative agents have been successfully substituted, initially with the anti-CD52 monoclonal agent alemtuzumab, and more recently with PTCY. ATG/ATLG nevertheless remains a proven and popular serotherapy, particularly in Europe, and is incorporated into several treatment regimens in both the MAC and RIC setting (256, 257, 258, 259, 260). Use in MAC regimens has historically been the reserve of alloSCT involving unrelated or mismatched donors, and in the latter is increasingly being supplanted by PTCY (261). Dose recommendations are better standardised in the MAC setting, where for ATLG a dose of 30-60mg/kg (equivalent to 4.5-7.5mg/kg ATG), usually over 3 days from -3 to -1 varying by donor source is recommended (262). Lower doses of ATLG have shown equal efficacy in paediatric patients, and is promising in adult settings, although the extension to RIC settings is less clear (263, 264, 265). For RIC regimens serotherapy is important in transplants using unmanipulated grafts for biphasic immune tolerance, first in reducing host rejection of the infused graft and secondly by *in vivo* depletion of alloreactive donor T cells which reduces GVHD, particularly chronic type (266). However, the balance of relapse risk is a significant consideration with RIC regimens and the optimal dose is not agreed (262, 267) (247).

ATG/ATLG has significant acute and potentially chronic side effects which are only partially predictable. A systemic inflammatory response syndrome (SIRS)-type infusion reaction is frequent and of variable intensity, necessitating high-dose steroid and antihistamine concurrent treatment. Typical symptoms and signs include fever, rigors, acute dyspnoea, gastrointestinal disturbance, electrolyte disturbance, rash, urticaria, serum sickness and in some cases overt anaphylaxis (268). This process appears to be a form of cytokine release syndrome (CRS), which in the era of CAR-T is now better recognized and described (269, 270). Further non-infusion related effects include non-specific haemolysis and hyperbilirubinemia. Those with myelofibrosis are at higher risk of acute liver injury due to ATG/ATLG, which can be marked but is usually self-limiting. This effect may be due to hepatic extramedullary haematopoiesis (271). Fever may result in additional antibiotic exposure and the attendant side effects thereof and contributes to early organ stress during transplantation.

ATLG has a long half-life and there is substantial inter-patient variability in its clearance, with some patients retaining lymphotoxic drug levels beyond 50 days after administration (252, 272). Immune reconstitution is therefore variable between patients, although modulated by other post-transplant variables, such as steroid exposure for treatment of GVHD. Pharmacokinetic studies using Thymoglobulin® have demonstrated that drug exposure is affected not only by the dosage and timing of administration, but also by individual patient factors such as weight and lymphocyte count (273, 274, 275, 276). Protracted immunosuppression leads to increased opportunistic infection, viral re-activation and increases the risk of disease relapse by reducing immune surveillance (277, 278). Early recovery of CD4⁺ T cells post-transplant is associated with improved survival and efforts to improve the balance of graft rejection, GVHD and physiological T cell function are an important step towards a successful long-term transplant outcome (279, 280). ATG/ATLG is an effective bridge in the early phase of transplant and clearly allows for successful engraftment and early graft propagation, however it comes with the risk of lingering toxicity.

Attempts to address this have come in the form of dose reduction strategies, dose sparing by combination with additional serotherapies or T cell modulators, individualised dose calculations and therapeutic drug monitoring (281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297). The standard dosing algorithm is presently determined by graft source and patient weight and therefore validated dosing nomograms are needed to guide optimal treatment. Two studies of lymphocyte count-guided ATG (Thymoglobulin®) in both an adult and paediatric setting encourages the clinical application of bespoke dosing, however the application of this in adult transplantation with ATLG is as yet unproven (298, 299).

PTCY based platforms may address some of these issues, and in the setting of haploidentical and HLA mismatched transplants show superior outcomes (300, 301, 302, 303). Manufacturing, pharmacodynamic and pharmacokinetic properties of cyclophosphamide are broadly predictable in comparison to ATG. However, the benefit of PTCY in the matched related and unrelated donor domain is less well defined and direct prospective comparator trials have not yet reported (304, 305, 306, 307, 308). Retrospective comparative studies do not demonstrate superiority in terms of relapse incidence, chronic GVHD or overall survival (309). Infectious outcomes after PTCY-based conditioning remain troublesome, including early bacterial infection in the setting of mycophenolate use and later with a not insignificant viral re-activation risk (310, 311).

Alemtuzumab is usually combined with the alkylator melphalan and it is less usual to see it combined with busulfan, although does see use in certain indications such as immunodeficiency syndromes (312, 313, 314). The difference between ATG/ATLG and alemtuzumab is therefore clouded by the additional variable of a frequently alternative chemotherapy backbone. There is no clear signal that any given conditioning approach in this domain is clearly superior, and therefore treatment choices are frequently based on institutional preference and experience (315). International data series using the various *in vivo* depletion strategies provide some evidence for guiding individualized treatment decisions, where one or another approach may be adopted based on the balance of risks and benefits for that patient. Institutional familiarity, however, is a metric that is not often calculable but needs to be considered when considering practice change, as many of the routes by which safety in transplant is achieved is by nursing and medical experience in administering, monitoring and treating side-effects of a familiar and established regime. Despite its drawbacks, ATG/ATLG based therapy is a useful benchmark against which contemporaneous and future therapy changes can be judged.

Immune reconstitution

Timely immune reconstitution post-transplant is an important milestone for minimizing serious infectious complications, improving the GVL effect and ultimately long-term recovery (316). The hallmarks of reconstitution include adequate recovery of T cell subpopulations, in particular CD4⁺ T cells, and appropriately functioning B cell and plasma cell populations capable of producing normal immunoglobulin levels (317). Cessation of immunosuppression and navigation of significant GVHD is frequently necessary to achieve these goals and thereafter successful completion of a post-transplant programme of re-vaccinations is an important proxy of physiological functioning of the donor immune system.

There are both time and treatment specific determinants of reconstitution and frequently there is an interplay of these variables (318). From the recipient perspective, prior treatment and disease burden are likely to have an impact, but in the setting of a successful engraftment, the donor and graft characteristics are likely of greater significance. The extent to which mature donor T cells in the graft contribute to longer term immune reconstitution is unclear, however they may aid successful engraftment and thereafter production of new generations of donor derived T cells which undergo antigen exposure and maturation in the host reticuloendothelial and thymic environment. *In vivo* T cell depletion therapy including ATG/ATLG is a key early transplant determinant of T cell survival, and the degree of impact varies between patients depending on the rate of elimination (319). The advent of both acute and chronic GVHD affect reconstitution, particularly in those who require treatment with systemic steroids and prolonged immunosuppression. There is ongoing interest and early phase work in modulating the non-selective effects of serotherapy by infusion of donor T effector memory (TEM) cells shortly after engraftment to rapidly reconstitute non-alloreactive regulatory T cells, which can improve infectious outcomes. These methods involve TEM selection with surface markers including CD45RA and CD62L to avoid naïve and central memory T cells.

In paediatric patients an association between CD4⁺ recovery of 50 cells per microlitre within 100 days of transplant and a favourable survival outcome has been shown, including improved survival after GVHD onset (280, 320). This indicates that even though T cells are known arbiters of GVHD, early recovery of regulatory T cells as a component of overall T cell recovery may be important in enhancing the GVL effect and leads to a net benefit. Clinically available tests for immune monitoring post-transplant are broadly limited to T cell subset and immunoglobulin quantification and there is therefore a need for international collaboration to rationally design additional tools for predicting and manipulating reconstitution (273).

Graft versus host & leukaemia

The therapeutic benefit of alloSCT with RIC platforms heavily relies on an immeasurable GVL effect (16, 20, 321). By proxy, the development of GVHD, particularly in its chronic form is shown to associate with better overall disease related outcomes, although with the attendant morbidity and sometimes mortality of GVHD itself when extensive, and indeed the treatment thereof. Navigating the balance of these two effects is frequently difficult and with high variability from patient to patient, as several factors determine the likelihood and strength of this phenomenon, including patient factors, donor and graft factors, conditioning, and post-transplant priming. Candidacy for alloSCT in older patients is frequently predicated on the response likelihood of the underlying disease and the physiological reserve of the patient to withstand organ toxicity and navigation of possible GVHD. GVHD of any grade is common in the RIC setting and also in advancing age, due in part to the universal use of PBSC stem cell grafts and frequent use of DLI (322). Many patients experience GVHD at low grades but a significant portion are at risk of serious morbidity from high grade acute GVHD and the development of life altering chronic GVHD. Quality of life is negatively affected in patients who experience GVHD and there are mixed reports that this affect is accentuated by age (323, 324, 325, 326, 327, 328, 329). Effective strategies to mitigate some of this risk include ATG/ATLG pre-stem cell infusion followed by further post-infusion T cell modulation with low-dose methotrexate or MMF and calcineurin inhibitors. Despite the older age cohort, these

therapies are generally well tolerated in those deemed fit enough for alloSCT. The complications of renal dysfunction and hypertension are more common as compared to MAC cohorts, in addition to the age-agnostic but rarer risks of microangiopathic haemolytic anaemia (MAHA) and posterior reversible encephalopathy syndrome (PRES). Allowing sufficient T cell expansion and activation to enact a GVL effect requires a tapering of immunosuppression, which exposes patients to GVHD when these medicines are weaned, typically between the 3rd and 6 months. In the highest risk diseases, early withdrawal of immunosuppression is frequently attempted but increases the risk of breakthrough GVHD. In some, despite active GVHD, the associated GVL effect is insufficient to control disease recurrence and patients can experience the difficult position of relapse and GVHD concurrently.

Prediction of GVHD in clinical practice is currently limited to an assessment of the categorical variables that increase risk, including HLA mismatching, unrelated donor use, PBSC grafts, female donors with male recipients and inadequate peri-transplant immunosuppression, particularly erratic calcineurin inhibitor levels in the first 30 days post-transplant (330, 331, 332, 333). An increased risk is also associated with the use of DLI and in those with *FLT3* mutations exposed to post-transplant maintenance sorafenib. Additional clinically applicable tools are needed to calculate risk on a patient basis using validated biomarkers. Cytokine profiles, T cell profiling and artificial intelligence monitoring of clinical parameters have all shown promise in predicting GVHD onset before clinical manifestation but are not validated in routine clinical practice to date (172, 334, 335, 336). Critical to the potential benefit of these strategies is the timely availability of results to allow for meaningful interventions to prevent significant GVHD onset, without a significant undermining the GVL effect.

The grading and staging of both forms of GVHD is aided by several validated scoring systems and helps guide treatment decisions (337, 338, 339). For acute disease requiring systemic treatment, corticosteroids are the standard first line of treatment and the most effective (340). The duration of treatment varies by response and combinations with additional agents including recommencement of calcineurin inhibitors, ruxolitinib and extracorporeal electrophoresis (ECP) are commonly used in steroid refractory disease (341, 342). Access to further lines of therapy is variable, and several drugs have either been approved in various jurisdictions or trialled for efficacy, including ibrutinib, imatinib, belumosudil and in gastrointestinal disease, TNF blockers (343, 344, 345). Many of these treatments have drug-specific side effects which may affect older patients more frequently and most treatments contribute to delays in immune reconstitution, thereby increasing the risk of opportunistic infections.

Infectious morbidity

Infection, organ toxicity and GVHD are the three biggest non-relapse related stressors that alloSCT candidates may encounter. In RIC transplantation, the patient's ability to navigate severe infection can pose a significant challenge to achieving a successful transplant outcome and comprises a large share of the NRM risk. A review of past history of infections during prior chemotherapy can offer a risk assessment for future events but is not always reflective and in some cases of MDS and many with MPN, the first experience of such a pronounced risk occurs during alloSCT. Infection is multifactorial and sometimes unpredictable but institutional

experience with, and audit of, established regimens such as FluBuATLG offer a framework for estimating risk.

Infection presents in three broad phases: immediate bacterial and fungal infection during neutropenia prior to engraftment, viral re-activation post-engraftment and opportunistic infection throughout, prior to late immune reconstitution. Early bacterial infection in RIC alloSCT is akin to the that seen with MAC alloSCT, where recipient disruption of the gastrointestinal and blood barrier, indwelling vascular catheters and environmental exposures are frequently implicated. NRM in the early phase of transplant is driven by these early infections and the accompanying sepsis syndrome, which in older patients can cascade into organ dysfunction and result in clinical decompensation. Sepsis may present differently in older patients, and the impact of co-morbidities and pre-transplant medications can impact recognition and the rapidity of the evolution of sepsis (346, 347, 348, 349, 350). Enhanced awareness of sepsis recognition and prompt management in the last decade has improved outcomes in acute bacterial infection in several domains including alloSCT. Advancements in antibiotic stewardship, microbiological testing and screening, radiological imaging and widespread protocolized antimicrobial algorithms have helped reduce risk in the early transplant period (351). However, the rise in multidrug resistant bacteria continues to threaten safe transplant practice (352, 353, 354).

The risk of invasive fungal infection (IFD) correlates closely with prolonged neutropenia and immunosuppression and patients with a history of AML and MDS are at particularly high risk from the time of induction all the way through transplant (355, 356, 357). Screening for IFD is limited to serial galactomannan and beta-d-glucan measurements, which do not always correlate with radiological and clinical outcomes. Modern mold-active antifungal prophylaxis offers significant risk reduction of IFD for patients undergoing alloSCT, in particular prior to engraftment and during treatment with high-dose steroids for GVHD (358, 359). Diligent use of antifungal prophylaxis during remission induction pre-transplant also helps limit the incidence of occult fungal disease at the time of transplant, although there is considerable variation in practice internationally (360). The rate of IFD in RIC alloSCT is similar to MAC alloSCT despite the reduction in direct mucosal damage and a somewhat shorter time to neutrophil engraftment, and appears to occur comparatively later, during the highest risk period for acute GVHD and early chronic GVHD, indicating the impact of immunosuppression and steroids. Breakthrough IFD in the setting of antifungal prophylaxis is associated with excess morbidity and mortality, often due to aggressive and resistant fungal species. The most common non-candida species of concern in European centres is *aspergillus fumigatus*, which often develops resistance to azole antifungals, limiting therapeutic options. Prolonged exposure to antifungal therapy is associated with an increased risk of side-effects and drug-interactions, which in older patients can prove challenging.

The second phase of infectious complications associated with alloSCT is viral re-activation, of which, cytomegalovirus (CMV), Epstein-Barr virus (EBV) and adenovirus (ADV) are the most frequently implicated (361, 362). The use of ATG/ATLG and other *in vivo* T cell depletion strategies are one of the key factors associated with an increased risk of viral re-activation (363). Pre-transplant screening of donor and recipient serostatus helps to stratify risk, and where possible, appropriate donor selection can reduce the risk of infection in seronegative recipients (364, 365, 366). For CMV, the success of prophylactic letermovir for seropositive recipients has reduced some of the risk and is well tolerated including in older patients (367). Protocolized polymerase chain reaction (PCR)-based monitoring for low level CMV, EBV and

ADV copy numbers during treatment with immunosuppression is critical to reducing mortality from uncontrolled viral proliferation, as it offers an opportunity to intervene with either reduction of immunosuppression or pre-emptive virus-directed therapy before evidence of end-organ damage occurs (368). Reduction in immunosuppression is an effective method of treating viral reactivation but is associated with a risk of inciting GVHD, the treatment of which in turn increases the risk of further viral re-activations. Antiviral therapy is associated with additional morbidity in older patients as the few available options are associated with toxicity to graft and renal function (ganciclovir/cidofovir/foscarnet) (369, 370, 371, 372, 373, 374). ATG/ATLG use is associated with an increased risk of post-transplant lymphoproliferative disorder (PTLD) driven by EBV reactivation, however the risk is low and with PCR-based monitoring, pre-emptive rituximab monotherapy is effective and well tolerated, and PTLD requiring multi-modal chemotherapy is rare (375, 376). Other viral infections which may add to the morbidity of alloSCT include herpes simplex virus (HSV), human herpes virus 6 (HHV6), BK virus and endemic and pandemic respiratory viruses. These viruses complicate all transplant formats, but in the RIC setting need to be considered in the context of the patient's fitness.

The third risk domain for infection post RIC alloSCT is opportunistic infection, particularly during post-transplant immunosuppression and GVHD therapy. The use of prophylactic antimicrobials such as co-trimoxazole, nebulized pentamidine, atovaquone and penicillin reduces the incidence of *pneumocystis jirovecii* and associated atypical fungi, toxoplasmosis and encapsulated bacteria prior to robust CD4 recovery and adequate post-transplant vaccination (377, 378). While the occurrence of atypical infection in older patients is a serious complication of transplantation, the risk is low in the setting of prophylactic measures and active surveillance.

DLI and relapse management

Across an array of RIC settings, the largest obstacle to long-term success in transplant is disease relapse. A median of almost one third of patients relapse despite alloSCT, frequently within the first two years of transplant and at higher rates than in the MAC alloSCT setting (65, 379, 380, 381, 382, 383). Reducing intensity and toxicity shifts more of the therapeutic strategy to the GVL effect, which varies by disease and patient, and in some is unable to overcome the kinetics of disease progression. Relapse post-transplant is an event that carries a poor prognosis. Patients are likely to have been exposed to therapy pre-transplant and have developed clonal progression or disease resistance mechanisms that narrow the pool of suitable therapies for relapse. Physiological reserve is diminished by the pre-transplant therapy and the transplant process itself and some patients are ineligible for an intensive attempt at reattaining remission. The pre-transplant risk profile of the underlying disease can help identify those at the highest risk of post-transplant relapse, however tools for dynamically mitigating this risk are lacking. Prophylactic maintenance to reduce relapse risk is currently limited to *FLT3* positive disease, where sorafenib has shown some benefit and studies for gilteritinib (MORPHO: NCT02997202) are ongoing (384, 385, 386). Azacitidine has shown some promise in those who do not have a targetable mutation but is subject to further clinical trials, including an important study combined with venetoclax (VIALE-T: NCT04161885) (387, 388). Additional trials using targeted therapies such as IDH1/2 inhibitors and TP53 restoration agents are similarly under review (389).

MRD monitoring is now routine post-transplant for those patients with specific disease mutations such as *NPM1*, core-binding factor and *KMT2A* fusions. Serial monitoring offers transplant physicians the opportunity to modulate the risk of impending overt relapse by adjusting immunosuppression or considering DLI. Unfortunately for many patients the baseline disease harbours no mutations that can currently be monitored by sensitive PCR-based assays, however advances in this field are expanding access steadily and in those with distinctive leukaemia-associated immunophenotypes, increasingly sensitive flow cytometric methods are helping to bridge the gap. Total and CD3⁺ chimerism monitoring has an established role in guiding decision making in RIC alloSCT, as mixed chimerism is more frequently seen due to non-myeloablative conditioning (390, 391, 392). Early chimerism assessment from 6 weeks post-transplant can guide immunosuppression weaning strategies and further again at 3 months. However, this form of monitoring is not always predictive of outcome, as some patients will relapse despite a full donor chimerism and some with a mixed donor chimerism do not subsequently relapse. Short tandem repeat (STR)-based chimerism assays in clinical practice are typically sensitive to a level of between 1 and 5%, which is orders of magnitude less sensitive than those seen with *NPM1* transcript monitoring (393). Relapse prediction by cytokine expression and T cell characterisation show promise but are not routinely available.

The success of RIC alloSCT is enhanced by judicious use of DLI to enhance the GVL effect (394, 395, 396, 397, 398). The largest risk with DLI is inducing significant GVHD, however DLI appears to be well tolerated including in older patients, and induced GVHD appears to be less problematic than spontaneous GVHD (399). There are three primary situations where DLI may be beneficial. The first is a prophylactic approach, where patients who have not experienced spontaneous GVHD, demonstrate full donor chimerism and are deemed at high risk of relapse based on disease characteristics, with DLI being used to help mitigate this risk. This method is currently being investigated by the ELIT-AML01 (ClinicalTrials.gov ID: NCT03597321) trial in France, and the results are eagerly awaited. Nevertheless, a number of patients have been treated in this manner previously and have experienced positive outcomes (400). The second and more established approach is pre-emptive, whereby the development of a mixed donor chimerism is an impetus for DLI use, particularly when the mixed chimerism state persists after cessation of immunosuppression. Chimerism guided DLI strategies account for the largest proportion of DLI use internationally as there is compelling evidence for a reduction in subsequent relapse risk for those who attain a full donor chimerism after pre-emptive DLI, as compared to those who never receive a DLI or do so but fail to achieve a full donor chimerism (401, 402). In the RIC cohort, the tolerability of induced GVHD post-DLI must be considered alongside the current dearth of salvage options for post-transplant relapse. The third approach is in the relapsed setting, where DLI is used as part of a salvage strategy in either overt disease relapse or in those with rising MRD (403, 404). DLI is typically insufficient alone in management of post-transplant relapse, however, improvement of the dismal prognosis of those with post-transplant relapse has been seen when combining DLI with disease directed therapy, particularly hypomethylating agents in myeloid disease (405, 406, 407, 408, 409). Due to the prevalence of higher risk disease profiles in older patients and cumulative toxicity accrued through induction and transplant, the appropriateness of these salvage options is variable.

Part 2: *The role of cytokines in reduced intensity conditioning allogeneic stem cell transplantation.*

Cytokine dynamism through transplant

Cytokines are small polypeptides secreted by a range of immunological and non-immunological cells which bind specific target receptors and instigate signal transduction. Target receptors can be on other cells or the secreting cells themselves and once engaged leads to downstream changes in transcription, protein expression and ultimately cell behaviour. Cytokines have direct and indirect effects depending on the molecule, either by direct stimulation of a particular metabolic pathway or by stimulation of an effector class of cells such as T cells, where they can encourage differentiation and proliferation. Cytokines are detectable in serum and variances in levels can be correlated with clinical particulars, such as disease activity, inflammation and GVHD (410). In addition to temporal and situational variances in secretion, differences in secretion can be seen on a patient basis due to polymorphisms in the genes encoding both cytokine and receptor. The inflammatory milieu is delicately balanced by opposing effects, which in health promote homeostatic functions, but during transplant are frequently unbalanced due to extrinsic stressors and host/donor interactions. Many cytokines demonstrate site specific and dose dependent effects, where a given concentration may have either inflammatory or anti-inflammatory consequences, or varying clinical manifestations based on organ site (411).

Cytokines are an attractive target for translational research as they can be measured using routine clinical samples, storage of those samples at low temperatures is uncomplicated and the freeze/thaw cycle is less impactful than with cellular analytes. Multiplex enzyme-linked immunosorbent assays (ELISA) and chemiluminescence techniques allow for high through-put of samples and the techniques for running such assays are user-friendly with significant shared knowledge from other clinical settings using these techniques. Some platforms offer a significant degree of automation, which ultimately improves turnaround time and costs. As compared to cellular analysis with flow cytometry, where expertise in manual gating is required, cytokine analysis is less nuanced and conversion of analysis outputs to quantifications is performed by designated programmes.

AlloSCT is an inherently inflammatory process which involves disease-related, conditioning-related and host/donor interactions. Understanding the key pro- and anti-inflammatory cytokines responsible for these processes and how they change over time is a key component in understanding the biology and variability of alloSCT. Pre-clinical and clinical data highlights the role of several inflammatory cytokines such as interleukin (IL) -1, 2, 6, 12, 17, 21, 22 & 33, tumour necrosis factor alpha (TNF α) and interferon-gamma (IFN γ) in addition to pleiotropic and anti-inflammatory cytokines including IL-4, 10 and transforming growth factor beta (TGF β). The interplay of these cytokines and their relationship to key transplant milestones is of interest in that they may potentially predict transplant complications and allow for intervention, but also may represent therapeutic targets in and of themselves (*Fig. 8*). T cell profiles during transplant are intrinsically linked to cytokine expression as both secreting sources of many cytokines but also as key targets for cytokine mediated effects. T cell differentiation, proliferation and exhaustion are driven by cytokines production by other T cells, endothelial, mucosal, epithelial and stromal cells and other haematological cells including myeloid cells, antigen presenting cells, NK cells, B/plasma cells and tissue

macrophages (412, 413, 414). This complex network of up and down regulation of activity is heavily dependent on cytokine signalling and in alloSCT is uniquely the product of combining two immune systems. GVHD occurrence is not simply the product of antigen and receptor interactions however, as the phenomenon can occur in the absence of tissue expression of alloantigen, which indicates a multimodal disease process (415). The impact of timing of measurement of serum cytokines is important, as analysis of cytokines in the early transplant phase is reflective of both residual host and engrafting donor profiles. Similarly, in later phases of transplant where mixed donor chimerism occurs, cytokine expression may reflect both recipient and donor immune activity.

Cytokine profiling has the potential to offer multi-domain assessments of the transplant process in sequential and real time models and in a reproducible manner. Correlation with studies of the innate and adaptive immune cell evolution through transplant can offer new insights into the quantitative and qualitative behaviour of a range of immune cells in alloSCT, where the cytokine secretome represents both the drivers of immune activation/proliferation and also the products thereof (416, 417, 418, 419, 420, 421, 422, 423). However, it is important to consider correlative analysis in the context in which it arises, with consideration of the timepoint in the transplant process, the relevant competing factors and the interdependent cytokine interactions and their frequently pleiotropic effects. Further complexity arises in the allogeneic transplant setting due to inherent minor immune antigen mismatches and single nucleotide polymorphisms (SNP) of genes for cytokines and their receptors and the myriad combinations between recipient and donor.

The use of cytokine profiles as a predictive tool has been attempted in previous studies (424, 425, 426). GVHD incidence and severity prediction is the most prominent application given the inherent inflammatory associations of many cytokines (334, 336, 427, 428, 429, 430, 431, 432, 433). Additionally, cytokine expression patterns may hint at other non-GVHD related outcomes such as NRM and relapse and potentially be quantifiable proxies of the GVL effect.

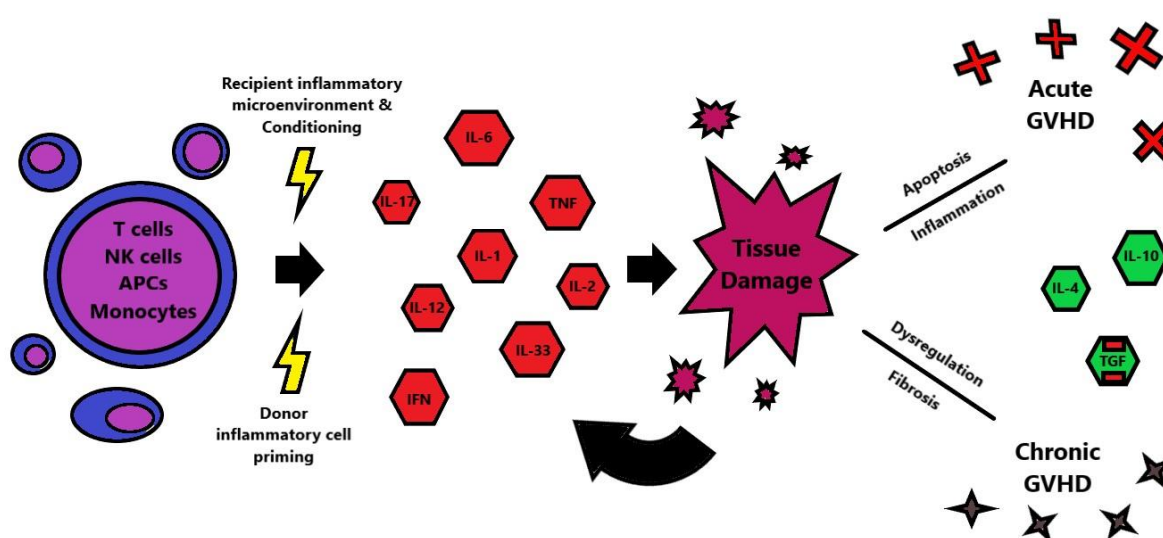


Figure 8: A simplified model for cytokine activity in GVHD.

Interferon-gamma (IFN γ)

IFN γ is a pleiotropic type II interferon, with a significant role in T cell differentiation and functional modulation, particularly Th1 and Tc1 differentiation (434). Several immune cells are involved in its synthesis, including both helper and cytotoxic T-cells, B cells, NK/T cells and antigen presenting cells (APCs). Signal transduction is through the STAT1 pathway following engagement with the IFN γ receptor and leads to induction of CXCL9. It exerts its effect by enhancing MHC class 2 antigens and inhibiting Th2 differentiation.

As a primarily pro-inflammatory cytokine, IFN γ exerts a significant effect on the development of GVHD and the potency of the GVL effect but can also have immunosuppressive activity depending on the context (435, 436). IFN γ demonstrates site specific effects, where pro-inflammatory signalling can lead to clinical acute GVHD in the gut but may be protective in the lung parenchyma (416, 437, 438, 439, 440, 441, 442, 443). Evidence of a role in immune mediated graft rejection points to the importance of this cytokine in recipient and donor interactions (444). The GVL effect of IFN γ stimulation is not necessarily due to direct cytokine engagement with the malignant cell surface, as absence of the IFN γ receptor does not preclude a GVL effect, but rather is likely mediated by IFN γ stimulation of T cells and APCs. Donor rather than host derived IFN γ appears to drive GVHD (445). Targeting of the IFN γ receptor as a preventative therapy for GVHD has been tested with baricitinib (JAK1/JAK2), demonstrating reduction in GVHD with preservation of the GVL effect (446, 447, 448).

Tumour necrosis factor alpha (TNF α)

TNF α is a key pro-inflammatory cytokine produced by monocytes/macrophages with a similar activity pathway to lymphotoxin alpha 3 (LT α 3) (449, 450). It signals through the TNF receptor and results in activation of the NF κ B, MAPK and pro-apoptotic caspase pathways. Like many other cytokines, individual polymorphisms in genes encoding the cytokine and its receptor lead to variation in sensitivity to its effects. In alloSCT its early presence is due to secretion by recipient myeloid cells during conditioning and then later by donor derived T cells (451, 452, 453). Inflammatory processes induced by TNF α contribute to GVHD with a particular tropism for skin and gut (454, 455, 456). It does not directly induce a GVL effect but does so indirectly in concert with antigen presenting cells and T cells (457, 458). Monitoring of TNF α during alloSCT has been shown to predict chronic GVHD (459).

Blockade of TNF α has been studied in several small studies showing some modest responses, particularly in gastrointestinal GVHD (460, 461, 462, 463, 464, 465, 466, 467). Alternative TNF family signalling including with LT α 3 may account for the overall disappointing results in targeting TNF α . Additionally, TNF α blockade is associated with an increased risk of opportunistic infection including fungal and mycobacterial infection, which is of concern in heavily immunosuppressed patients. New generation inhibitors may fare better but are still associated with suboptimal response (468, 469).

Interleukin 1 β (IL-1 β)

Interleukin 1 is a predominantly pro-inflammatory cytokine family, of which IL-1 α and IL-1 β are the best studied. IL-1 β is an endogenous pyrogen primarily secreted by macrophages/monocytes and antigen presenting cells following stimulation by alarmins through toll-like receptors. There is also some production by mature B and NK cells and epithelial cells. Signalling by IL-1 β depends on the MYD88 pathway and its release alongside IL-18 is downstream of the inflammasome complex. IL-1 β promotes secretion of further pro-inflammatory cytokines, particularly TNF α , IL-6 and IL-12. IL-1 β promotes immune cell migration by enhancing endothelial adhesion molecules and results in localization of inflammatory processes. IL-1 β has a significant role in the development of GVHD however variation is seen depending on polymorphisms in the gene encoding the cytokine and its receptor (IL-1R) (470, 471). IL-1 β does not appear to have a distinct impact on the GVL effect. Targeting IL-1 β activity in GVHD in pre-clinical models have not translated to demonstrable clinical benefit (472, 473, 474, 475). The use of anakinra in autologous transplant is being studied as a potential modulator of gastrointestinal inflammation and may have future applications in the allogeneic transplant domain if successful (476, 477, 478).

Interleukin 2 (IL-2)

IL-2 is a pleiotropic cytokine with pro-inflammatory effects mediated by stimulation of T cell proliferation, enhanced NK cell cytolytic activity and expansion of regulatory T cells (479, 480). Its production is stimulated by CD4⁺ T cells following priming of the major histocompatibility complex (MHC)/antigen pathway by APCs. IL-2 signalling following engagement with the IL-2 receptor (IL-2R/CD25) is through phosphoinositol 3-kinase (PI3K), JAK/STAT and RAS/MAPK pathways (481). In alloSCT the role of IL-2 is twofold, where it promotes the development of GVHD and also helps mediate a GVL effect through stimulation of CD25⁺ regulatory T cells (482, 483, 484). Standard GVHD prophylaxis with calcineurin inhibitors work partly by inhibiting IL-2 signalling by blocking calcineurin phosphatase, which is required for the intracellular communication of the IL-2 signal. Therapeutic targeting of the IL-2 pathway is limited by this dichotomous relationship, where improvements in GVHD will negatively affect the GVL effect. The effect of exogenous IL-2 administration can reduce GVHD by expansion of regulatory T cells, which limit GVHD and preserve the GVL effect, but dose and timing is likely to contribute to the balance of effect of this pleiotropic cytokine, making it difficult to utilize in clinical practice. Basiliximab, which targets CD25 and competes with endogenous IL-2, has shown benefit in solid organ transplantation and has been studied in steroid refractory GVHD, where it can reduce T cell proliferation, however it does not have an established role in alloSCT and there is concern for the potential of impairing regulatory T cell activity and increasing the risk of relapse (485, 486, 487).

Interleukin 4 (IL-4)

IL-4 is a pleiotropic cytokine that promotes the differentiation of naïve T helper cells to Th2 cells, and although it has pro-inflammatory properties, particularly in allergy, it can reduce pro-inflammatory Th1 differentiation. It is produced further by Th2 cells promoting positive feedback but also by granular cells including eosinophils, mast cells and basophils. IL-4

mediates tissue repair in conjunction with other typical anti-inflammatory cytokines including TGF β , IL-10 and IL-13. Suppression of Th1 differentiation and reduction in IFN γ production is a key effector pathway for IL-4. Following stimulation of the IL-4 receptor (IL-4R α), signalling occurs via the JAK/STAT pathway (479). IL-4 secreted by bone marrow stromal cells may play a role in abnormal megakaryocytic activity and thrombocytopenia in AML (488). In alloSCT host NK cells play a role in IL-4 mediated CD4⁺ regulatory T cell expansion leading to a GVHD protective effect (489). IL-4 can reduce acute GVHD via anti-inflammatory activity, however a mouse knockout model shows total absence of IL-4 results in GVHD as absence of IL-4 results in expansion of Th17 T cells in place of Th2 (421, 490, 491, 492, 493). IL-4 may increase the risk of chronic GVHD later in transplant, particularly in the lung, through IL-13 induction and the promotion of fibrosis (494, 495, 496).

Interleukin 6 (IL-6)

IL-6 is a pro-inflammatory cytokine secreted by a range of cells including macrophages/monocytes, dendritic cells, B and T cells, fibroblasts and some non-haematological cells in response to antigen stimulation of toll-like receptors. It encourages T cell proliferation and differentiation towards cytotoxic T lymphocytes (CTL), upregulates Th17 differentiation and suppresses regulatory T cell differentiation. Following engagement with the IL-6 receptor (IL-6R α /CD126) signals are transmitted through the JAK/STAT3 and MAPK cascades (497). Increased IL-6R α expression in some myelodysplastic syndromes may drive disease progression (498). In alloSCT IL-6 is associated with GVHD development (452, 499, 500, 501, 502, 503, 504). Polymorphisms in the gene encoding IL-6 result in a differential susceptibility to GVHD between patients (505). IL-6 blockade for risk reduction and treatment of GVHD has been studied with mixed results but may have a role in defined circumstances (506, 507, 508, 509, 510, 511, 512, 513, 514).

Interleukin 10 (IL-10)

IL-10 is an anti-inflammatory cytokine with immunosuppressive properties secreted by regulatory T cells, B cells, dendritic cells and monocytes. IL-10 reduces macrophage and dendritic cell activity, suppressing the presentation of antigens to T cells and thereby downregulating the instigation of inflammatory processes (452). It has regulatory effects on TNF α and suppresses the production of Th1 cytokines including IFN γ in addition to direct antiproliferative effects on T cells, the Th17 axis and promotes the propagation of regulatory T cells, particularly FoxP3⁺, CD25⁺ cells (515). In alloSCT IL-10 negatively affects the incidence and severity of GVHD (516, 517). Polymorphic changes in the encoding gene as with many other cytokines can affect clinical outcomes (518, 519). IL-10 does not appear to be a therapeutic target however, with mouse models demonstrating fulminant GVHD and decreased survival when exogenous IL-10 was administered (520, 521). Nevertheless, identifying low IL-10 levels in alloSCT may predict future GVHD risk.

Interleukin 12p70 (IL-12p70)

IL-12p70 is the active heterodimeric form of the IL-12 cytokine comprising a p40 and a p35 subunit and is produced by antigen presenting cells, macrophages, neutrophils, dendritic cells and Langerhans cells (522, 523). IL-12 stimulates proliferation and cytotoxicity of NK cells and Th1 cells and mucosal associated invariant T (MAIT) cells. Subsequent production of IFN γ and TNF α drives the inflammatory cascade. Following stimulation of the heterodimeric IL-12 receptor (IL-12R β 1+2) signal propagation is by STAT4 in the JAK/STAT pathway. In alloSCT IL-12 promotes the development of GVHD but also results in a GVL effect (445, 524, 525, 526, 527, 528, 529). There is evidence of a time-sensitive relationship between GVHD and IL-12, where initially it appears to be associated with reduced GVHD risk but rapidly becomes positively associated with its development thereafter (434). In keeping with this time-sensitivity, IL-12 as a therapeutic target in clinical practice is limited (530).

Interleukin 17A (IL-17A)

IL-17, most commonly referring to IL-17A as the most potent of the family, is a pro-inflammatory cytokine produced by CD4⁺ Th17 cells and CD8⁺ T cells in addition to IFN γ producing CD4⁺ T cells and MAITs. The IL-17 receptor (IL-17R) stimulates Act1/STAT6, NF κ B and MAPK pathways to promote Th17 differentiation, induction of granulopoiesis by G-CSF and CXCL family production and IL-6 production (531, 532). The activity of IL-17 is affected by the inflammatory microenvironment (434). In alloSCT IL-17 plays an important role in both acute and chronic GVHD development, in which the interaction of recipient derived IL-17 produced by MAIT cells particularly after endothelial disruption during the early phase of transplant is likely to play a significant role (533, 534, 535, 536, 537, 538). IL-17 is a potential therapeutic target for prevention and treatment of GVHD with IL-17 inhibitors such as secukinumab or targeting down-stream signal mediators such as ROR γ t (539, 540, 541, 542).

Interleukin 33 (IL-33)

IL-33 is a pleiotropic cytokine in the IL-1 family produced in endothelial, epithelial and fibroblast-like cells and acts as an alarmin, which is released by injured tissues rather than secreted. The receptor for IL-33 is suppression of tumourgenicity 2 (ST2), which is present on Th1, Th2, regulatory T cells, CD8⁺ T cells, NK cells, innate lymphoid cells, neutrophils, eosinophils, basophils and mast cells (543). ST2 is present in soluble form and acts as a dummy receptor for IL-33, or bound on the above-mentioned effector cells, whereby it stimulates MYD88 dependent signalling (544). IL-33's association with GVHD in alloSCT is time-dependent, where early production in transplant results in increased TNF α production and drives GVHD (545, 546, 547, 548, 549, 550, 551). In the pre-allograft setting, increased IL-33 is associated with a protective effect due to expansion of recipient regulatory T cells (434, 552). Therapeutic targeting of IL-33 signalling relies on targeting ST2, where removal of the dummy receptor can increase exposure to IL-33 and reduce IL-17 and IFN γ as mediators of GVHD (553, 554, 555, 556). Due to pleiotropic activity, dose and timing are important considerations. Commercially available anti-IL-33 drugs such as tozorakimab and anti-ST2 drugs such as astegolimab have not been tested in GVHD models (557, 558).

Other cytokines: TGFβ, IL-21 & IL-22

TGFβ is regulatory cytokine produced by T cells, myeloid cells, and is also found in platelets. It induces numerous cellular changes including proliferation and differentiation through a SMAD protein pathway (559). The effect of TGFβ varies depending on the profile of other cytokines present. In alloSCT it protects against acute GVHD by downregulation of inflammatory T cell responses to alloantigen presentation. However, it has profibrotic effects which leads to the development of chronic GVHD, limiting its potential as a therapeutic target (560, 561).

IL-21 is a pleiotropic cytokine produced by CD4⁺ T cells and NK cells which promotes differentiation and proliferation of NK, T, B and dendritic cells and signals through a JAK/STAT pathway. IL-21 can drive chronic GVHD by its effect on B cell alloantibody production but also has a regulatory role as a strong inducer of IL-10 (562, 563). IL-22 is a subset of the IL-10 family and signals through a JAK/STAT pathway. It has both proinflammatory and regulatory roles and has been identified in both acute and chronic GVHD, but this effect is likely to be time and context dependent, where the presence of additional cytokines may alter the specificity (564, 565).

Summary

AlloSCT is an effective therapy for myeloid malignancies, and frequently the only treatment likely to induce long-term disease control. The combination of fludarabine, busulfan and ATLG as the FluBu3ATLG RIC regimen has been a mainstay from early clinical application of RIC based transplantation over two decades ago and remains a relevant and popular choice in modern practice. Favourable rates of NRM offer an avenue for young patients with co-morbidities and older patients to access alloSCT, who would otherwise be ineligible for standard MAC regimens. Demographic projections indicate an ever-expanding demand for RIC alloSCT and therefore a pressing need for practice optimisation. In the face of competition from alternative platforms, the FluBu3 regimen is tasked with justification of its place amongst the competitors and furthermore with identifying areas of weakness that can be improved. Optimisation of the regimen requires both retrospective review and prospective assessment of adjunctive measures; conditioning individualisation, peri-transplant monitoring algorithms and preventative management of transplant complications.

The opportunity for optimisation relies firstly on the expansion of clinical tools for prognosis and risk assessment. In myeloid disease sophistication of diagnostics is allowing for bespoke disease characterization and prognostication at the time of diagnosis, however additional tools are needed at alloSCT to better reflect the dynamism of the individual patient journey through transplant and beyond. In developing these tools, consideration must be given to equity and resource management as in many alloSCT programmes demand outstrips available resources. Therefore, development of prognostic tools and refinements in practice must include low cost and time efficient measures, which lends importance to the exploration of low-intensity assessments such as the EASIX score. Further predictive tools using biomarkers to anticipate key transplant outcomes, including GVHD, relapse, NRM and survival are needed in the clinic. Cytokine panels have the potential to identify the nuances of immune activity in transplant and can be measured at serial timepoints. Some of the key inflammatory cytokines implicated in transplant complications are now available in clinical laboratories managing inflammatory disorders and there is scope for further expansion of the available analytes.

Interrogation and correlation of cytokine expression and clinical outcomes in real-world datasets in alloSCT may help incorporate these tests into transplant algorithms.

The second area for potential optimisation relates to donor medicine, where practices are in evolution. The relative weighting of donor and recipient variable matching and particularly the potential impact of donor age on outcome post-transplant must be examined in this reduced intensity context. The consequences for all RIC alloSCT programmes include donor availability and an increasing demand on international registries. It is therefore important to identify if these findings are manifest in additional datasets as no randomized clinical trials are currently available.

Relapse post-alloSCT is an area of unmet clinical need, and efforts to prevent its occurrence drive the need to optimise this conditioning platform. An unacceptably high number of patients endure either relapse or treatment related mortality post-transplant and there is a large scope for improvement on current achievements across all current regimens. Given the aggressive nature of myeloid disease requiring alloSCT, proactive prevention of relapse represents the ideal strategy. International use of DLI is variable but its highest potential can be exploited in the RIC alloSCT domain and synergises well with the FluBuATLG approach. How best to employ DLI in a rational and anticipatory manner is an area of ongoing investigation. Managing relapse after its occurrence is similarly an area in need of further exploration, with promising signals for combinations of cellular and pharmaceutical therapies.

The emergence and popularity of PTCY-based regimens poses the largest challenge to the FluBuATLG platform. Acute toxicity and longer-term suppression of immune reconstitution and knock-on effects on infection risk and relapse are significant concerns following exposure to ATLG, with frequent clinical consequences for patients. Nevertheless, ATLG is effective at mitigating severe chronic GVHD, which in its extensive form can have profound deleterious effects for patients. In the fully HLA matched setting, it has not yet been convincingly shown that moving to a PTCY approach is mandated, but clearly stronger direct comparator data is needed. Individualised ATLG dosing and schedules have the potential to extend the role of ATLG in transplant medicine if these refinements can translate to improved outcomes. Determining the various drivers of immune reconstitution following ATLG will identify facets of the FluBuATLG platform most amenable to optimisation by rationally designed clinical trials.

Chapter 2

Retrospective review

Abstract

Fludarabine, busulfan (total dose 8mg/kg) and anti-T lymphocyte globulin (FluBu3ATLG) for RIC haematopoietic stem cell transplantation is a long-established conditioning regimen for older patients with myeloid disease. However, RIC transplantation is in evolution, including alternative chemotherapy backbones and GVHD prevention strategies. We examined the performance of this combination in modern practice in 143 sequentially treated patients from 2015-2021. Grade II-IV acute GVHD was common at 46.2%, however grade III-IV disease (11.2%) and chronic GVHD (16.8%) had a low cumulative incidence. 2-year overall survival (OS) was 70% at a median follow-up of 26.3 months. Increasing donor age was not associated with a worse survival outcome at any age-cut-off. Non-relapse mortality plateaued at 13.5% at 2-years. The cumulative relapse incidence at 2-years was 31.7% and RFS was 68.1% and 58.9% at 1- and 2-years respectively. Increased relapse risk was noted in those with graft cryopreservation and with higher graft T cell content ($p=0.02$), therefore fresh stem cell grafts are endorsed. Relapse was significantly reduced in those with mixed donor chimerism after day 90 by adopting a liberal pre-emptive donor lymphocyte infusion (DLI) strategy ($p=0.008$). The use of DLI as part of post-relapse salvage was effective, with improved OS of over 1 year ($p=0.01$). Importantly, advancing recipient age was not a predictor of any major transplant outcome in this cohort of older, frailer patients. The EASIX score complemented this platform by predicting OS at the pre-transplant ($p=0.01$), GVHD onset ($p=0.05$) and 1-year time-points ($p=0.009$) and predicted subsequent relapse at the 1-year mark ($p=0.01$). FluBu3ATLG supported by modern supportive care is well tolerated by older patients, with modest toxicity, favourable long-term outcomes and stands as a benchmark against which changes in RIC practice can be compared.

Introduction

RIC alloSCT activity, particularly for older patients, has expanded consistently over the last 2 decades (128, 130). Non-ablative doses of fludarabine (Flu) and busulfan (Bu), combined with rabbit anti-T-lymphocyte globulin (ATLG) effectively achieve engraftment of donor stem cells while limiting acute and late toxicity for those who would be otherwise ineligible for standard myeloablative regimens (3). The dose and schedule of busulfan is readily adaptable to produce varying intensity of conditioning, from non-myeloablative (Flu/Bu2) to reduced intensity (Flu/Bu3) and myeloablative (Flu/Bu4) (6). This combination is popular in European practice and FluBu3 with ATLG has been the standard reduced intensity conditioning regimen for myeloid diseases in clinical use at our institution for over 20 years.

The regimen format has remained mostly unchanged against a backdrop of two decades of transplant research. Recent interest in RIC alloSCT optimisation however has the potential to significantly alter this treatment platform's position in the therapeutic hierarchy. Alternative chemotherapy backbones, *in vivo* T cell depletion strategies, GVHD preventative strategies, evolving recipient candidacy criteria, donor selection criteria and post-transplant consolidation strategies all serve to offer increasing choice and individualisation in the RIC domain (41, 183, 304). The investigation of these changes is underpinned by a pressing need to increase availability to previously excluded patient groups and cater to increasing demand, driven by aging demographics (187). However, a paucity of direct comparator randomised

control trials hampers comparison of alternative approaches. Allogeneic transplantation is a complex process, and an array of static and dynamic variables determine outcome, ultimately leading to the difficulty in defining the optimal treatment approach.

In this aspect of the study, we interrogate the performance of this RIC platform in a cohort of uniformly treated patients in the modern era. The domains reviewed include an overall assessment of the regimen in addition to topical unanswered questions in RIC alloSCT. We examine five primary domains and their respective impacts on long-term outcomes post FluBu3ATG alloSCT: aging and frailty, the role of ATLG and immune reconstitution, donor and graft characteristics, relapse prevention and the effect of DLI and risk prediction using the EASIX model. As a reflection of established practice, this dataset provides insights into the incremental effects of two decades of refinements using a uniform platform. The results of this study provide a standard against which we can benchmark future practice changes and inform the analysis and future directions discussed in chapter 3.

Methods

Study Design

A retrospective study was conducted of 143 sequential patients undergoing RIC alloSCT from 2015 to 2021 in the National Adult Stem Cell Programme at St. James' Hospital in Dublin, Ireland. Clinical details of transplant recipients were collected using electronic, paper records, the National Integrated Medical Image System (NIMIS) and the laboratory information system (LIS). A database was created cataloguing 260 unique variables from pre-transplant to last follow up, with a censor date of 21st December 2022.

Patients and donors

Participants who had WHO-defined AML, MDS or MPN and underwent a RIC alloSCT and provided consent for data analysis at the time of transplantation were included. Age inclusion eligibility included any recipient over the age of 18. Donor characteristics and graft composition and processing data were collected in all available cases. Graft data included donor age and gender, graft cryopreservation status, CD34⁺ dose and viability, erythroid and granulocyte colony forming unit measurement and graft T cell content (CD3⁺, CD4⁺, CD8⁺ and CD4⁺/CD8⁺).

Conditioning regimen and immunosuppression

The RIC regimen included was fludarabine (180mg/m² over 6 days from day -9), busulfan (8mg/kg intravenously over 3 days from day -5) and *in vivo* T cell depletion with rabbit ATLG (30mg-40mg/kg for sibling donors and 60mg/kg for unrelated donor over 3 days from day -3). GVHD prophylaxis was methotrexate (30mg/m² split in 3 doses at day +1, 3 and 6) and a calcineurin inhibitor (ciclosporin or tacrolimus). Stem cell source was fresh or cryopreserved unmanipulated peripheral blood stem cells from sibling or unrelated donors with $\geq 9/10$ HLA-A, B, C, DRB1 and DQ matching.

Outcomes

Primary outcomes assessed were overall survival, non-relapse mortality, cumulative relapse incidence, acute GVHD incidence, chronic GVHD incidence, CD4⁺ and immunoglobulin reconstitution at day 100 and day 180, DLI exposure and viral re-activation incidence. Relapse and NRM were considered as competing events. Additional outcomes measured included engraftment time and rates, graft failure, sinusoidal obstructive syndrome incidence, calcineurin toxicity and secondary malignancy. Acute GVHD was graded according to the MAGIC criteria or Glucksberg scale. Chronic GVHD was graded according to the NIH criteria. The first day of acute GVHD was used to calculate cumulative incidence curves for that grade. Donor chimerism at 45 and 90 days was categorised as full (>97%), mixed (3-97%) or graft rejection (<3%).

Statistical methods

Demographic and categorical characteristics were described using frequencies, medians and ranges for continuous variables and proportions for categorical variables. Survival outcomes were calculated using Kaplan-Meier estimates. Cumulative incidence of NRM (with relapse as competing risk), relapse (with death in remission as a competing risk), acute and chronic GVHD (with death as a competing risk) and immune reconstitution of CD4⁺ T cells and immunoglobulins (with death as a competing risk) were calculated using Fine and Gray's test (competing risks model). Univariable analyses were performed using a Cox model for OS and Gray's test for relapse, NRM and GVHD. A Cox proportional-hazards model was used for multivariable regression. A backward-stepwise approach was used to determine independent variables considered significant and variables recognized as potential prognostic factors were screened using LASSO regression and included in the Cox model. Selection of variables was proportionate to number of recorded events to avoid overfitting of the model. Proportional hazard ratios and 95% confidence limits are provided as a summary measure in the absence of binary outcomes. Comparison of categorical variables was conducted using the chi-squared test and for means using the t-test (Mann-Whitney U for non-parametric data). Comparison of medians was conducted using the Kruskal-Wallis H test for non-parametric data and the ANOVA for comparing categorical and continuous variables. Correlation of quantitative variables was conducted using Spearman's correlation coefficient (ρ) and the Holm-Bonferroni was applied to account for multiple comparison testing. A significance alpha of 0.05 was adopted. Statistical analysis was conducted using STATA software (version 18.0 MP-Parallel edition, StataCorp, Texas, USA).

Results

Demographics

143 patients who received a RIC allograft with FluBuATLG conditioning between 2015 and 2021 were identified and included for analysis (see Table 2 for summary of demographics). There were 57 women and 86 men (ratio 1:1.5). The median age at diagnosis was 58 (range 26-71) and the median age at transplant was 59 (range 27-72). The median time to transplant from diagnosis was 291 days (range 47-3483). 3 patients received a prior autologous transplant for myeloma (n=1), or lymphoma (n=2) and no patients had a prior allogeneic transplant. Pre-transplant remission induction treatment was performed at 14 national and

cross-border centres (*Fig. 9*). The median age in those with AML was 59, 60 in those with MDS and 59 in those with MPN.

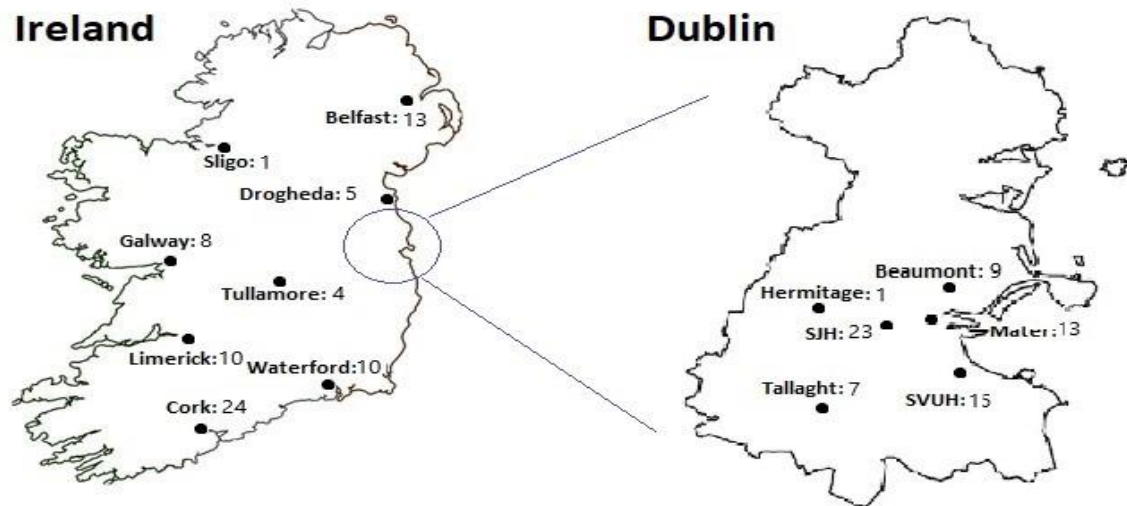


Figure 9: National and cross-border referral sources.

Table 2: Demographics

Number of patients	143
Time to transplant from diagnosis	Median: 291 days, range 47-3483
Sex	Male: 86 Female: 57
Age profile	Median: 59, range 27-72 <50: 10 50-54: 21 55-59: 41 60-64: 52 65-70+: 19
Disease class	AML: 72 MDS: 40 MPN: 31
CIBMTR Disease risk index	Favourable: 10 Intermediate: 90 Adverse: 43
Stem cell source	Sibling: 62 Unrelated: 81
Graft cryopreservation	16/143
HLA Matching	10/10: 132 9/10: 11
ABO Matching	Major mismatch: 42 Minor mismatch: 29
Age-adjusted HCT-CI	Median: 2, range 1-8
GVHD	Acute: 85/143 Chronic: 24/143
DLI	Prophylactic: 2/54 Pre-emptive: 36/54 Therapeutic: 16/54

Disease status at transplant and pre-transplant treatments

72 patients were transplanted for AML, 40 for MDS and 31 for MPN. 54 patients with AML were transplanted in CR1, with a further 18 in CR2. No patients received a transplant beyond CR2. 11 patients with MDS were transplanted in CR1, 9 patients did not receive any disease directed therapy pre-transplant, 13 were considered improved compared to diagnosis, 1 was considered refractory to therapy (ATG) and 6 had stable disease post-treatment. In the MPN group only 1 patient did not receive pre-transplant therapy. 16 patients had improved disease, 7 had stable disease, 6 had progressive disease, and 1 received a transplant in aplasia post-chemotherapy.

69 patients with AML had prior treatment with intensive cytotoxic chemotherapy including standard anthracycline (daunorubicin) and cytosine arabinoside (cytarabine) (DA), liposomal daunorubicin and cytarabine (CPX-351) and fludarabine, cytarabine, idarubicin and GCSF (FLAG-Ida/FLAG). 3 patients received azacitidine either alone or in combination with ruxolitinib or venetoclax, and a further 10 patients received it subsequent to intensive chemotherapy for initial remission induction. The anti-CD33 monoclonal antibody gemtuzumab ozogamicin was used in 4 patients, the *FLT3* inhibitor midostaurin in 1 and the Bcl-2 inhibitor venetoclax in 5.

19 patients with MDS had prior treatment with azacitidine, 9 had cytotoxic chemotherapy with anthracycline and cytosine arabinoside and the remainder had no treatment or supportive treatments including growth factors or immunosuppression including ATG, steroids and ciclosporin.

17 MPN patients had ruxolitinib as part of their pre-transplant disease therapy, 7 had azacitidine, 2 had intensive cytotoxic chemotherapy and the remainder had other therapies including hydroxyurea monotherapy, pegylated-interferon and steroids.

Acute myeloid leukaemia

Using the diagnostic criteria available during the period of observation (The 4th Edition of the World Health Organization Classification of Hematolymphoid Tumors), the subclassifications of AML included AML NOS (n=35), AML with myelodysplasia-related changes (n=21), AML with recurrent genetic abnormalities (n=10) and therapy-related myeloid neoplasms (n=6).

Cytogenetic profiling was available in 69 of 72 patients, with 3 failed karyotyping at diagnosis (Table 3). The most prevalent cytogenetic profile was a normal karyotype (n=36). Other abnormalities included complex karyotype (n=6), t(9;11) (n=4), trisomy 8 (n=3), +1 (n=2), -7 (n=2), trisomy 13 (n=1), t(8;21) (n=1), t(3;16) (n=1), t(2;5) (n=1), t(11;19) (n=1), t(11;16) (n=1), near tetraploidy (n=1), hyperdiploidy (n=1), -y (n=1), partial loss of chromosome 21 (n=1), inversion16 (n=1), +11q/21 (n=1), del9q21 (n=1), -6q (n=1), add7 (n=1) and +11 (n=1).

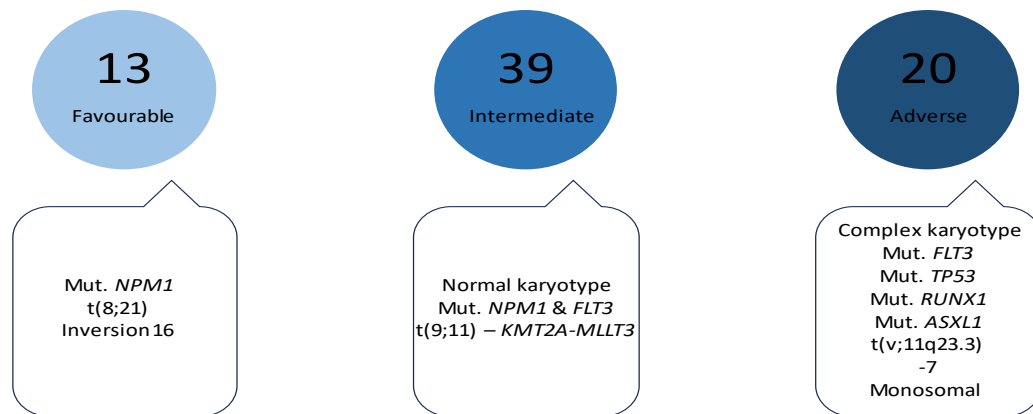
PCR single gene testing results for *FLT3* were available for 65 patients and results for *NPM1* were available for 66. A next generation sequencing molecular analysis panel (Oncomine®: 46 genes) became available for 20 of 72 latter patients. The advent of routine clinical molecular profiling beyond *FLT3/NPM1* emerged during the study period and was not available for many

of the patients in the earlier phases. Additionally, risk stratification based on molecular abnormalities was not standardised at the time of diagnosis of most patients and would not have determined the decision to transplant in these cases. Where testing was available, molecular lesions identified in this cohort pre-transplant included *NPM1* (n=19), *FLT3* (n=12), *JAK2* (n=5), *TET2* (n=5), *SRSF2* (n=4), *RUNX1* (n=3), *ASXL1* (n=2), *BCOR* (n=2), *CBL* (n=2), *U2AF1* (n=1), *TP53* (n=1), *IDH1* (n=1), *STAG2* (n=1), *NRAS* (n=1), *NF1* (n=1), *DDX41* (n=1) and *DNMT3A* (n=1).

The European Leukaemia Network (ELN) 2017 guidelines on the risk stratification of AML were the contemporaneous guidelines for this group of patients. Using the available cytogenetic and molecular data, 13 were considered favourable risk, 39 intermediate risk and 20 adverse (Fig. 10). These data are however incomplete, as earlier patients may have harboured high-risk molecular abnormalities included in the 2017 guidelines such as *TP53*, *ASXL1* and *RUNX1*, but did not have available testing for these mutations. All patients who received a transplant with a favourable disease profile at diagnosis did so due to relapsed disease, either with haematological relapse in the earlier patients or with MRD-based or haematological relapse in the later patients. *FLT3-ITD* testing during this period was qualitative with only the latest patients having data available on allelic ratio/VAF. Therefore, a small sub-cohort of patients (n=8) with co-mutations in *FLT3* and *NPM1* were considered intermediate risk for transplant decision purposes, but some of these may have had a low allelic ratio and be considered as favourable risk in the context of the 2017 guidance.

The ELN 2022 guidelines have now superseded the 2017 guidelines for risk stratification at diagnosis, and thus aid in the decision to transplant. With available information, application of this version of the guideline downgrades the risk category from adverse to intermediate in 3 patients (all due to the reclassification of *FLT3-ITD* as an intermediate risk molecular abnormality in the absence of a high-risk cytogenetic abnormality), upgrades 3 from intermediate to adverse (due to mutations in *BCOR*, *SRSF2* and *RUNX1*) and 1 from favourable to adverse (due to a *U2AF1* mutation).

Diagnostic criteria have similarly changed subsequently from the WHO 4th edition classification of myeloid neoplasms to the 2022 5th edition and there is now also the International Consensus Classification (ICC) of AML (2022). The primary changes in the WHO classification refer to cytogenetic abnormalities and hierarchical diagnostic labels, and the ICC adopts a similar structure but with the new category of MDS/AML for a blast count of 10-20% and with significant consideration of gene abnormalities in the definition of myelodysplasia-related disease. All patients in the study cohort were considered AML if blast count at diagnosis was >20% or if there was an AML defining cytogenetic abnormality. The additional categories in this cohort using the ICC criteria would be *AML with myelodysplasia-related gene mutations* and *AML with myelodysplasia-related cytogenetic abnormalities*, however, insufficient diagnostic data are available to accurately re-classify this cohort of patients and all further interpretations are done in the context of contemporaneous guidance (WHO 4th edition, ELN 2017).



Acute Myeloid Leukaemia: ELN 2017 Risk Categorisation

Figure 10: AML risk categories (ELN 2017 guidelines).

Myelodysplastic syndromes

The subclassifications of MDS in this group includes MDS with excess blasts (n=17), MDS with multilineage dysplasia (n=17) and therapy-related myeloid neoplasm (n=6). Cytogenetic data were available on 38 patients. The most common profile was a normal karyotype (n=16). Other cytogenetic abnormalities included complex karyotype (n=4), 20q- (n=4), -7q (n=2), t(3;21) (n=1), +y (n=1), trisomy 8 (n=1), t(6;9) (n=1), +9q22 (n=1), -7 (n=2), i17q (n=1), 11q- (n=1), trisomy 15 (n=1), -5q (n=1) and trisomy 9 (n=1). Molecular data were available for 13 patients. Abnormalities identified included *ASXL1* (n=4), *DNMT3A* (n=3), *TET2* (n=2), *SETBP1* (n=2), *SF3B1* (n=2), *SRSF2* (n=2), *RUNX1* (n=2), *IDH2* (n=1), *U2AF1* (n=1), *KRAS* (n=1), *BCOR* (n=1), *NRAS* (n=1), *TP53* (n=1), *EZH2* (n=1) and *STAG2* (n=1). Risk stratification of MDS utilised the Revised International Prognostic Scoring System (IPSS-R). The median IPSS-R was 4.1 (Intermediate) with a range of 0.4 to 9.5. 2 patients were considered very low risk (IPSS-R <1.5), 9 were low (1.5 to 3), 13 intermediate (3.1-4.5), 10 high (4.6-6.5) and 6 very high risk (>6.5) (Fig. 11).

The WHO 2022 classification of myeloid neoplasms now defines myelodysplastic neoplasia based on exposure to prior cytotoxic therapy, which identifies the distinct category of patients who develop therapy associated MDS rather than the previous category of therapy-related myeloid neoplasm, which was a subsection of the AML diagnostic criteria. Additional categories of patients in this cohort now defined by the WHO 2022 criteria would include *MDS with low blasts and isolated 5q deletion* (n=1) and *MDS with low blasts and SF3B1 mutation* (n=1). The 17 patients classified as MDS MLD by the 2016 criteria would now be considered *MDS with low blasts* (n=16) or *MDS, hypoplastic* (n=1). The 17 patients with MDS with excess blasts would remain in the counterpart *MDS with increased blasts* category by WHO criteria, but according to the ICC 2022 recommendations, 15 of these 17 patients would be classified as MDS/AML.

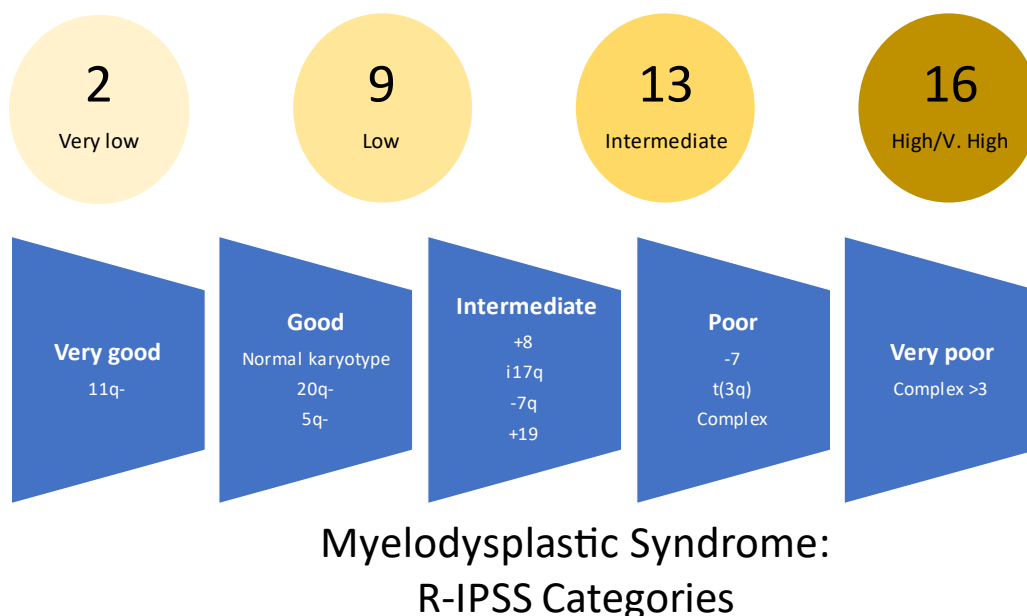


Figure 11: MDS risk categories (IPSS-R).

Myeloproliferative neoplasms and overlap syndromes

The subclassifications of MPNs by the WHO 2016 criteria included myelofibrosis (n = 23), and MPN, unclassifiable (n = 2). A further 5 patients with MDS/MPN overlap syndromes were included in this group, including proliferative chronic myelomonocytic leukaemia (n = 5) and atypical chronic myeloid leukaemia, *BCR-ABL1 negative* (n = 1). Further clinical subclassification of these myelofibrosis patients showed 6 patients had known pre-existing polycythaemia vera or essential thrombocythemia prior to transformation to myelofibrosis and 17 had primary myelofibrosis. Cytogenetic analysis was available on 16 patients, with one showing a secondarily acquired t(9;22) and the remainder displaying a normal karyotype. Several patients had failed cytogenetic analysis due to dry aspirates in the setting of marrow fibrosis.

MPN-driver mutation analysis for *JAK2*, *CALR* and *MPL* were available for all patients, and in later years extended molecular analysis was available for 9 patients. In the patients with myelofibrosis, the most common mutations identified were *JAK2* (n=13), *CALR* (n=7) and *MPL* (n=1). Two patients with myelofibrosis had none of the 3 cardinal driver mutations. Additional mutations seen in the myelofibrosis patients included *ASXL1* (n=3), *NRAS* (n=2), *TET2* (n=1), *RUNX1* (n=1) and *DNMT3A* (n=1). In the patients with CMML and atypical CML, the mutations seen included *TET2* (n=3), *ASXL1* (n=2), *SRSF2* (n=2), *SETBP1* (n=1), *TP53* (n=1), *BCOR* (n=1), *CBL* (n=1), *RUNX1* (n=1), *NRAS* (n=1) and *JAK2* (n=1).

Risk stratification prior to transplantation used the DIPSS/DIPSS Plus score for myelofibrosis, showing 4 patients had intermediate–1 disease, 15 had intermediate–2 and 4 had high risk disease (Fig. 12). In those with CMML, both the Dusseldorf score and CPSS-Mol were applied where sufficient molecular data was available. 1 had low risk disease, 3 had intermediate risk and 1 had high risk disease.

Using updated classification with the WHO 2022 edition, the patients with myelofibrosis would remain categorised as so with no further subclassification. Similarly, there would be no change by the ICC 2022 classification guidelines. According to the WHO, a minor change would apply to the 2 cases of MPN-U, now classified as MPN – not otherwise specified, whereas this minor alteration is not seen in the ICC guidelines. The patient with atypical CML, *BCR-ABL1 negative* is now classified as myelodysplastic/myeloproliferative neoplasm with neutrophilia by the WHO and remains as atypical CML by the ICC. The patients with CMML would remain unchanged.

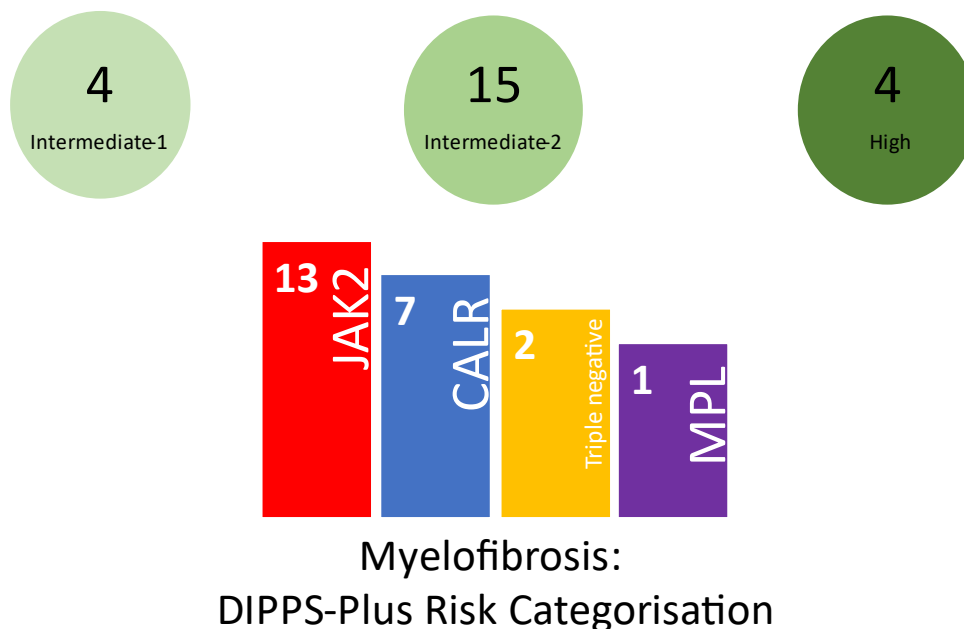


Figure 12: Myelofibrosis risk categories (DIPPS-Plus).

Age profile and transplant-related risk stratification

Age & weight profile

The median age at transplant was 59, with a range of 27 to 72. 93% of patients were aged over 50. Within five-year age groups, the largest cohort of patients were between the ages of 55 and 65 (Fig. 13). The age profile of transplant recipients in this cohort increased from a median age of 56.5 years in 2015 to 61 in 2021. The median time to transplant from initial diagnosis was 291 days. Time to transplant was 180 days in AML, 371 days in MDS and 391 days in MPN. The median weight was 80kg, range 46kg to 142.2kg. Transplants per year increased over the span of observation from 18 in 2015 to 34 in 2021.

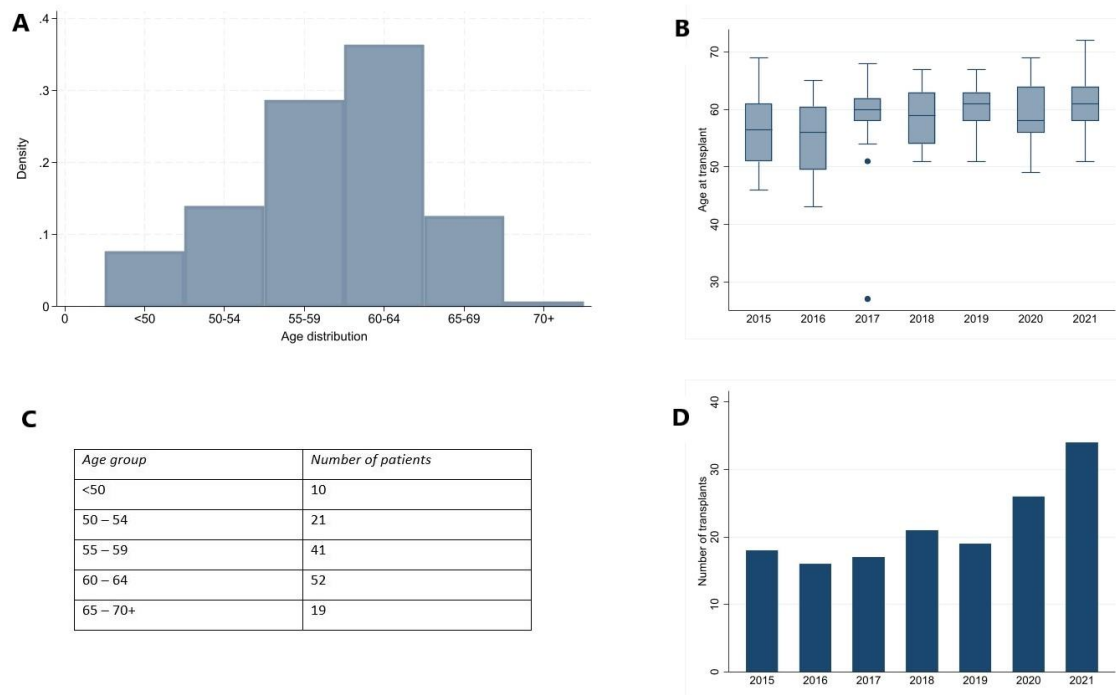


Figure 13. A: Age distribution. B: Age evolution over course of observation period. C: Absolute numbers of age groups. D: Number of FluBuATLG transplants per calendar year, 2015-2021.

Disease and patient risk scores

The CIBMTR disease risk index was calculated for all patients. 10 patients were classified as low risk. Of these patients, 8 had AML with an *NPM1* mutation and no additional adverse risk abnormalities and were transplanted in CR2 following overt or molecular relapse. The remaining 2 patients had relapsed favourable risk disease with t(8;21) and inversion 16 and were also transplanted in CR2. 90 patients were classified as intermediate, including AML (n=41), MDS (n=15) and all MPN patients (n=31). 43 were considered high risk and included AML (n=20) and MDS (n=23) with high-risk cytogenetic abnormalities.

The median KPS, as assessed immediately pre-transplant was 80, with a range of 60 to 100. The median KPS did not differ by disease group and there was a small difference in KPS between age groups. The median KPS for those <50 was 70, 80 ages 50– 54, 70 ages 55– 59, 80 ages 60 – 64 and 80 ages 65 – 70+.

The age-adjusted HCT-CI was recorded in all patients, with only 1 recipient capable of scoring < 1 due to age < 40 (this patient however did not score < 1 due to additional co-morbidities). The median age-adjusted HCT-CI score was 2 and the range was 1 – 8 (Fig. 14). A score of 2 using this prognostication system predicts a 2-year non-relapse mortality rate of 18% and a 2-year overall survival of 70%.

The median score in the AML subgroup was 2 with a range of 1 - 8. The median score in the MPN group was 2, with a range of 1 - 6. Patients with MDS had a slightly higher burden of co-morbidities pre-transplant with a median score of 3 and a range of 1 – 5.

In those aged <50, the median score was 3, range 1 – 4. In those aged 50 – 54 the median was 3, range 1 – 8. In those 55 – 59 the median was 2, range 1 – 8. In those 60 – 64, the median was 2, range 1 – 8. In those 65 – 69 the median was 1, range 1 – 5 and in the patient >70 the score was 4.

The median left ventricular ejection fraction pre-transplant was 56%, with a range of 40% to 75%. 9 patients had an ejection fraction <50%, 8 of whom were treated with anthracyclines as part of induction therapy and one patient who had azacitidine only. The median FEV1 was 101% with a range of 64 to 143%. The median DLCO/VA was 89% with a range of 40 to 128%. The median creatinine clearance was 103 mls/min with a range of 39 to 189 mls/min. The median serum ferritin pre-transplant was 1542 ng/ml, with a range of 6.7 to 14,417 ng/ml. 45 patients had a serum ferritin of >1,000 ng/ml at the time of transplant.

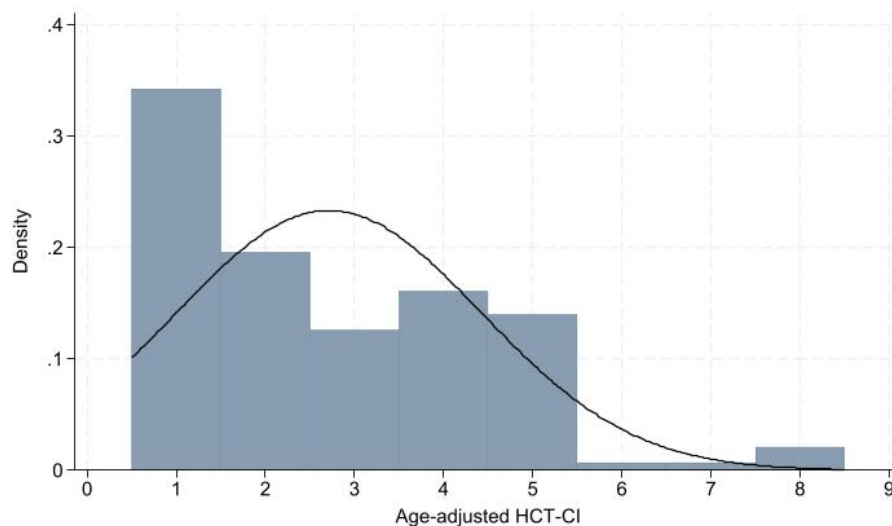


Figure 14: Age-adjusted HCT-CI median and range.

Donor source and characteristics

81 patients received a graft from an unrelated donor and 62 from a sibling. Female recipients were more likely to have a suitable sibling donor than males ($p=0.02$). The median age of all donors was 38 years, range 18-65. The median age was 26 in unrelated donors, range 18-64, and 54 in siblings, range 24-65. 84 pairs were sex matched and there were 22 female donors for male recipients and 37 male donors for female recipients.

There were 11 cases of HLA allele mismatch, all of which were permissive single antigen mismatches. 9 of the HLA mismatched grafts were from unrelated donors (B (n=4), C (n=2), DQ (n=2) and A (n=1)), and 2 from siblings (both A mismatches). All patients received PBSC grafts harvested from donors using standard weight based GCSF priming. 16 grafts were cryopreserved prior to re-infusion and thawed at the time of transplant; the remainder were infused fresh within 24 hours of collection. The indication for cryopreservation was primarily logistical due to the COVID-19 pandemic, and the institutional preference was for fresh product. There were 42 major ABO mismatched pairs, 29 minor ABO mismatches and 72 with

full ABO matching. Allografts from unrelated donors showed a trend towards higher rates of ABO mismatching ($p=0.07$).

Graft characteristics and cryopreservation

Graft composition information was available in all cases, with further extended T cell phenotyping in 140/143 grafts. The median CD34⁺ viability as tested by ethidium bromide acrinide orange (EBAO) staining was 99% (range 81.8-100) and the median CD34⁺ cell dose infused was $5 \times 10^6/\text{kg}$ (range 1.77 - 6.28). The median burst forming unit-erythroid (BFU-E) was $60.19 \times 10^4/\text{kg}$ (range 5.69 - 213.64). The median colony-forming unit granulocyte-macrophage (CFU-GM) was $48.66 \times 10^4/\text{kg}$ (range 11.38 - 171.58). The graft CD3⁺ median was $17.14 \times 10^7/\text{kg}$ (range 4.87 - 129.92). Further T cell subset analysis showed a median CD4⁺ count of $11.09 \times 10^7/\text{kg}$ (3.12 - 70.18), CD8⁺ of $6.37 \times 10^7/\text{kg}$ (1.34 - 61.06) and CD4⁺/CD8⁺ of $0.24 \times 10^7/\text{kg}$ (0.02 - 5.29) respectively.

Median CD34⁺ graft content was lower in those above the age of 35 ($p=0.015$) and was lowest in the oldest donor age group (median CD34⁺ $5 \times 10^6/\text{kg}$ with donor age <25 vs. $4.64 \times 10^6/\text{kg}$ with donor age >60). The median infused CD34⁺ count was similar in cryopreserved and fresh grafts (4.84 vs. 5). The median CD34⁺ EBAO viability was lower in cryopreserved grafts compared to fresh (92% vs. 99%, range 81.8 - 98.8 vs. 95.6 - 100). Despite this, a one-day reduction in time to neutrophil and platelet engraftment was seen in the cryopreserved group (17 vs. 18 days and 19 vs. 20 days respectively). The median BFU-E was slightly higher in cryopreserved grafts (68.18) compared to fresh grafts (57.65), but the variance was not significant ($p=0.95$). The median CFU-GM was similar in cryopreserved and fresh grafts (48.65 vs. 48.91).

The cryopreserved grafts had significantly higher CD3⁺ content as compared to fresh grafts (26.51 vs. 16.86, $p=0.003$). Similarly, T cell subclasses followed a similar distribution of higher content in cryopreserved grafts including CD4⁺ content (16.75 vs. 10.6, $p=0.01$), CD8⁺ content (9.45 vs. 5.78, $p=0.001$) and dual positive CD4⁺/CD8⁺ content (0.38 vs. 0.22, $p=0.003$).

Median time to transplant in the cryopreserved group was 437 days. Time to transplant was 344 days in AML, 412 days in MDS and 582 days in MPN. Disease classification and risk groups were similar between non-cryopreserved and cryopreserved groups. In non-cryopreserved groups the disease classes were 51.1% AML, 27.5% MDS and 21.2% MPN. In the cryopreserved group the diseases were 43.7% AML, 31.2% MDS and 25% MPN. CIBMTR DRI groups for non-cryopreserved cases were 7.8% low, 61.4% intermediate, 30.7% high. In cryopreserved cases the DRI groups were 75% intermediate and 25% high.

Conditioning format, engraftment and rejection

Transplants with sibling donors ($n = 62$) were conditioned with fludarabine $180\text{mg}/\text{m}^2$ as $30\text{mg}/\text{m}^2$ daily for 6 days from day minus 9, busulfan $8\text{mg}/\text{kg}$ given as $0.8\text{mg}/\text{kg}$ in 10 doses from day minus 5 to day minus 3 (FluBu3). 19/61 patients with a sibling donor in the first phase of the observation period received $10\text{mg}/\text{kg}$ ATLG on days minus 4 to minus 1, and the subsequent patients received $10\text{mg}/\text{kg}$ rabbit ATLG on day minus 3 to day minus 1. Transplants with unrelated donors ($n=81$) received ATLG on day minus 3 to day minus 1 at a dose to $20\text{mg}/\text{kg}$ with the same fludarabine and busulfan dosing.

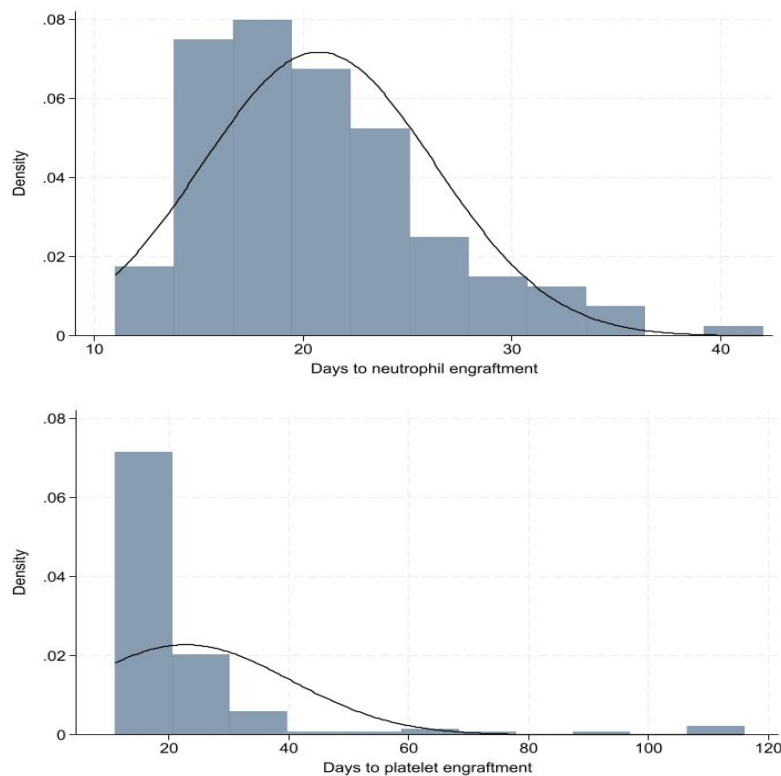


Figure 15. Left: Days to neutrophil engraftment, median and range. Right: Days to platelets engraftment, median and range.

Neutrophil engraftment was universal at a median of 20 days, range 11 – 42 days. Platelet engraftment occurred in 96.5% at a median of 18 days, range 11 – 116 days (*Fig. 15*). Time to neutrophil engraftment did not correlate with increasing age, with a median time of engraftment by age group of 24 days in those <50, 16 days aged 50-54, 21 days aged 55-59, 20 days aged 60-64 and 17 days aged 65 or above. Similarly, no significant difference in time to engraftment was seen in the different disease subtypes, with a median of 19 days in AML, 21 days in MPN and 19 days in MDS. Patients with primary myelofibrosis had a longer time to engraftment of 22 days but all achieved primary engraftment. In the cohort of patients who received a cryopreserved graft the median time to neutrophil engraftment was reduced at 19 days.

Platelet engraftment similarly was not significantly affected by age, and time to engraftment appeared reduced in the oldest age group. Median time to engraftment in those aged <50 was 18.5 days, 16 days in those aged 50-54, 18 days in those aged 55-59, 19 days in those aged 60-64 and in those aged >65 it was 14 days. Primary failure of platelet engraftment occurred in 4 patients, 3 of whom had MDS and 1 had myelofibrosis. Median time to engraftment based on disease subtype was 17, 18 and 22 days in AML, MDS and MPN respectively. Those with primary myelofibrosis had a slightly longer time to engraftment of 23 days. One patient who received a cryopreserved graft experienced failure of primary platelet engraftment. Failure of

primary platelet engraftment was not associated with advanced donor age, with 75% of failures occurring with a donor age <25 years.

Median time to neutrophil engraftment was 19.5 days in those transplanted using an unrelated donor (range 11-36) and 20.5 days with sibling donors (range 13-42). Median time to platelet engraftment with an unrelated donor was 19 days (12-116) and 16 days (11-110) with a sibling donor. All primary failures of platelet engraftment occurred with an unrelated donor. There was no correlation between donor age and time to neutrophil engraftment ($p(140) = 0.075$, $p=0.38$). Similarly, donor age did not correlate with time to platelet engraftment ($p(137) = -0.07$, $p=0.41$).

There were no cases of primary graft failure in this cohort. Secondary graft failure was seen in 36 patients, of which 33 were due to relapsed primary disease. Of the 3 patients who experienced late graft failure without relapse, 1 occurred in the setting of fatal multi-organ failure, sepsis and refractory gastrointestinal GVHD in a patient transplanted for myelofibrosis, 1 in the setting of disseminated adenoviral re-activation with fatal pneumonia in a patient transplant for MDS and 1 in the setting of refractory severe gastrointestinal and liver GVHD in a patient transplanted for AML. No cases of sinusoidal obstructive syndrome were diagnosed in this cohort of patients.

ATLG and GVHD prophylaxis

All patients received post-transplant methotrexate 10mg/m² on days +1/3/6 and the calcineurin inhibitors used for GVHD prophylaxis were ciclosporin (n=91) or tacrolimus (n=52) at transplant physician's discretion. A target trough serum level of 100-150 µg/L for ciclosporin and 5-10 ng/ml for tacrolimus was recommended. 124 of 143 patients had a recorded CNI level >10% above target within the first 30 days of stem cell infusion, on a median of 2 occasions with a range of 0 to 6.

Anti-T lymphocyte globulin use was universal as part of the conditioning protocol. 22 patients in the earlier part of the cohort were treated with *Fresenius*® brand rabbit ATLG and the remainder with *Grafalon*®. Those transplanted with sibling grafts (n=61) received 10mg/m² ATG on days minus 3 to minus 1 inclusive, and 20mg/m² for those with grafts from unrelated donors (n=82). The median total dose of ATLG was 3920mg, range 1380mg to 8250mg. The median absolute peripheral lymphocyte count on the first day of ATLG exposure was 0.1 x 10⁹/L, with a range of 0 to 1.9 x 10⁹/L. 121/143 patients had a lymphocyte count of 0.1 x 10⁹/L or less at this time point, following lymphodepletion with fludarabine.

Overall survival

OS at 1- and 2-years was 78.7% and 70% respectively. Median OS has not been reached at a follow up of 26.3 months (*Fig. 16*). There were 46 observed deaths within the observed period. Causes of death in the cohort included 17 non-relapse mortality events and 29 post-relapse deaths. In those who died post-relapse all were directly attributable to the relapse, including bone marrow failure complications, infection, and bleeding. A summary of univariable and multivariable survival analyses is provided in table 3. OS did not differ between sex (*Fig. 17*), age groups (*Fig. 17*) or class of disease (*Fig. 18*). Within the myelofibrosis subgroup, disease risk group by DIPPS-Plus strongly correlated with survival outcome (*Fig. 19*). In AML, no difference in survival was seen between those transplanted in CR1 vs. CR2+.

The median whole-cohort age-adjusted HCT-CI score of 2 predicted 70% 2-year OS, which was closely reflected in the actual outcome of 70% 2-year OS in this real-world patient group. Individual age-adjusted HCT-CI scores did not however stratify the risk of mortality (*Fig. 20*). The KPS as measured immediately pre-transplant did not predict overall survival in this cohort, with scores above and below a median score of 80 showing similar overall survival outcomes (*Fig. 21*). The CIBMTR DRI predicted a statistically significant difference in 2-year overall survival when comparing low and intermediate categories to the high-risk category (*Fig. 22*). 2-year overall survival in the high-risk group was 57%, as compared to 74% in the intermediate group and 80% in the low-risk group.

There was no statistically significant difference in OS between those who received a sibling or an unrelated donor stem cell graft (*Fig. 23*). OS was not worse in those who received a single antigen HLA mismatched graft, although numbers were low (*Fig. 24*). Minor ABO antigen mismatch was associated with an increased risk of all-cause mortality in this cohort ($p=0.01$), with major mismatch showing a trend towards increased mortality also ($p=0.06$). When comparing ABO matched transplants to any mismatch type, there was a significantly reduced overall survival in those with a mismatch (*Fig. 25*). No correlation between donor age and survival outcomes was seen (*Fig. 26 and 27*). Graft CD3⁺, CD4⁺ and CD4⁺/CD8⁺ content did not correlate with survival outcomes, however an association between increased graft CD8⁺ content and decreased OS was noted (*Fig. 28*). Graft cryopreservation was not associated with a decreased overall survival ($p=0.69$). There was no difference in OS between those who had ciclosporin as the calcineurin inhibitor of choice as compared to tacrolimus ($p=0.42$).

OS was not significantly different in those who did or did not develop any-grade acute GVHD (*Fig. 29*). Improved OS was however seen in those who developed chronic GVHD (*Fig. 30*). A mixed chimerism at day 90 post-transplant was not associated with a survival disadvantage in this cohort of patients ($p=0.90$). Additionally, those who received a donor lymphocyte infusion for any indication (prophylactic, pre-emptive or therapeutic) had an increased survival benefit ($p=0.001$). In patients who had a mixed donor chimerism beyond day 90, the use of a pre-emptive DLI was associated with a significant survival benefit compared to those with mixed chimerism who did not receive a DLI (*Fig. 31*). Similarly, in those who relapsed, receipt of a DLI as part of a salvage strategy was associated with a significant improvement in overall survival (mean survival 758 days vs. 1218 days) (*Fig. 32*).

T cell subset analysis was available on 52 patients at the day 100 time point. A recovery to $> 50 \times 10^6/L$ was not associated with an increased survival in this subgroup ($p=0.6$). Similarly, a recovery to $> 100 \times 10^6/L$ was also not associated with a survival benefit ($p=0.66$). Survival was not affected by total lymphocyte, CD3⁺ or CD8⁺ recovery at this time point. Immunoglobulin recovery data was available on 70 patients at day 100. Lower IgG recovery was associated with reduced overall survival ($p=0.03$), independently of IgA or IgM recovery. At day 180, T cell analysis was available on 86 patients. Total lymphocyte and T cell subset recovery was not associated with survival outcomes. At this time point 92 patients had immunoglobulin data, and an IgG level of $< 5 \text{ g/L}$ was associated with an increased risk of subsequent mortality (*Fig. 33*). At 1 year, data for T cell subsets ($n=90$) and immunoglobulins ($n=90$) did not predict an association with subsequent mortality.

Invasive fungal disease, determined as probable or confirmed, and requiring systemic therapy, was associated with reduced overall survival (*Fig 34*). CMV seropositivity in recipients was not associated with reduced survival and viral re-activation, as detected by PCR monitoring post-transplant, was not associated with reduced survival for CMV, ADV or EBV. Other

opportunistic infectious including BK, respiratory viruses, toxoplasmosis, and *pneumocystis jirovecii* were not associated with reduced survival.

At a cut-off of 3, the EASIX score, recorded immediately pre-conditioning, was predictive of a reduced 2-year overall survival (Fig. 35). The score at the day 0 time point was unable to predict survival outcomes. At the onset of acute GVHD and a median cut-off of 1.97, an increased EASIX score was predictive of reduced overall survival (Fig. 36). At 1-year post-transplant, at a median cut-off of 1.2, a higher EASIX score was strongly associated with an increased risk of subsequent mortality by 2 years (Fig. 37). Univariable cut-offs for prediction of mortality, EASIX and graft T cell content are summarized in tables 4 and 5.

A Cox multivariable regression model including significant univariable mortality predictors alongside screening by LASSO regression identified a model including ABO mismatch, DLI use, chronic GVHD, CIBMTR-DRI group and IFD. After model fitting, all these variables except for the CIBMTR-DRI risk group retained significance. A post-hoc global test confirmed the proportional hazard assumption was not violated (Table 4).

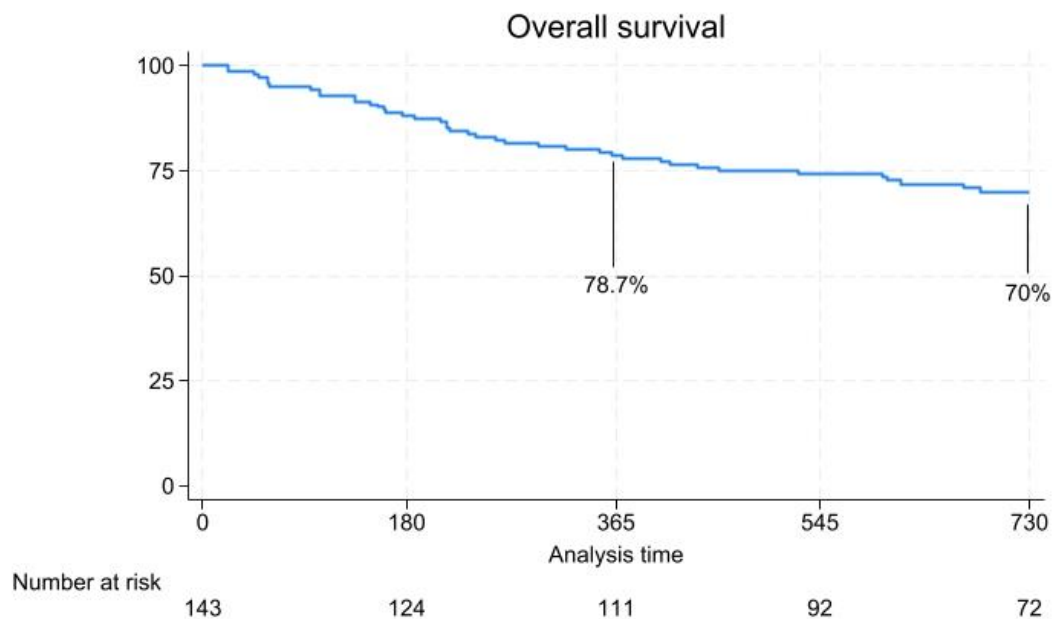


Figure 16: Overall survival at 2-years.

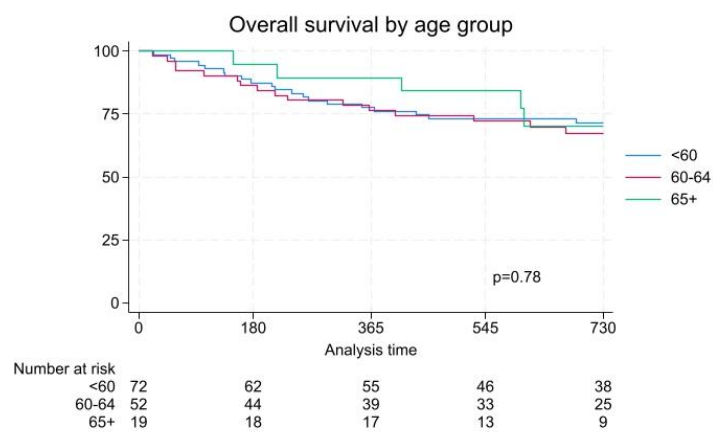
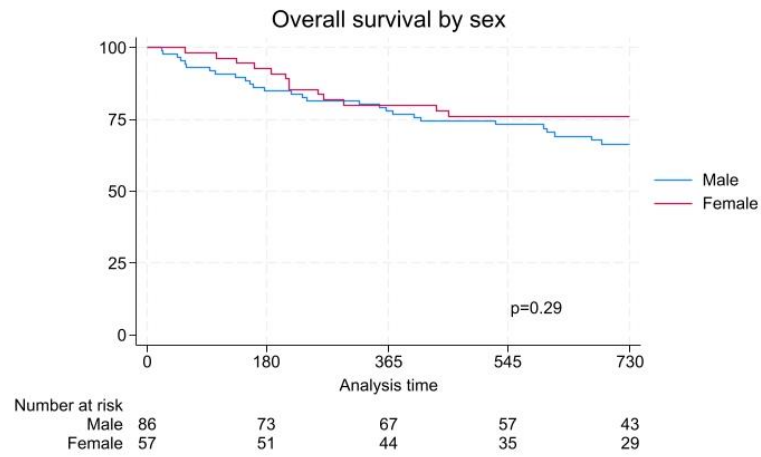


Figure 17: Overall survival by recipient sex and by recipient age group; <60, 60-64, 65+.

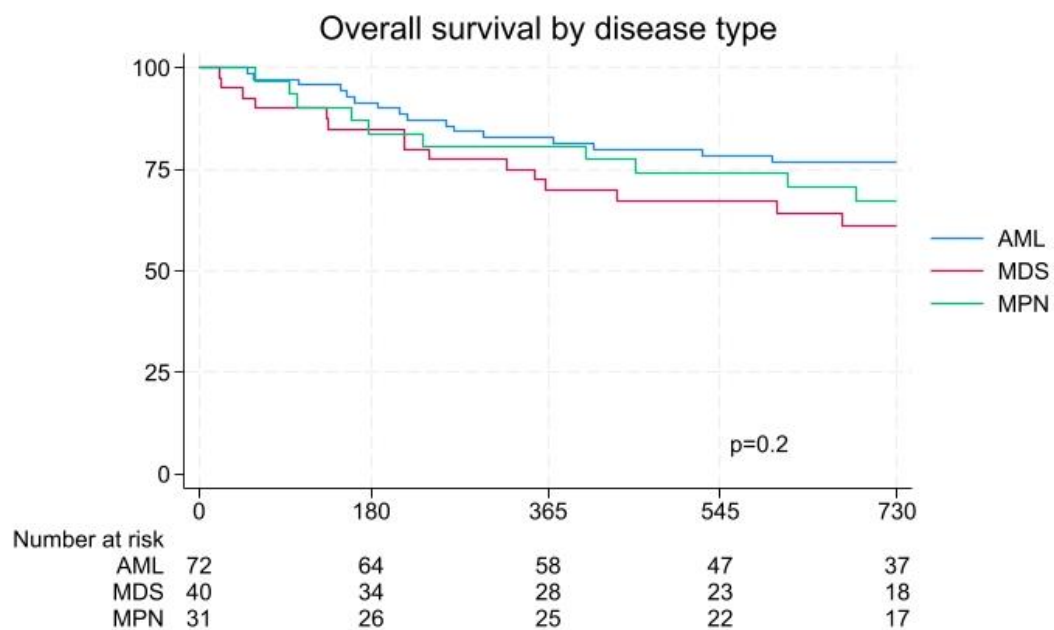


Figure 18: Overall survival by disease subtype; AML, MDS and MPN.

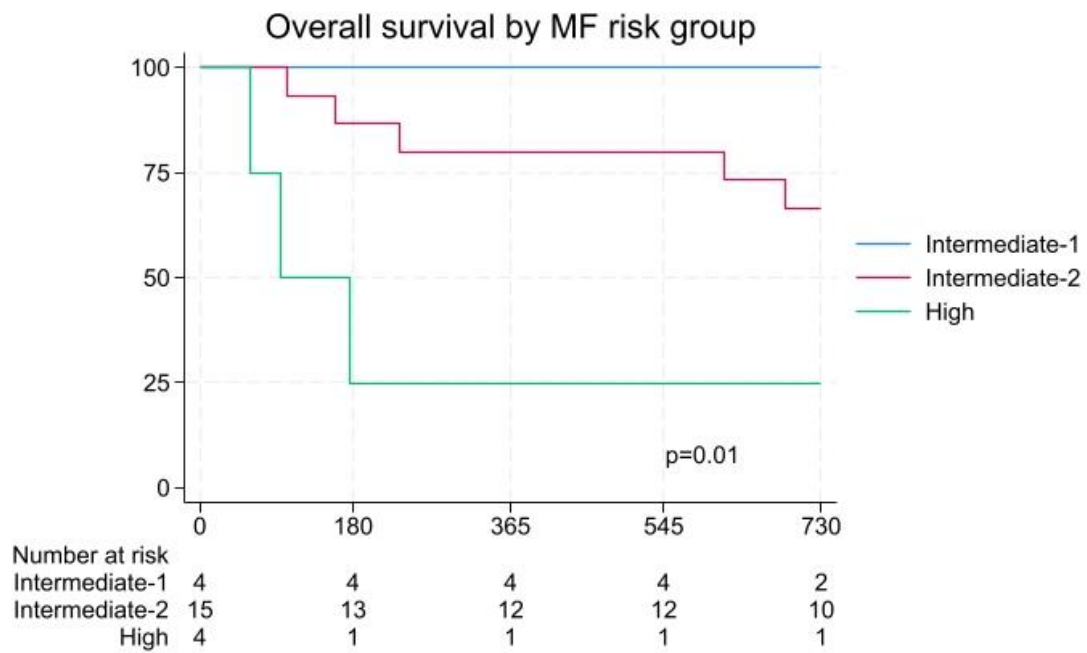


Figure 19: Overall survival by MF risk group; intermediate-1, intermediate-2 and high.

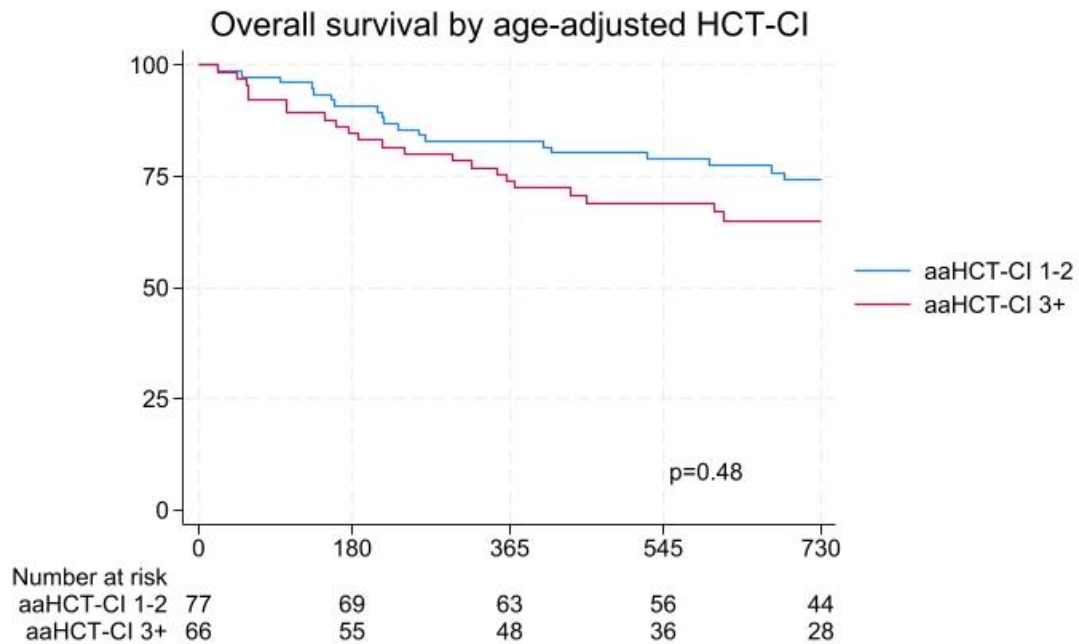


Figure 20: Overall survival by age-adjusted HCT-CI at cut-off of 3.

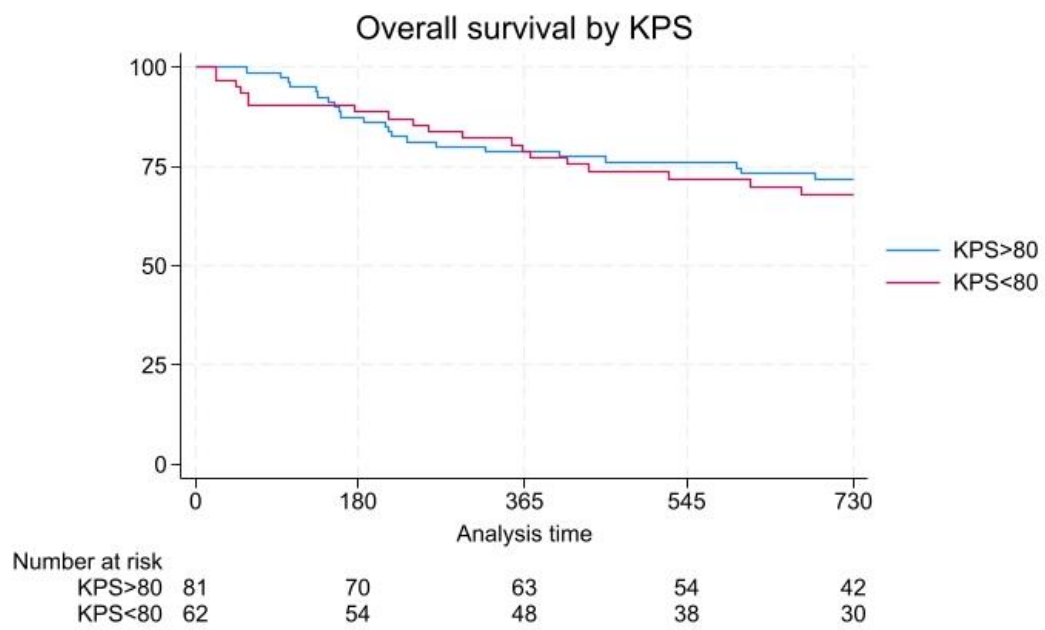


Figure 21: Overall survival by Karnofsky Performance Score at cut-off of 80.

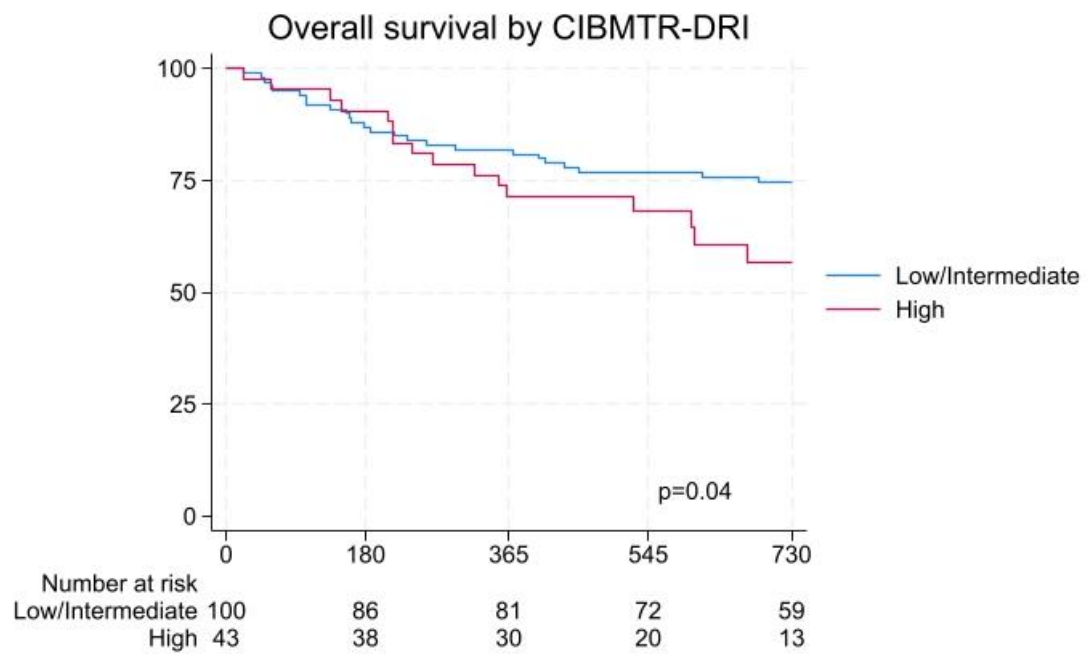


Figure 22: Overall survival by CIBMTR-DRI; low and intermediate vs. high.

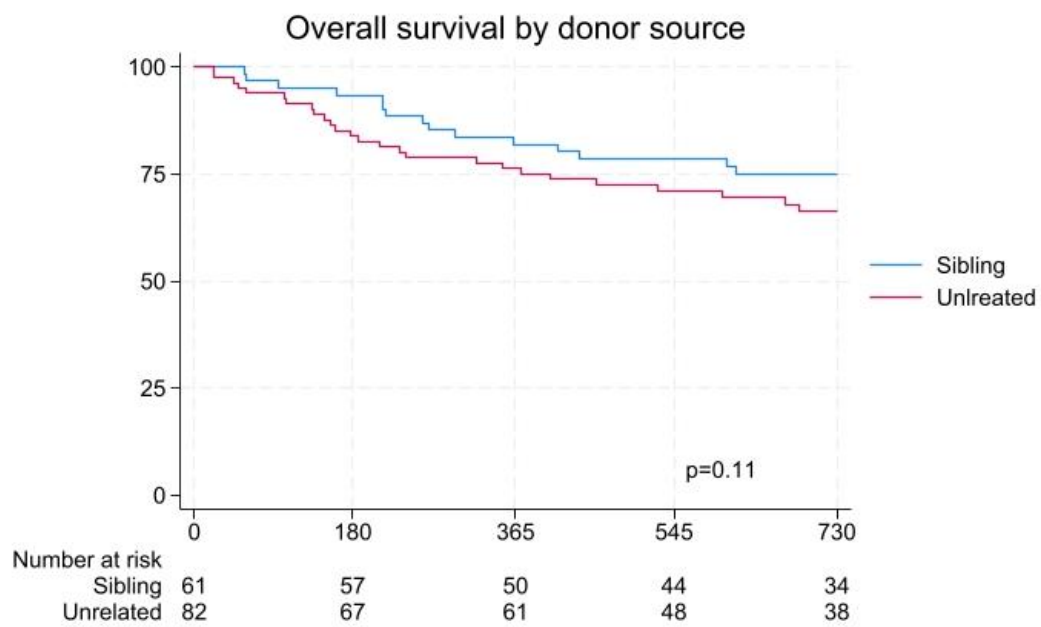


Figure 23: Overall survival by donor source; sibling vs. unrelated.

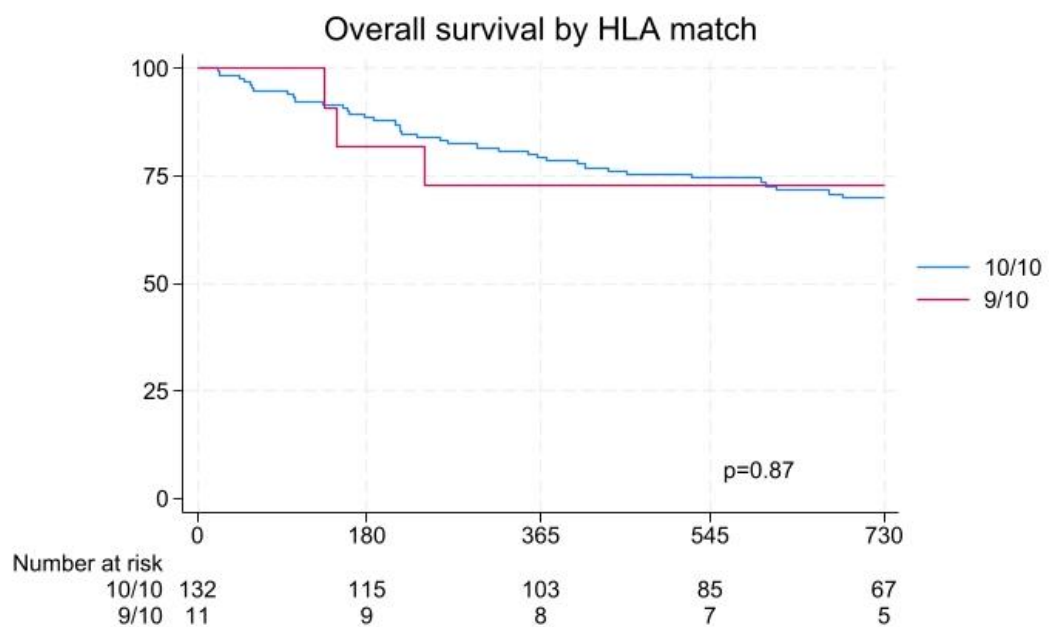


Figure 24: Overall survival by HLA match.

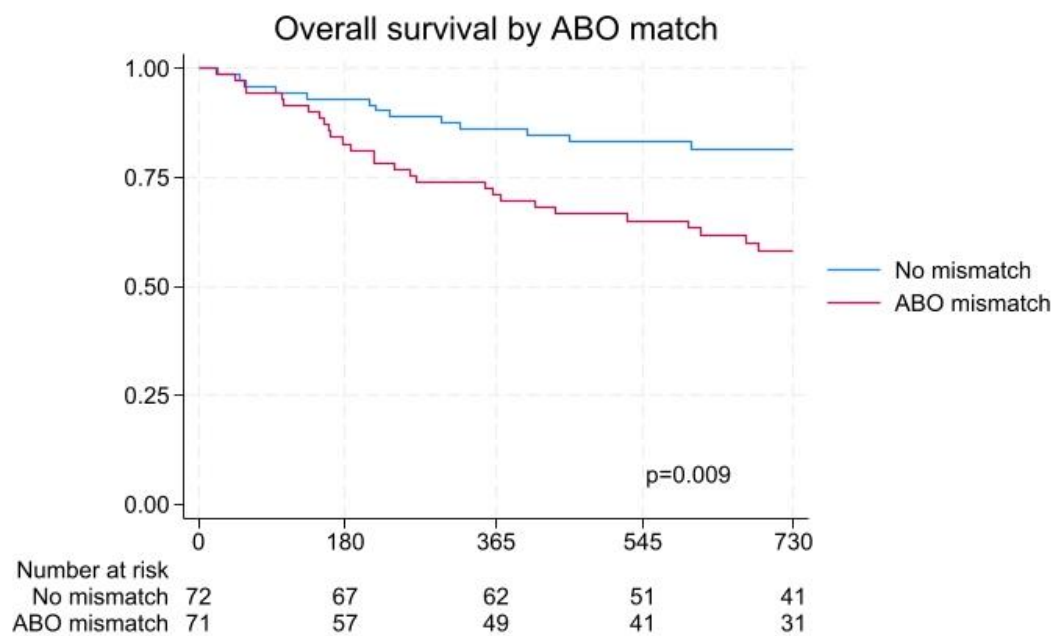


Figure 25: Overall survival by ABO match.

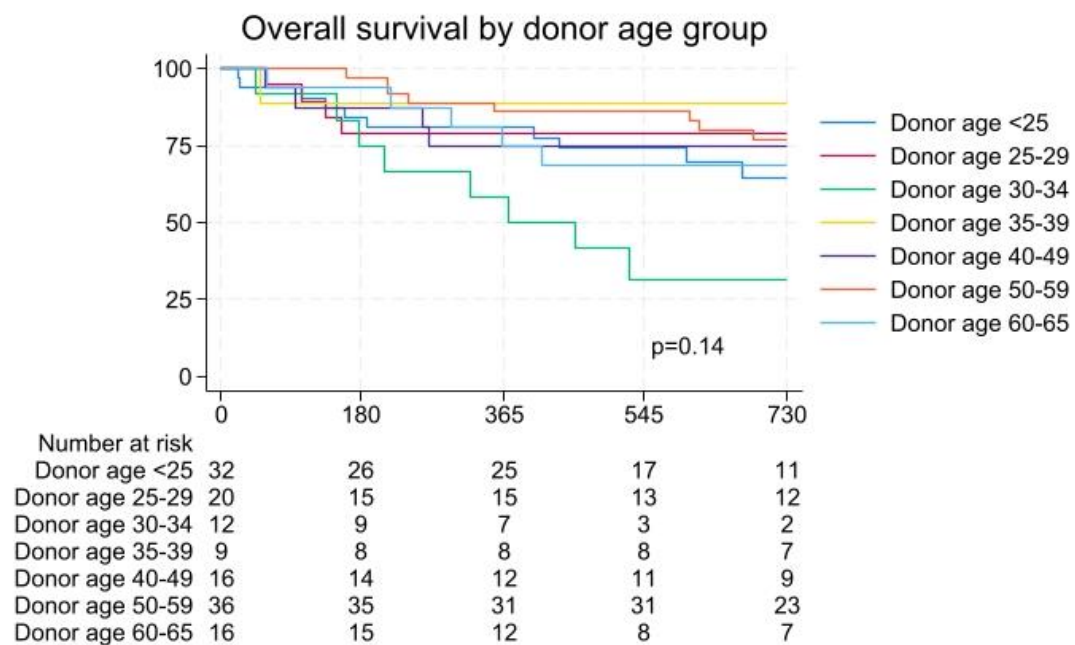


Figure 26: Overall survival by donor age group.

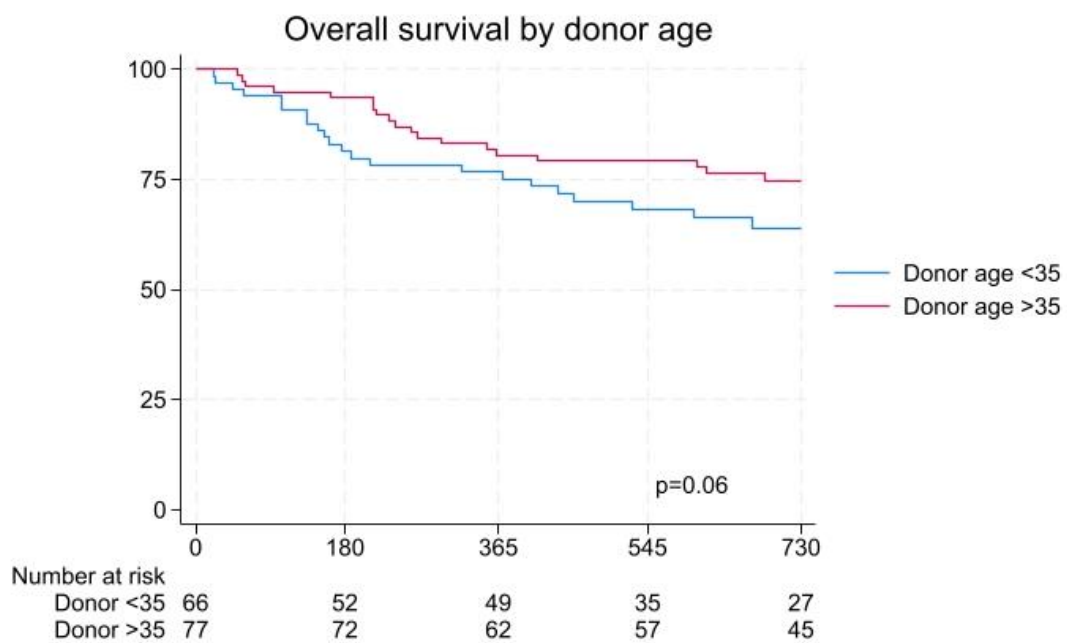


Figure 27: Overall survival by donor age above and below 35.

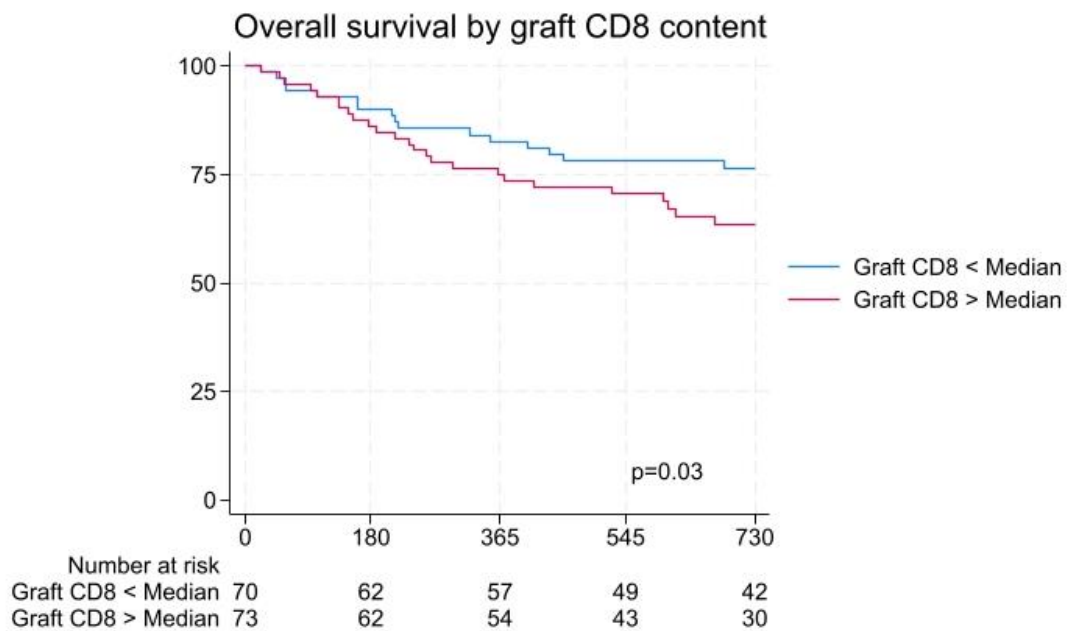


Figure 28: Overall survival by graft CD8⁺ T cell content above and below median of $6.37 \times 10^7/\text{kg}$.

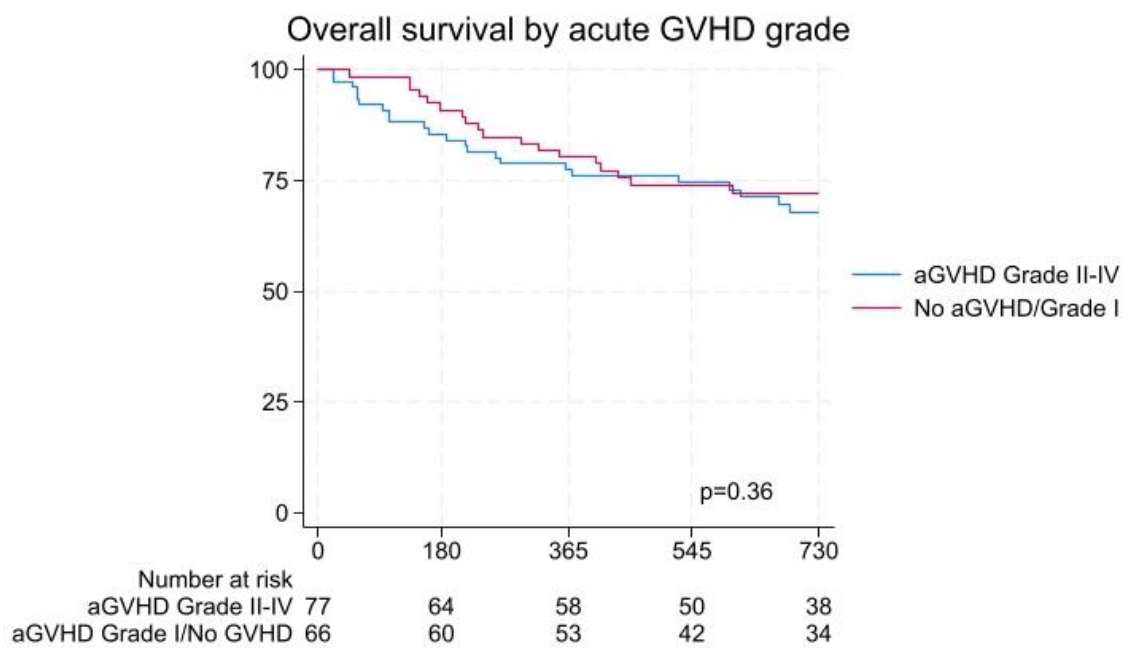


Figure 29: Overall survival by acute GVHD grade II-IV.

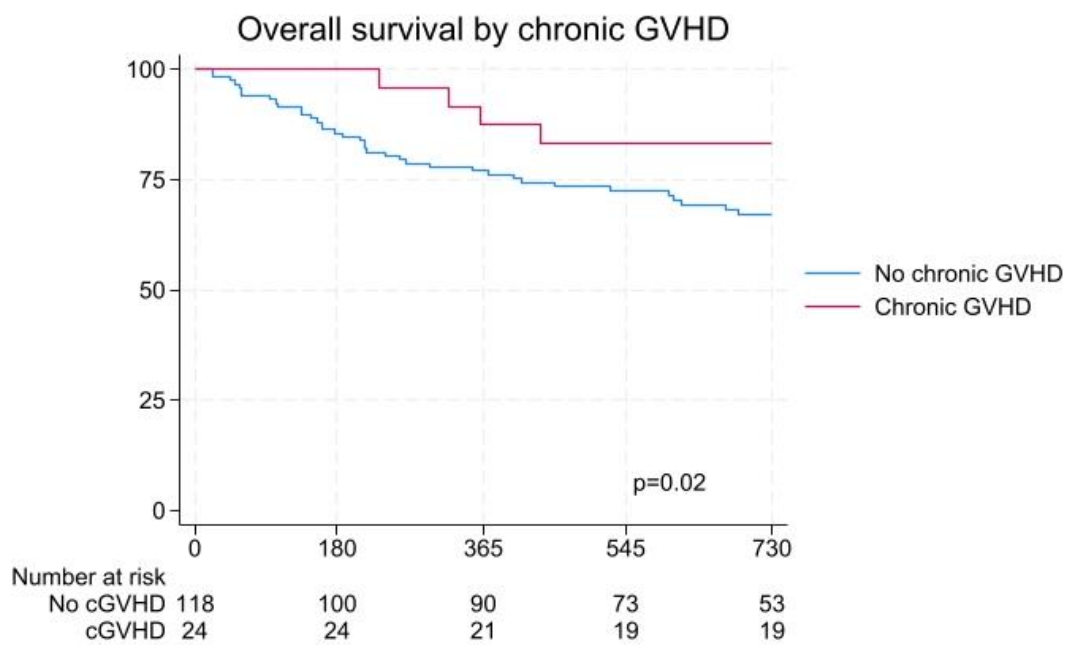


Figure 30: Overall survival by chronic GVHD.

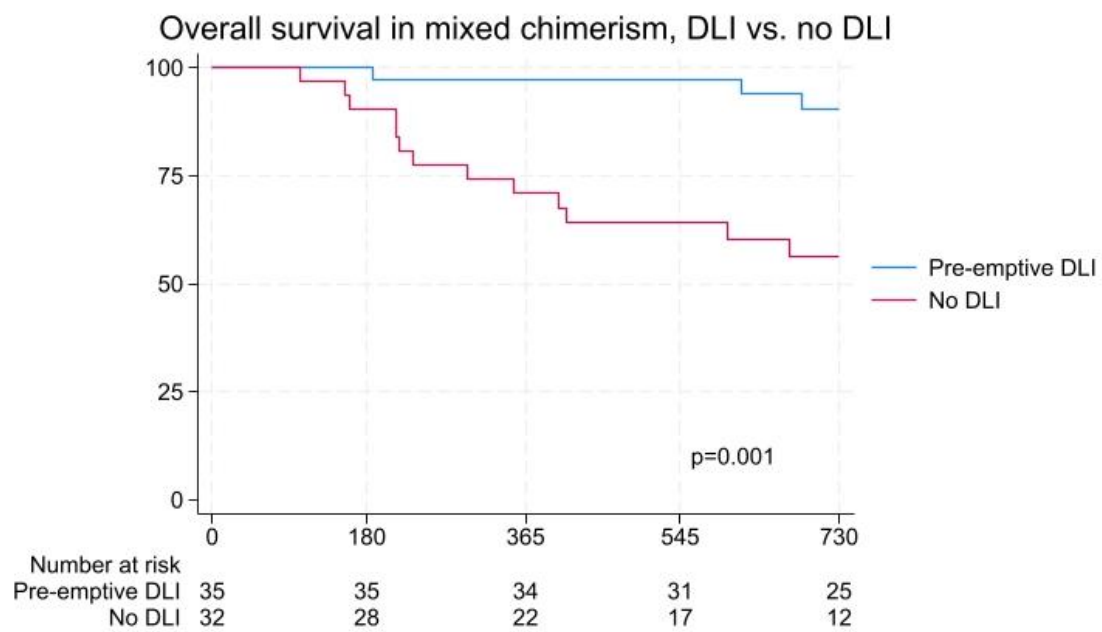


Figure 31: Overall survival by mixed chimerism beyond day 90; DLI vs. no DLI.

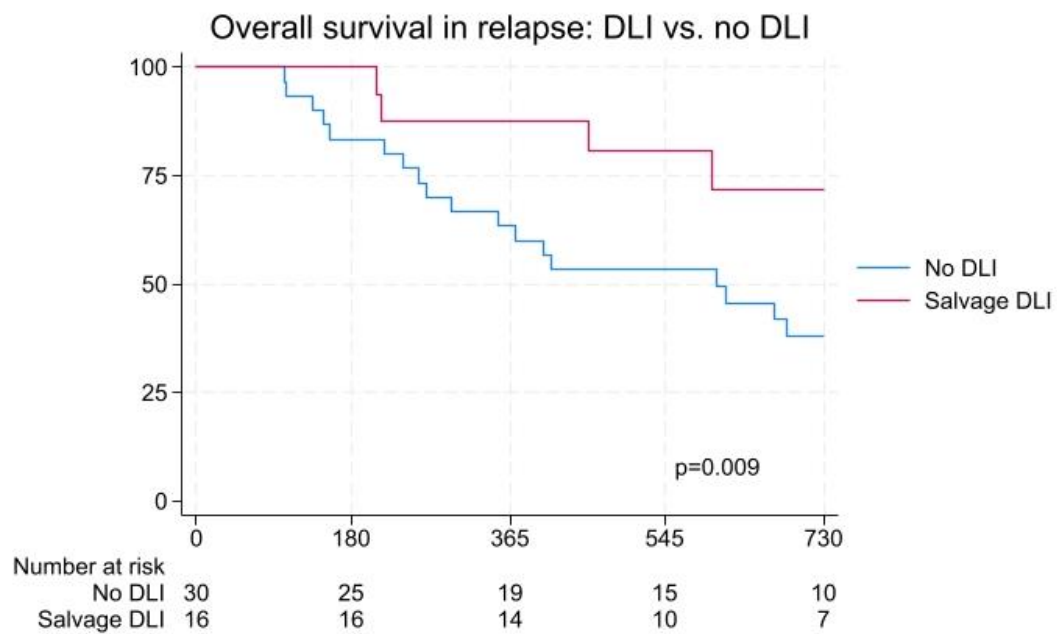


Figure 32: Overall survival post-relapse; DLI vs. no DLI.

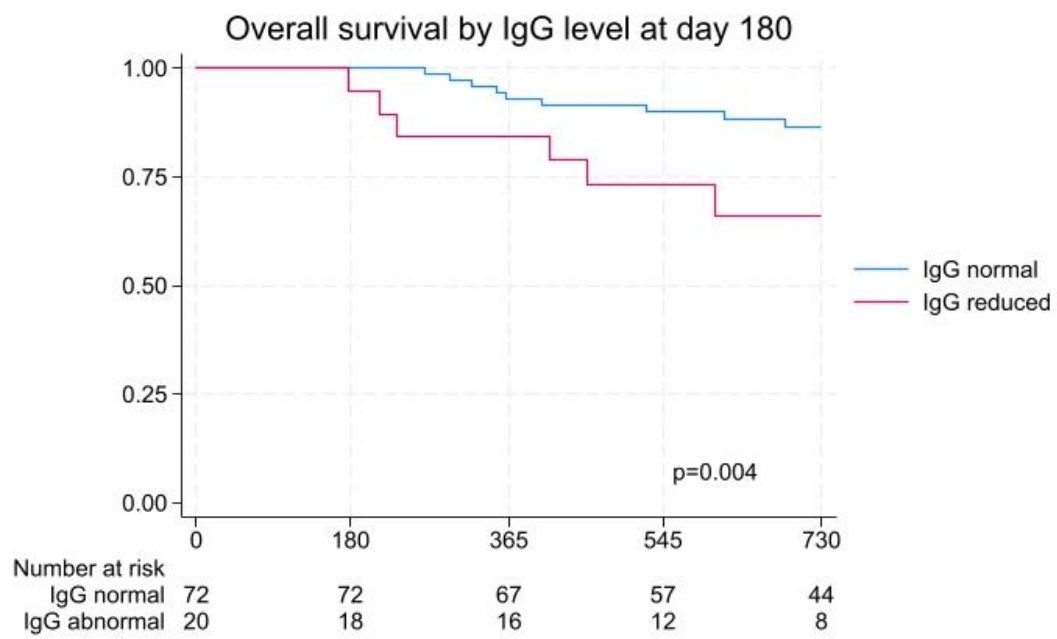


Figure 33: Overall survival by IgG recovery at day 180.

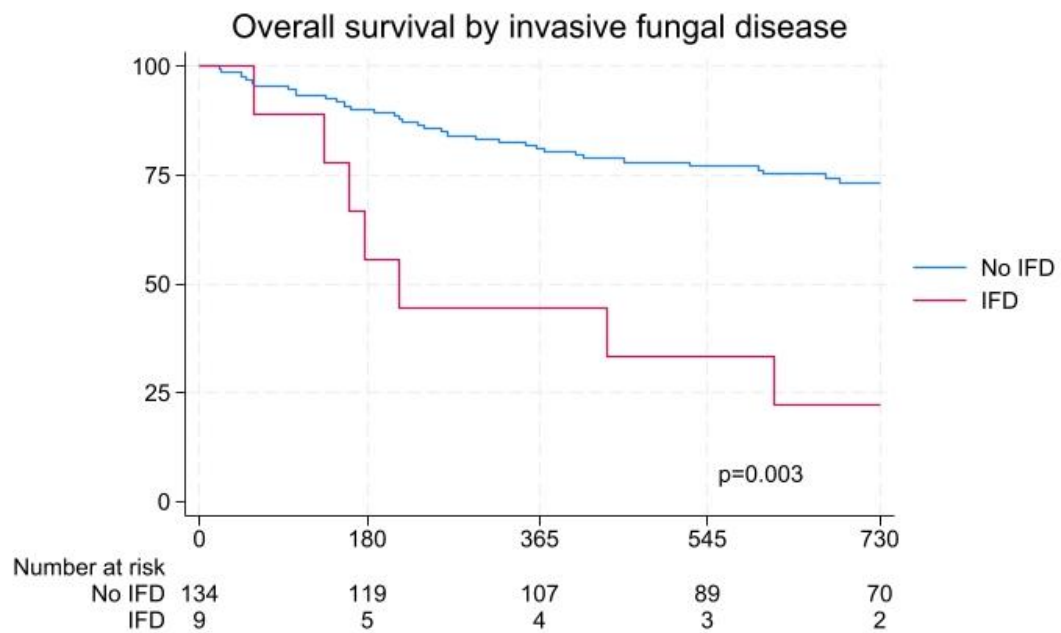


Figure 34: Overall survival by invasive fungal disease occurrence.

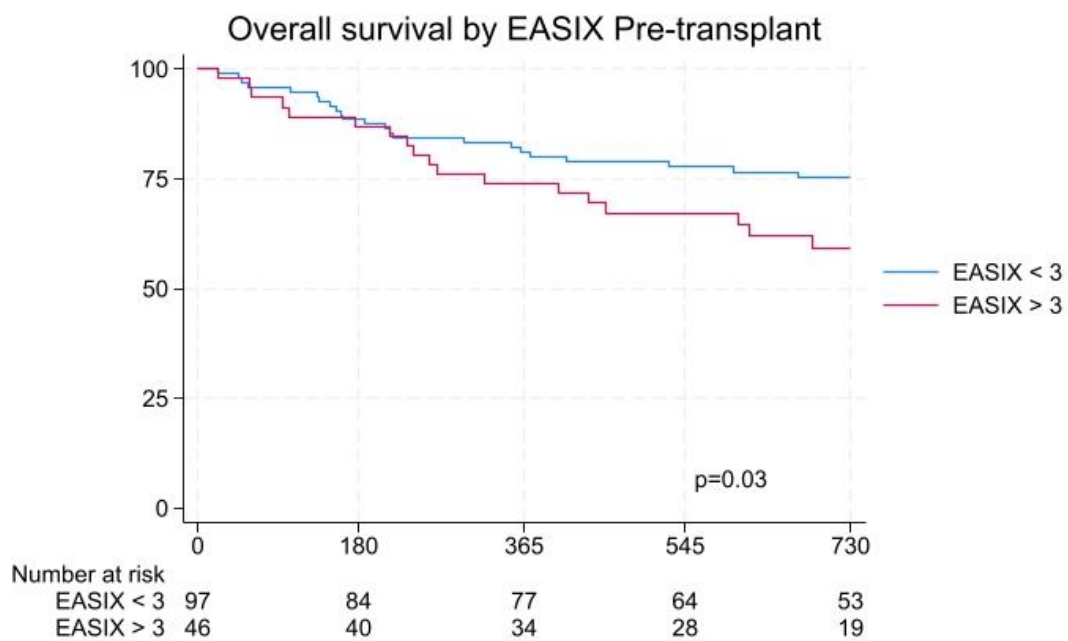


Figure 35: Overall survival by EASIX score pre-transplant at cut-off of 3.

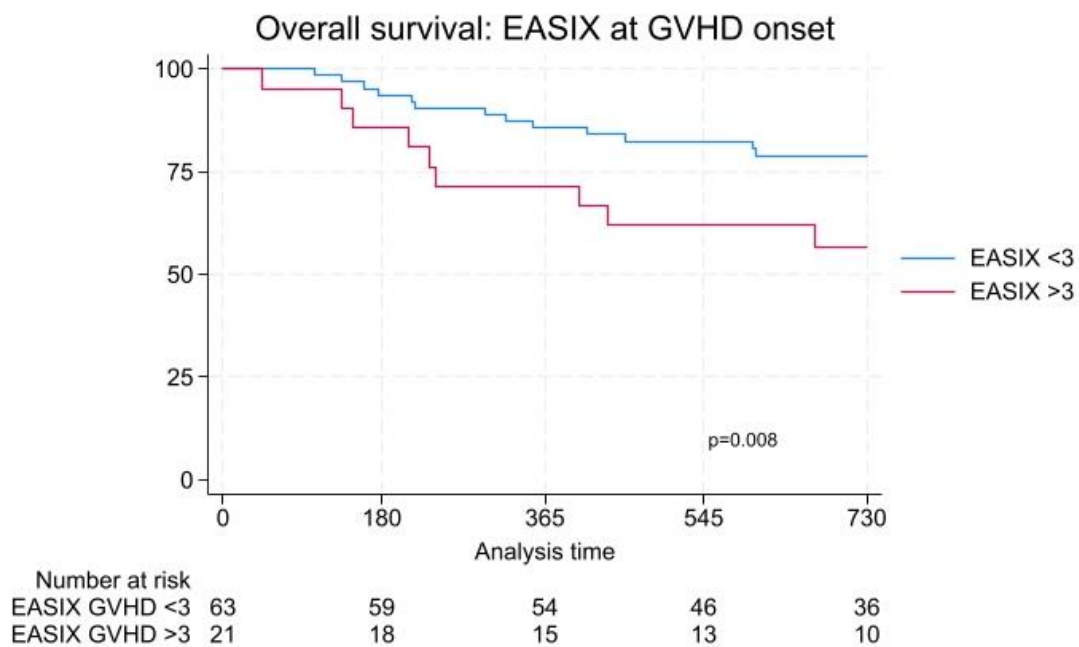


Figure 36: Overall survival by EASIX score at GVHD onset above and below cut-off of 3.

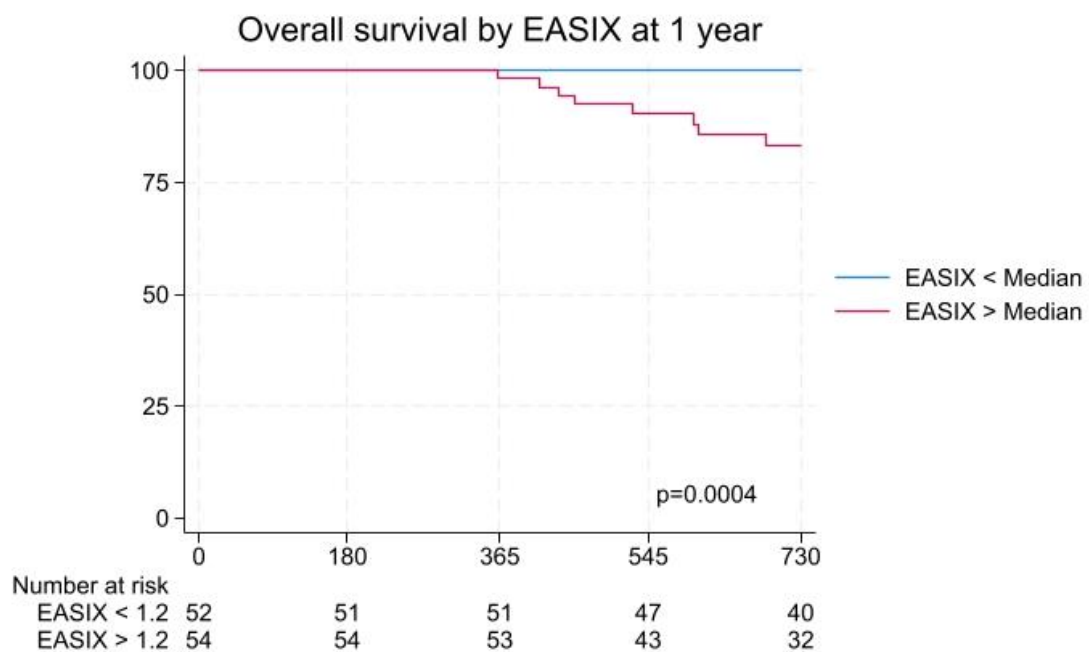


Figure 37: Overall survival by EASIX score at 1-year post-transplant at median of 1.2.

Table 3: Overall survival: univariable and multivariable predictors

Variable	Univariable analysis			Multivariable analysis		
	Hazard ratio	95% CI	P value	Hazard ratio	95% CI	P value
Male recipient	0.72	0.39 - 1.33	0.29			
Recipient age	1.05	0.70 – 1.57	0.78			
Recipient age > 65	0.79	0.37 – 1.68	0.54			
KPS < 80	1.08	0.60 – 1.93	0.78			
Disease subtype	1.24	0.88 - 1.76	0.20			
aaHCT-CI > 3	1.22	0.69 - 2.19	0.48			
CIBMTR Adverse risk	1.86	1.02 - 3.41	0.04	1.85	0.98 – 3.48	0.058
Pre-transplant ferritin > 1,000 ng/ml	1.00	0.99 – 1.01	0.37			
Unrelated donor	1.62	0.88 - 2.97	0.11			
Male donor	0.93	0.48 - 1.76	0.82			
Donor age < 60	1.19	0.5 - 2.82	0.68			
Donor age < 30	0.83	0.45 - 1.52	0.55			
Female donor male recipient	1.65	0.92 – 2.95	0.08			

Cryopreserved graft	1.20	0.47 - 3.08	0.70			
ABO mismatch	2.19	1.19 - 4.03	0.01	2.17	1.17 - 4.02	0.01
HLA mismatch	0.91	0.28 - 2.93	0.87			
ALC at first ATLG > 0.1 x 10⁹/L	0.95	0.43 - 2.14	0.91			
ALC pre-conditioning	1.08	0.84 - 1.40	0.51			
Tacrolimus vs. ciclosporin	1.26	0.70 - 2.29	0.43			
Acute GVHD (grade II-IV)	0.76	0.42 - 1.37	0.36			
Acute GVHD (grade III-IV)	1.94	0.90 - 4.17	0.11			
Chronic GVHD	0.36	0.13 - 0.98	0.04	0.35	0.12 - 0.97	0.04
Day 100 CD4 > 50	0.69	0.18 - 2.64	0.60			
Day 100 CD4 > 100	0.76	0.21 - 2.65	0.66			
Mixed chimerism at day 90	0.99	0.51 - 1.89	0.97			
DLI (all indications)	0.30	0.14 - 0.62	0.001	0.28	0.13 - 0.61	0.001
Pre-emptive DLI for mixed chimerism	0.18	0.05 - 0.55	0.003			
Relapse	3.69	2.02 - 6.73	<0.0001			
Salvage DLI	0.31	0.11 - 0.83	0.02			
IgG < 5g/L by day 180	2.74	1.08 - 7.00	0.03			
CMV reactivation	0.99	0.53 - 1.84	0.98			
EBV reactivation	1.23	0.69 - 2.20	0.47			
ADV reactivation	1.60	0.67 - 3.79	0.31			
Invasive fungal disease	4.12	1.83 - 9.31	0.001	5.04	2.11 - 11.99	<0.001

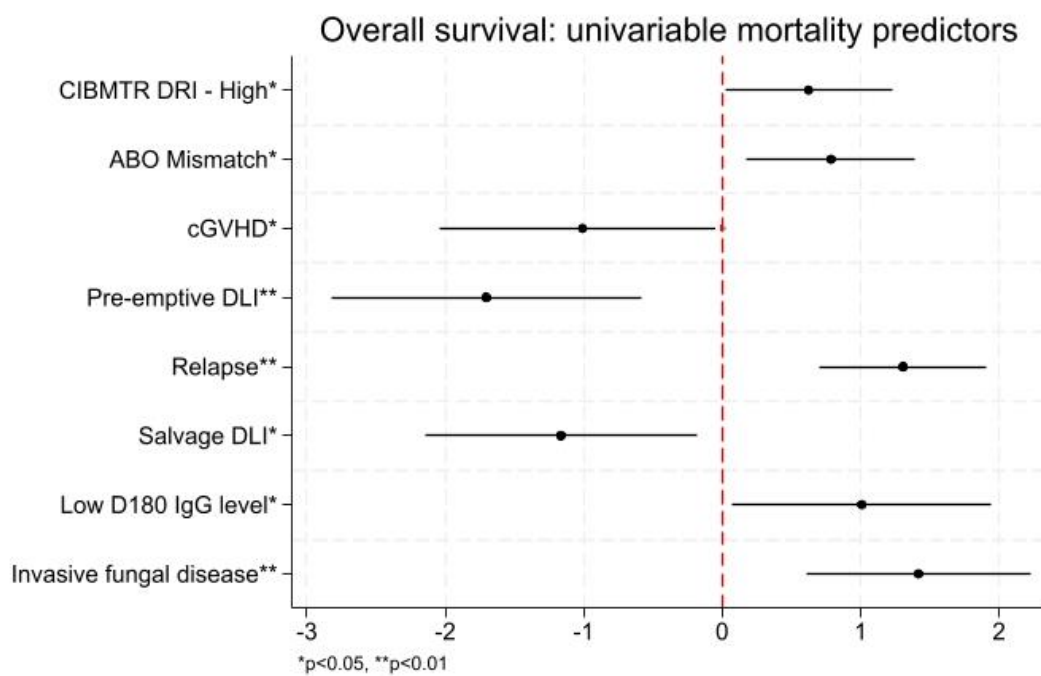


Figure 38: Coefficient forest plot for overall survival in univariable analysis.

Table 4: Overall survival: univariable EASIX Modelling

Variable	Hazard ratio	95% CI	P value
EASIX Pre-transplant	1.12	1.05 – 1.20	0.004
Mean	1.95	1.09 – 3.48	0.02
Mean + 1 SD	1.83	0.88 – 3.79	0.12
Median	1.31	0.73 – 2.23	0.35
25th Centile	1.05	0.53 – 2.07	0.88
75th Centile	1.87	1.02 – 3.40	0.04
EASIX Day 0	1.01	0.99 – 1.02	0.12
Mean	1.13	0.63 – 2.07	0.67
Mean + 1 SD	2.19	0.86 – 5.57	0.13
Median	0.91	0.51 – 1.61	0.73
25th Centile	1.79	0.84 – 3.85	0.11
75th Centile	1.32	0.70 – 2.48	0.39
EASIX at GVHD onset	1.03	0.99 – 1.06	0.16
Mean	2.97	1.33 – 6.63	0.008
Mean + 1 SD	2.25	0.67 – 7.56	0.23
Median	2.20	0.94 – 5.15	0.05
25th Centile	2.47	0.74 – 8.29	0.10
75th Centile	2.96	1.33 – 6.63	0.01
EASIX at 1 year	1.23	1.12 – 1.35	0.0004
Mean	8.67	2.66 – 28.24	0.0002
Mean + 1 SD	7.83	2.37 – 25.79	0.004
Median	13.9	1.80 – 107.11	0.004
25th Centile	-	-	0.003
75th Centile	7.21	2.21 – 23.45	0.0005

Table 5: Overall survival Graft T cell content

Variable	Hazard ratio	95% CI	P value
Graft CD3⁺	1.01	0.99 – 1.03	0.10
Mean	1.01	0.55 – 1.85	0.96
Mean + 1 SD	1.95	0.98 – 3.85	0.07
Median	1.56	0.86 – 2.83	0.13
25th Centile	1.28	0.63 – 2.59	0.48
75th Centile	1.33	0.71 – 2.48	0.36
Graft CD4⁺	1.02	0.99 – 1.04	0.22
Mean	0.82	0.44 – 1.52	0.52
Mean + 1 SD	1.18	0.64 – 2.16	0.58
Median	0.99	0.55 – 1.77	0.97
25th Centile	1.72	0.79 – 3.71	0.14
75th Centile	1.44	0.77 – 2.70	0.25
Graft CD8⁺	1.03	1.01 – 1.06	0.04
Mean	1.18	0.66 – 2.14	0.57
Mean + 1 SD	1.82	0.84 – 3.91	0.15
Median	1.88	1.03 – 3.43	0.03
25th Centile	1.18	0.58 – 2.39	0.63
75th Centile	1.67	0.92 – 3.07	0.10
Graft CD4⁺/CD8⁺	1.18	0.78 – 1.78	0.48
Mean	1.24	0.67 – 2.32	0.49
Mean + 1 SD	1.62	0.72 – 3.65	0.26
Median	0.95	0.53 – 1.71	0.87
25th Centile	1.28	0.69 – 2.39	0.43
75th Centile	-	-	-

Non-relapse mortality

The cumulative incidence of non-relapse mortality (NRM) at 1- and 2-years was 12.5% and 13.5% respectively (*Fig. 39*). A summary of univariable and multivariable predictors of NRM are provided in Table 6. NRM was similar in both sexes (*Fig. 40*) and across all age groups (*Fig. 41*). There were 17 NRM events in this cohort occurring at a median of 160 days, range 22-437. The median age in this group was 60, with a range of 27 – 64. A proportionately higher NRM rate was seen in those with MDS (n=9) and MPN (n=5) as compared AML (n=3), despite a similar median age in these groups of 60, 61 and 58 respectively ($p=0.01$) (*Fig. 42*).

Pre-transplant assessments of risk including the age adjusted HCT-CI, KPS and iron overload were not predictive of NRM in this cohort of patients (*Fig 43 - 45*). The median age-adjusted HCT-CI score in the NRM group was 3, as compared to 2 in the remainder of the cohort. In AML, favourable risk disease, all of whom arrived at transplant in CR2, was associated with an increased risk of NRM (*Fig. 46*), and in myelofibrosis, high risk disease was associated with an increased risk (*Fig. 47*). Disease risk stratification in MDS did not correlate with NRM risk (*Fig. 48*). The CIBMTR DRI similarly did not correlate with NRM outcomes.

No NRM events occurred in any of the HLA mismatched transplants. A minor ABO mismatch was associated with an increased risk of NRM (*Fig. 49*), whereas major mismatch was not. Cryopreservation of the stem cell graft was not associated with increased NRM as compared to fresh product (*Fig. 50*). Low graft T cell content below median in both CD4⁺ and CD4⁺/CD8⁺ compartments was associated with an increased risk of NRM (*Fig. 51 and 52*).

8 patients in the NRM group developed acute GVHD and this was contributory to death in 6 cases with 1 further patient experiencing an engraftment-type syndrome with acute respiratory distress syndrome. While NRM was not increased in those who developed any grade of acute GVHD, grade III - IV GVHD was associated with an increased NRM risk (*Fig. 53*). Chronic GVHD was not associated with an increased risk of NRM ($p=0.69$).

Viral infection was implicated in 6 cases of NRM, including CMV pneumonitis, ADV pneumonitis, influenza B and Sars-CoV-2. Bacterial sepsis accounted for 3 deaths, overwhelming *pneumocystis jirovecii* infection in 1 and fatal invasive fungal infection in 2. 1 patient died from liver cirrhosis of unknown aetiology, with biopsies not consistent with GVHD and 1 patient died from secondary malignancy. NRM was not increased in CMV, ADV or EBV re-activation as detected by PCR surveillance (*Fig. 54 - 56*). Treatment for suspected or confirmed invasive fungal disease was strongly associated with an increased NRM risk (*Fig. 57*).

The EASIX score at the time-points of pre-transplant, at day 0, and at the onset of acute GVHD did not predict an increased risk of NRM, although a trend towards a higher incidence of NRM at the onset of acute GVHD was seen in those with higher scores (*Fig. 58-60*). Failure to recover IgG levels by day 180 post-transplant was associated with an increased risk of NRM (*Fig. 61*). EASIX and graft T cell content cut-offs for NRM prediction are summarized in Table 7 and 8.

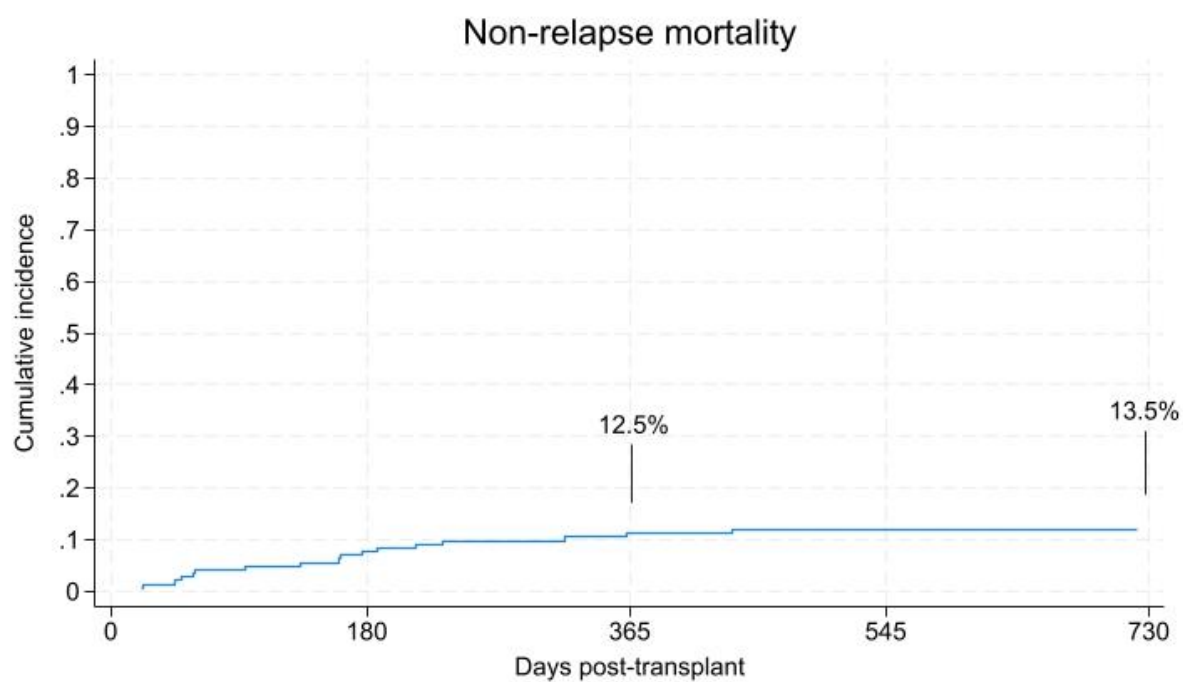


Figure 39: Cumulative incidence of non-relapse mortality.

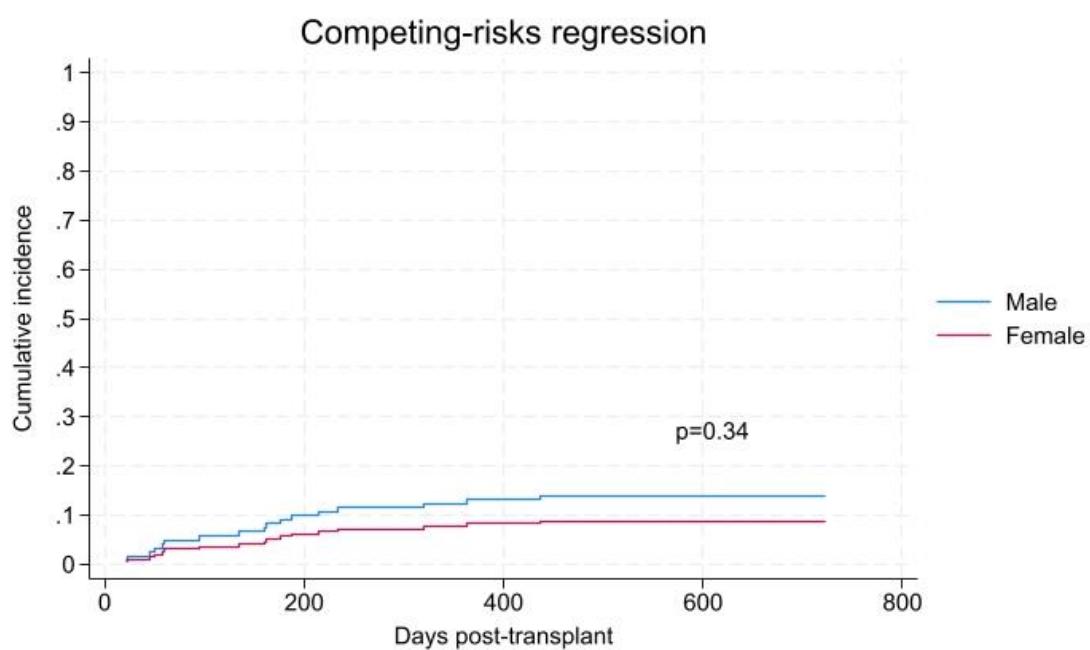


Figure 40: Cumulative incidence of non-relapse mortality by recipient sex.

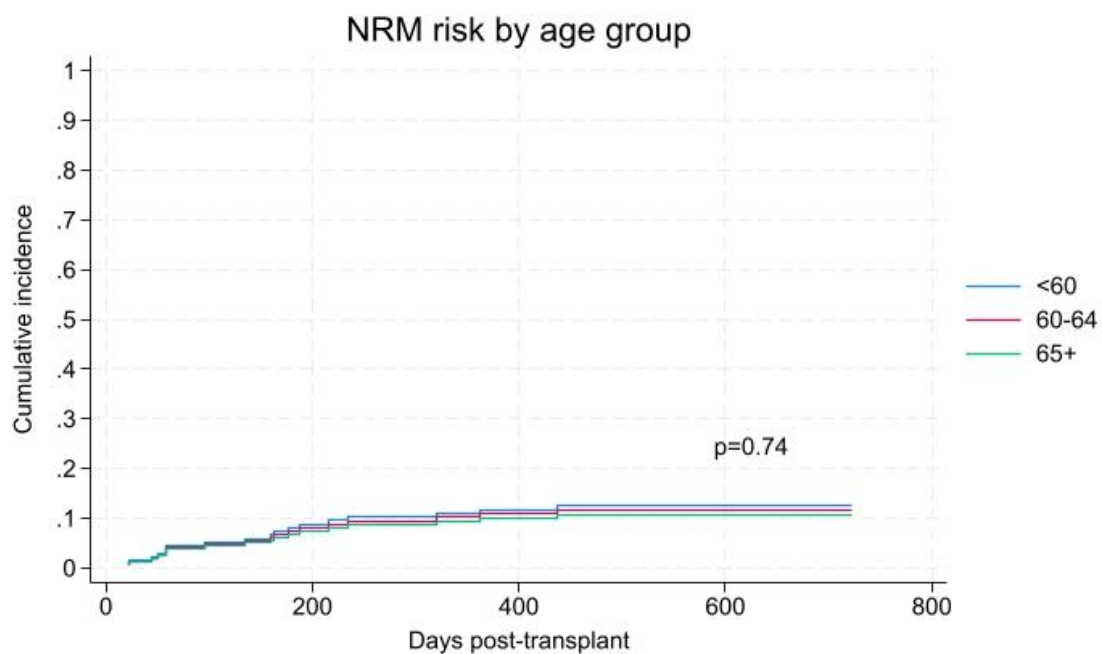


Figure 41: Cumulative incidence of non-relapse mortality by recipient age groups; <60, 60-64 and 65+.

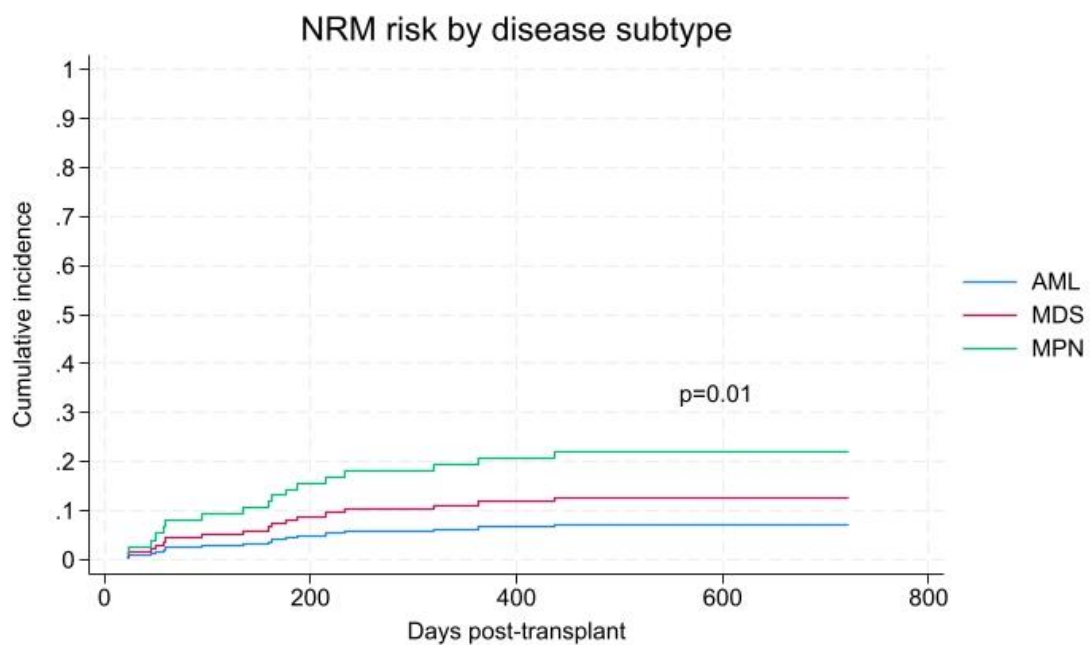


Figure 42: Cumulative incidence of non-relapse mortality by disease subtype; AML, MDS and MPN.

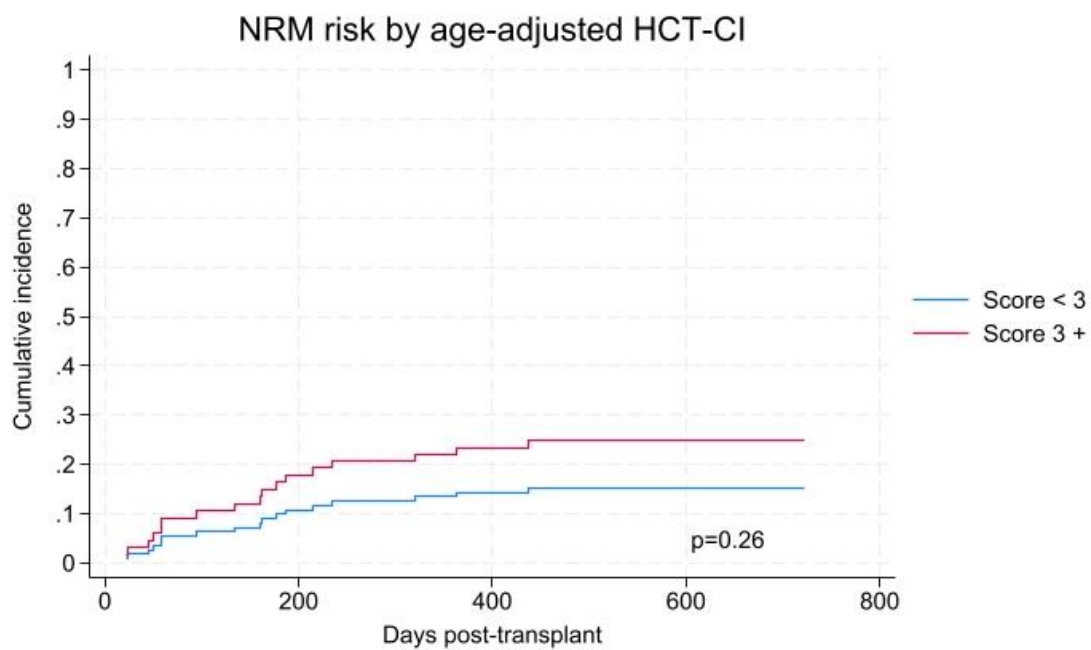


Figure 43: Cumulative incidence of non-relapse mortality by age-adjusted HCT-CI score above at a cut-off of 3.

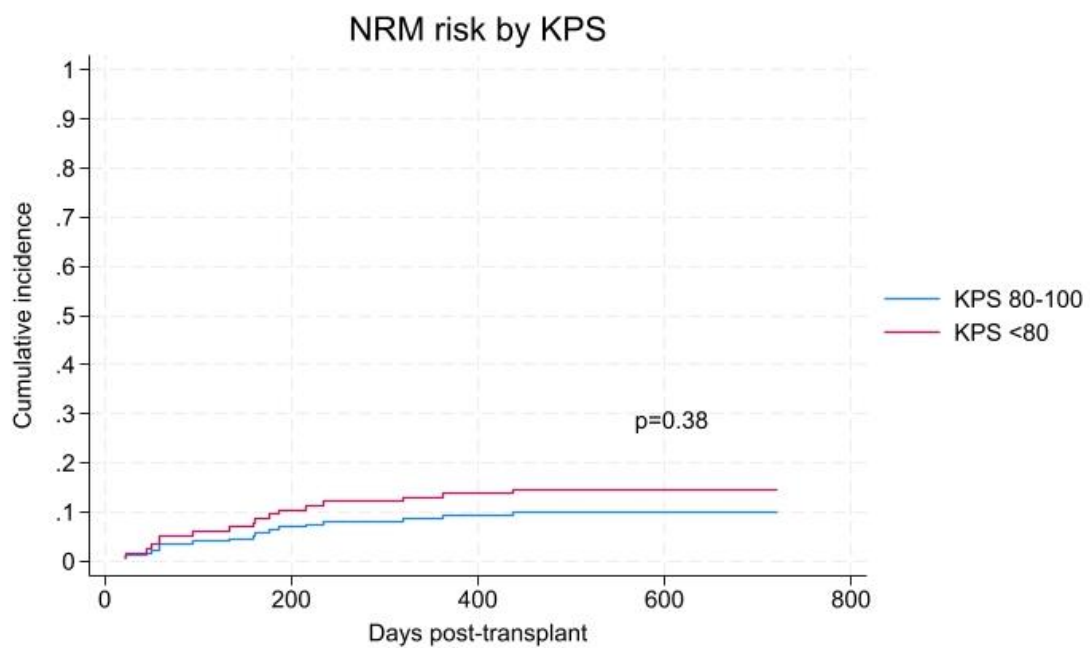


Figure 44: Cumulative incidence of non-relapse mortality by Karnofsky Performance Score at a cut-off of 80.

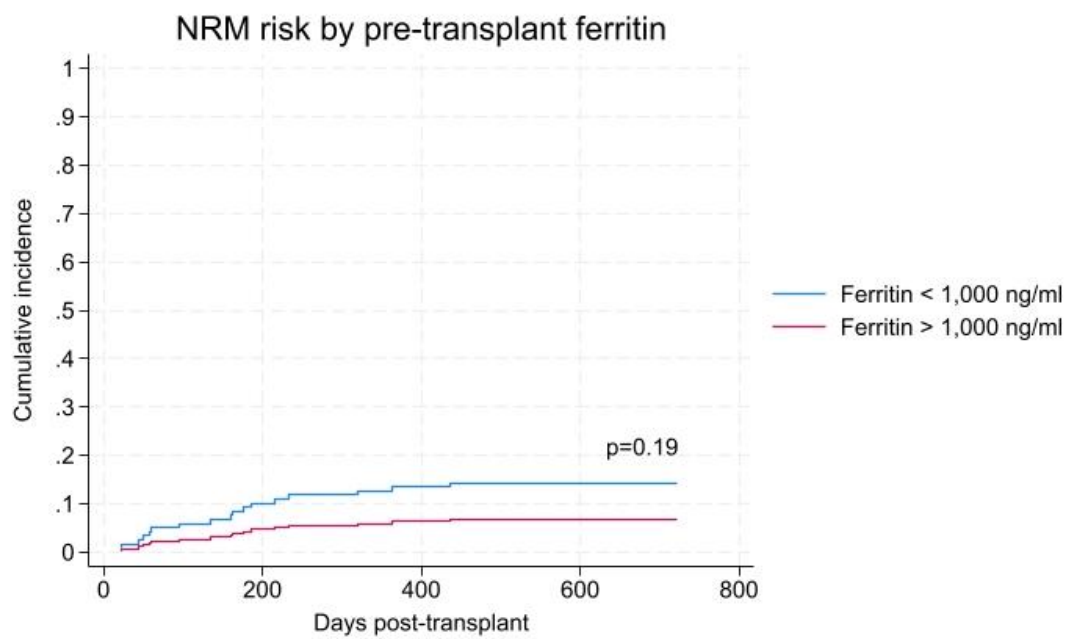


Figure 45: Cumulative incidence of non-relapse mortality by pre-transplant ferritin at cut-off of 1,000 ng/ml.

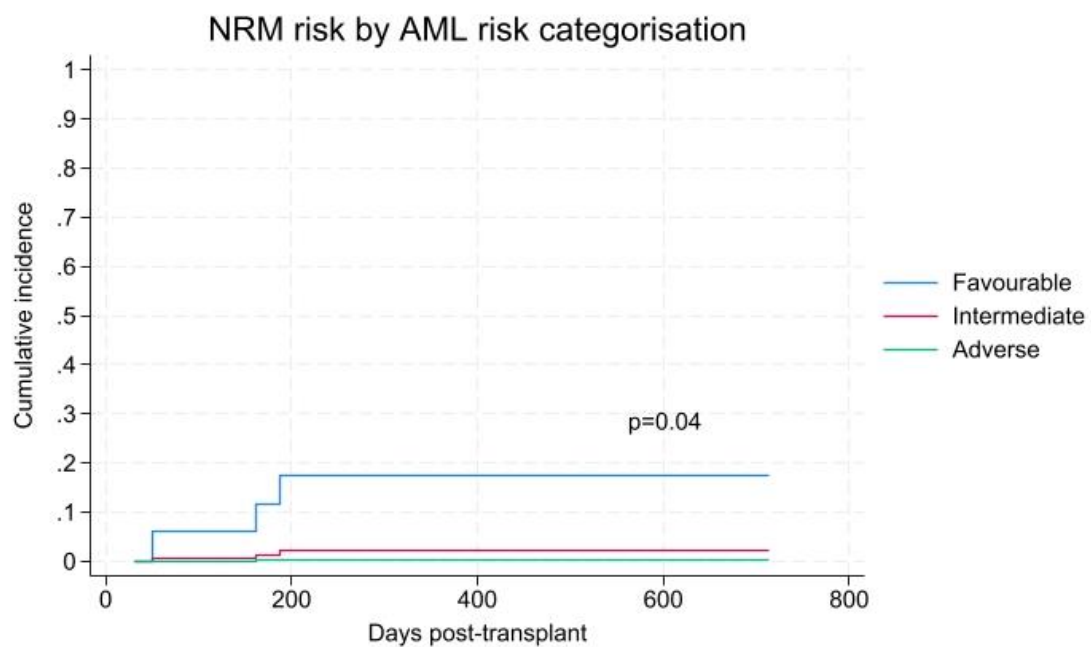


Figure 46: Cumulative incidence of non-relapse mortality by AML ELN 2017 risk classification group; favourable, intermediate and adverse.

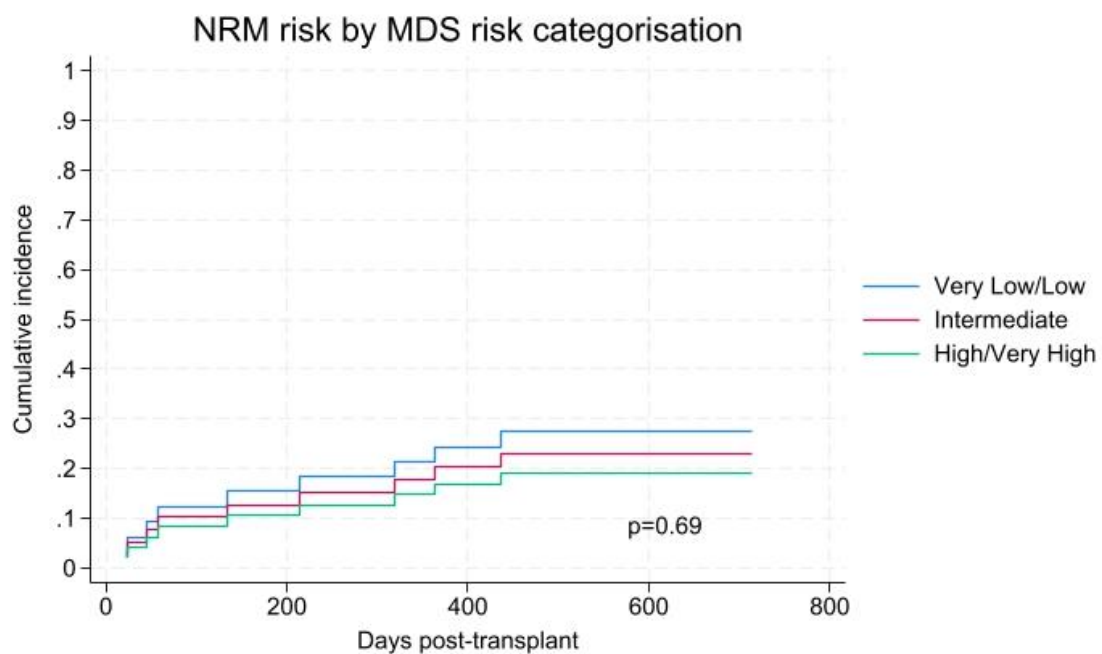


Figure 47: Cumulative incidence of non-relapse mortality by MDS R-IPSS risk group; very low and low, intermediate and high and very high.

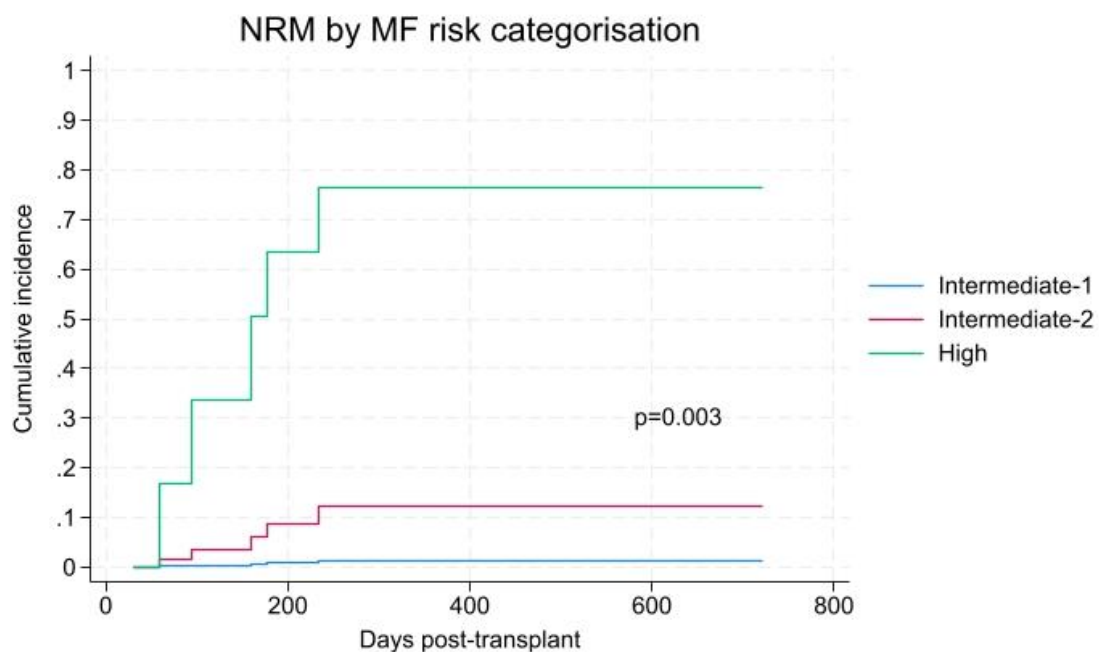


Figure 48: Cumulative incidence of non-relapse mortality by MF DIPSS-Plus risk group; intermediate-1, intermediate-2 and high.

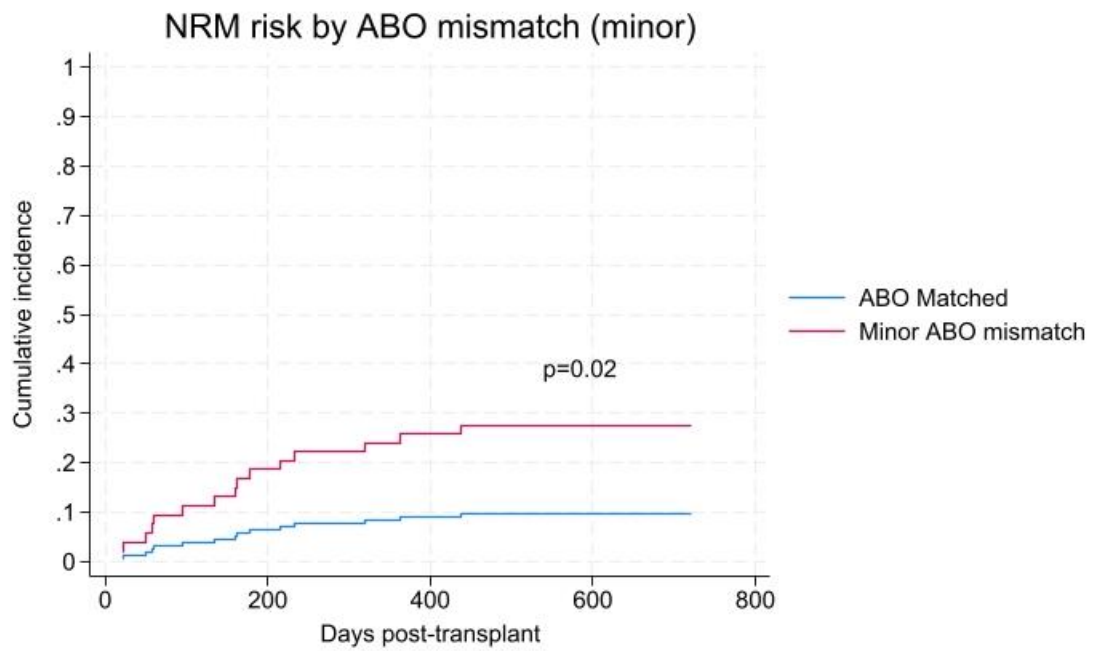


Figure 49: Cumulative incidence of non-relapse mortality in ABO minor mismatch.

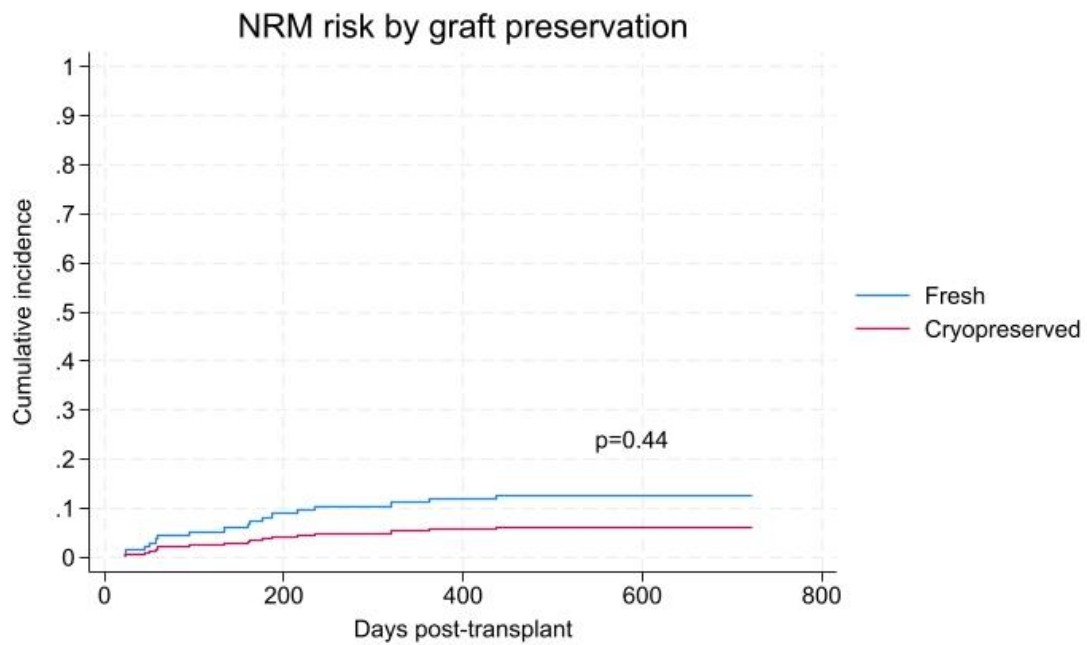


Figure 50: Cumulative incidence of non-relapse mortality by graft preparation; fresh vs. cryopreserved.

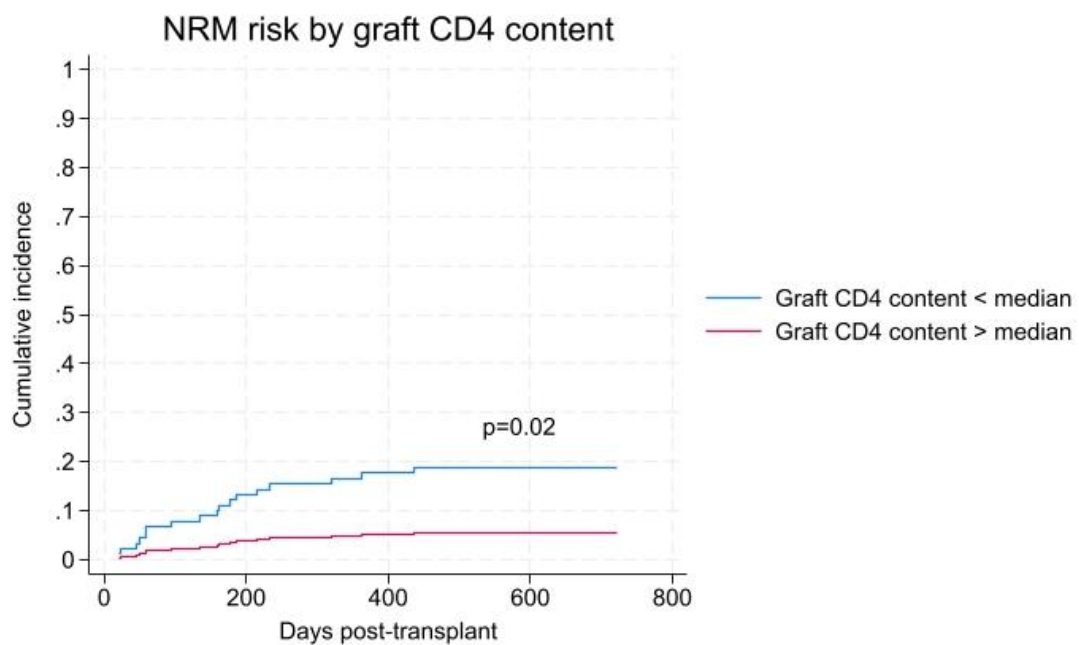


Figure 51: Cumulative incidence of non-relapse mortality by graft CD4⁺ T cell content above and below median of $11.09 \times 10^7/\text{kg}$.

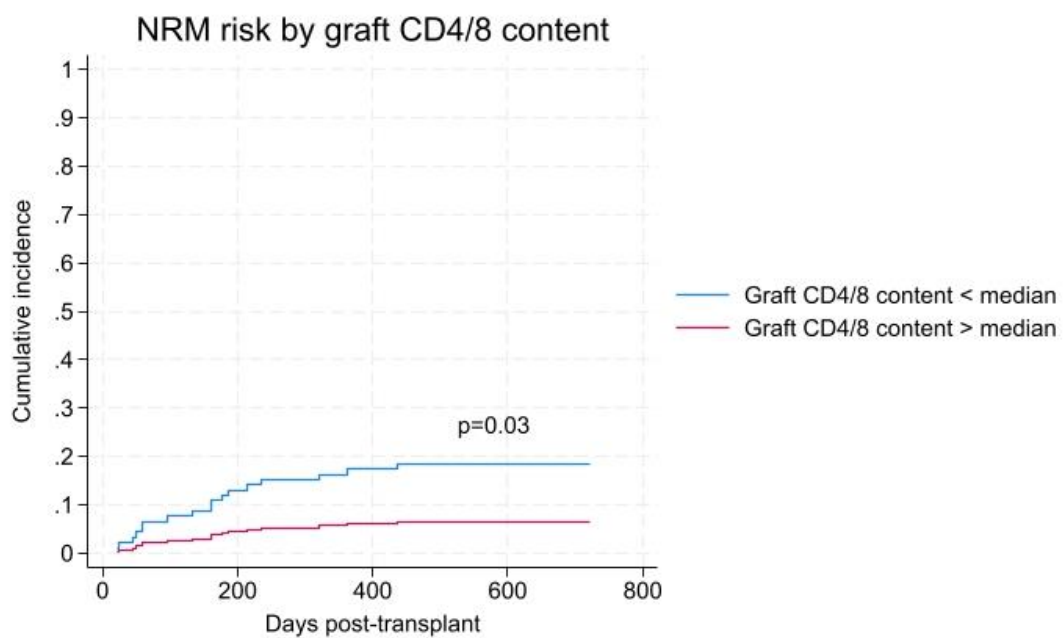


Figure 52: Cumulative incidence of non-relapse mortality by graft CD4⁺/CD8⁺ T cell content above and below median of $0.24 \times 10^9/\text{kg}$.

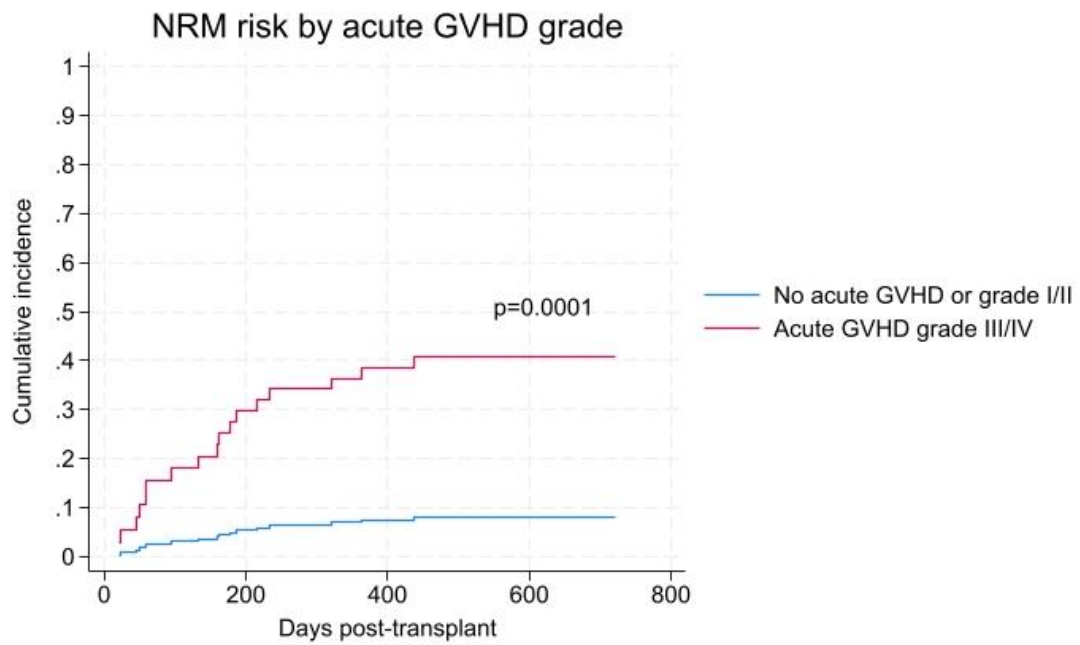


Figure 53: Cumulative incidence of non-relapse mortality by grade III-IV acute GVHD.

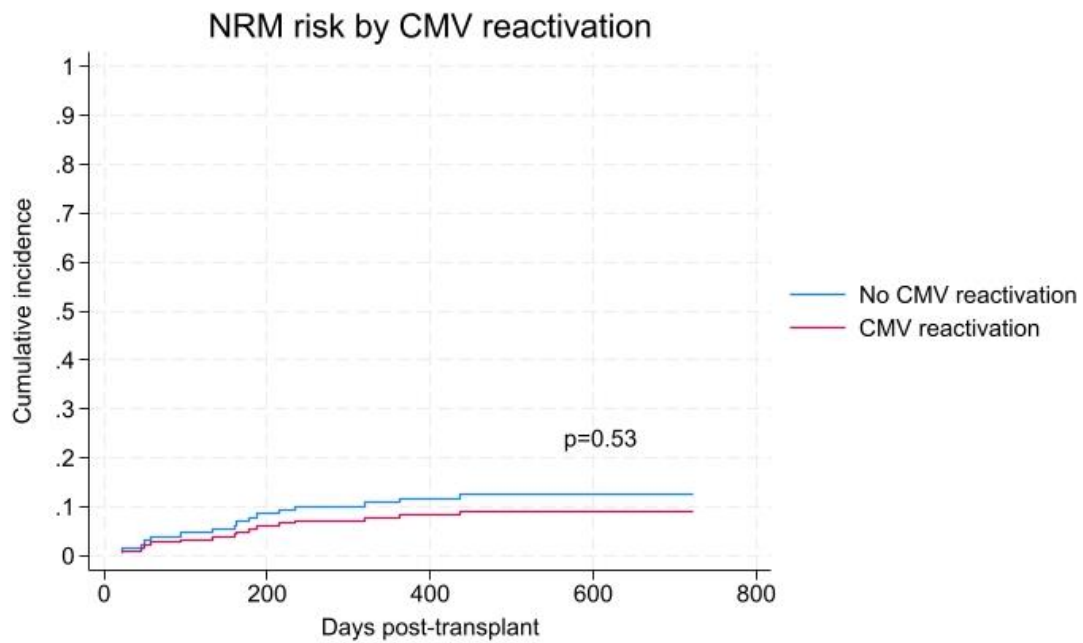


Figure 54: Cumulative incidence of non-relapse mortality by CMV re-activation.

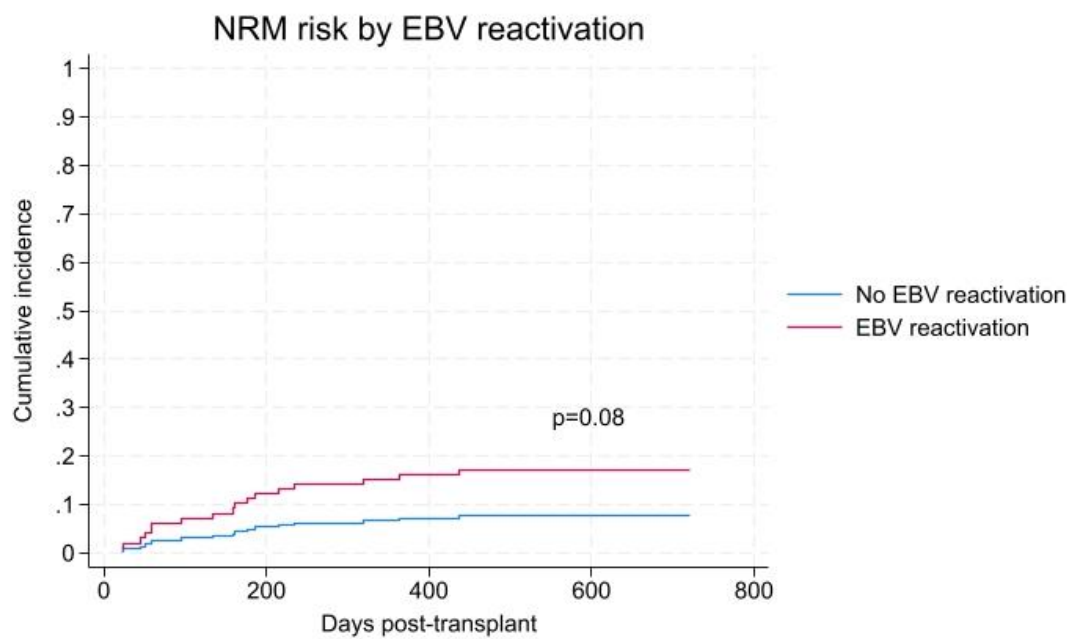


Figure 55: Cumulative incidence of non-relapse mortality by EBV re-activation.

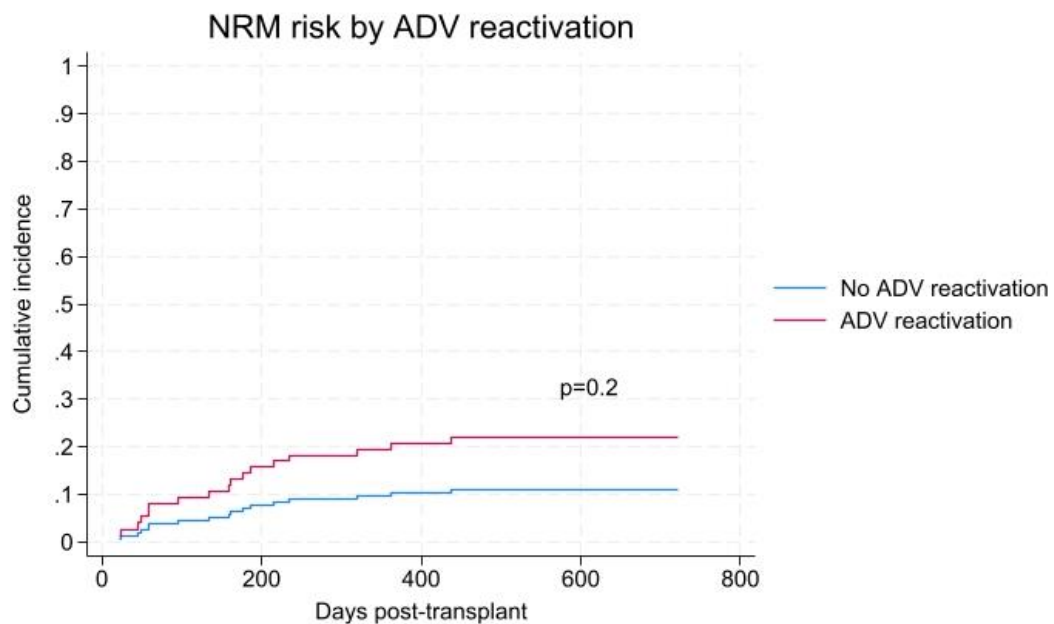


Figure 56: Cumulative incidence of non-relapse mortality by ADV re-activation.

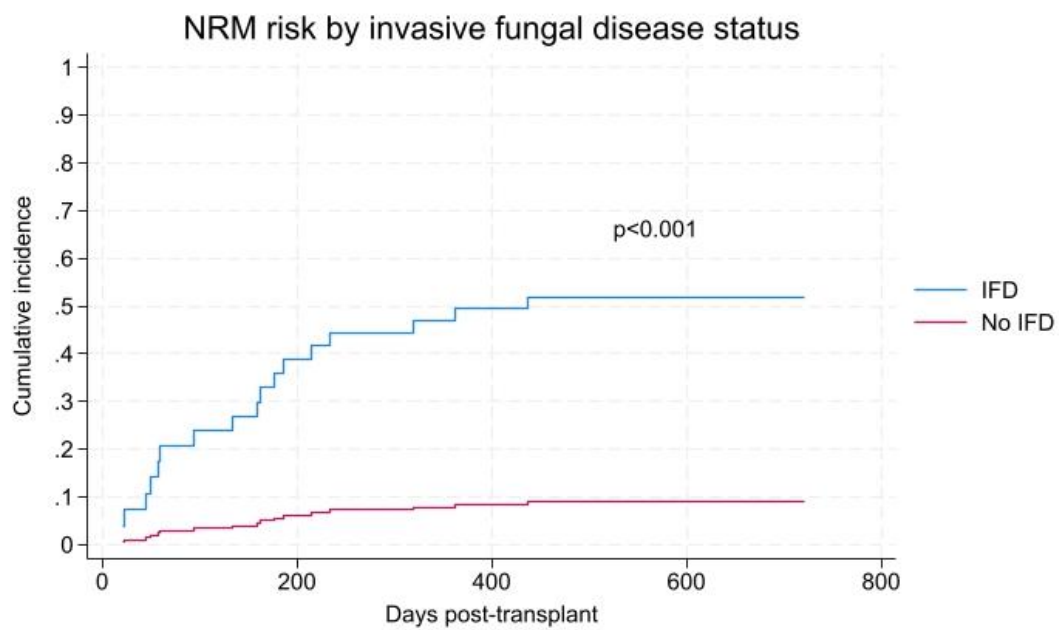


Figure 57: Cumulative incidence of non-relapse mortality by invasive fungal disease occurrence.

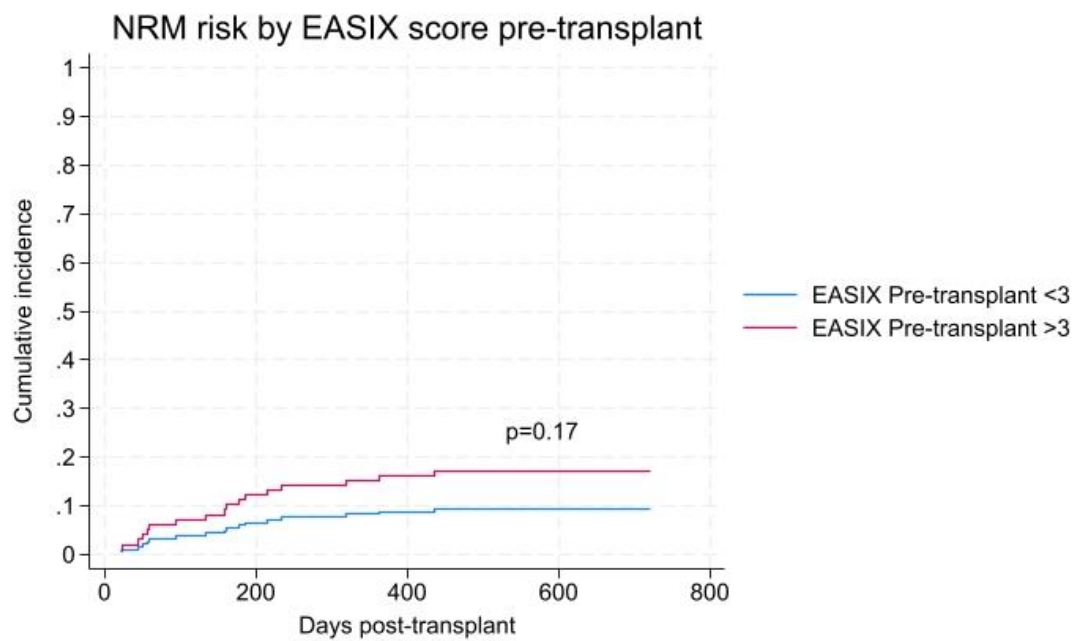


Figure 58: Cumulative incidence of non-relapse mortality by EASIX score pre-transplant at cut-off of 3.

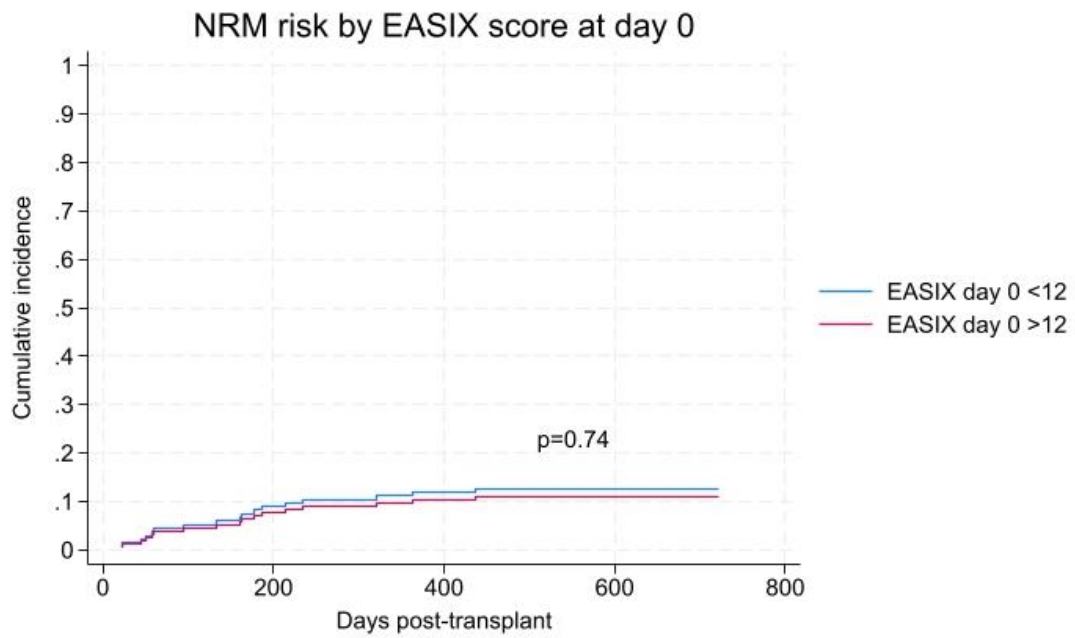


Figure 59: Cumulative incidence of non-relapse mortality by EASIX score at day 0 at cut-off of 12.

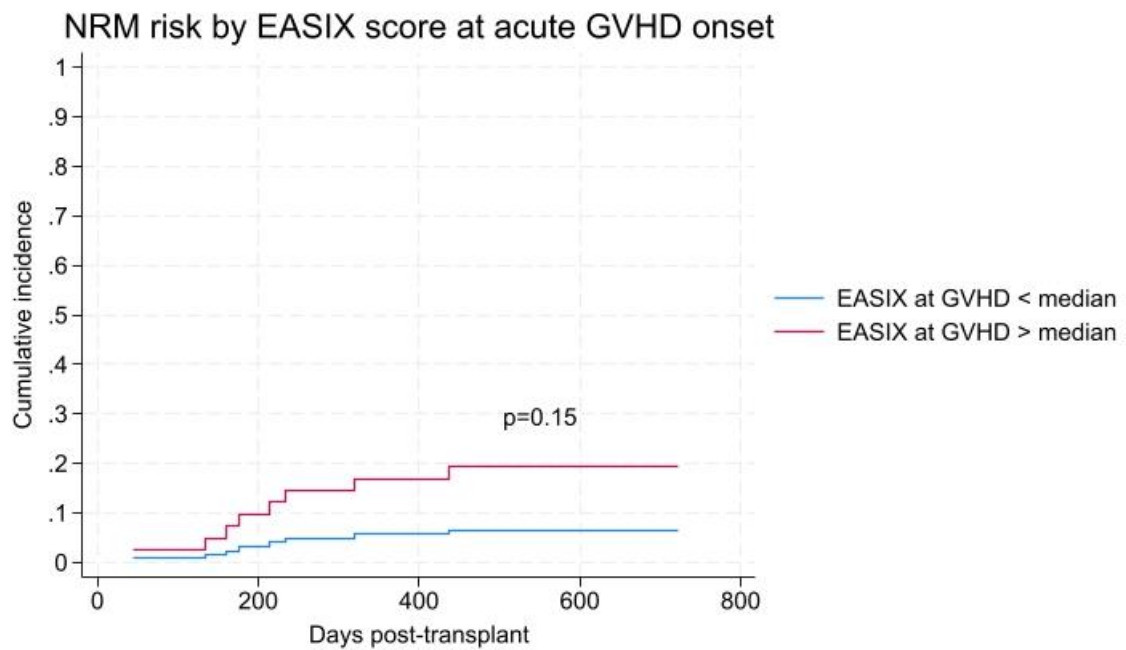


Figure 60: Cumulative incidence of non-relapse mortality by EASIX score at onset of GVHD at cut-off of median of 1.97.

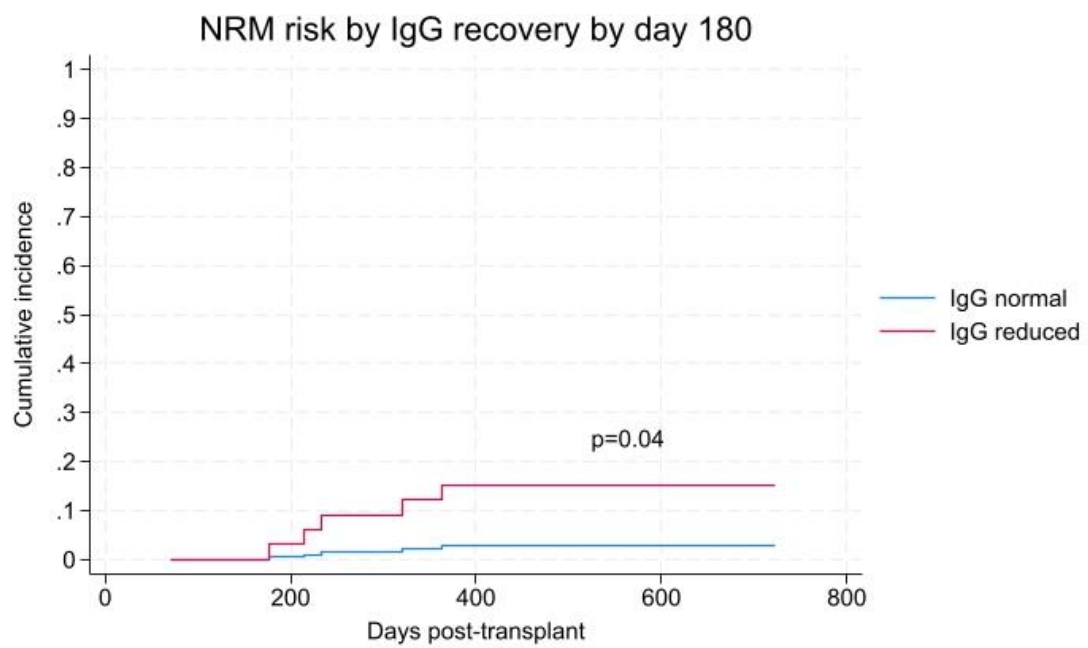


Figure 61: Cumulative incidence of non-relapse mortality by IgG level at day 180 post-transplant.

Table 6: Non-relapse mortality: univariable and multivariable predictors

Variable	Univariable analysis			Multivariable analysis		
	Hazard ratio	95% CI	P value	Hazard ratio	95% CI	P value
Male recipient	0.61	0.22 - 1.71	0.34			
Recipient age	0.92	0.56 - 1.52	0.74			
MDS or MPN	1.84	1.15 - 2.94	0.01			
CIBMTR DRI	0.84	0.34 - 2.09	0.71			
AML ELN 2017: Favourable	9.09	1.06 - 7.69	0.04			
Myelofibrosis	10.97	2.26 - 53.33	0.003			
DIPPS-Plus: High						
Age-adjusted HCT-CI >3	1.73	0.66 - 4.53	0.26			
KPS <80	1.52	0.59 - 3.95	0.38			
Pre-transplant ferritin < 1,000 ng/ml	0.45	0.13 - 1.52	0.19			
Unrelated donor	1.45	0.54 - 3.86	0.46			
Female donor to male recipient	2.30	0.73 - 7.20	0.15			
Donor age < 25	0.55	0.20 - 1.48	0.23			
Donor age < 40	0.53	0.19 - 1.42	0.20			
Fresh graft vs. cryopreserved	0.46	0.06 - 3.30	0.44			
ABO minor mismatch	3.12	1.14 - 8.55	0.02			
CMV mismatch	1.39	0.46 - 4.19	0.56			
High graft CD4 ⁺ content	3.69	1.21 - 11.29	0.02	2.77	0.95 - 8.33	0.06
Acute GVHD II-IV	1.93	0.75 - 4.97	0.17			
Acute GVHD grade III-IV	6.29	2.47 - 15.99	0.0001	3.62	1.36 - 9.64	0.01
Chronic GVHD	1.43	0.49 - 4.15	0.50			
Invasive fungal disease	7.71	2.88 - 20.69	<0.001	4.13	1.58 - 10.78	0.004
IgG < 5 g/L by day 180	5.93	1.02 - 34.43	0.04			

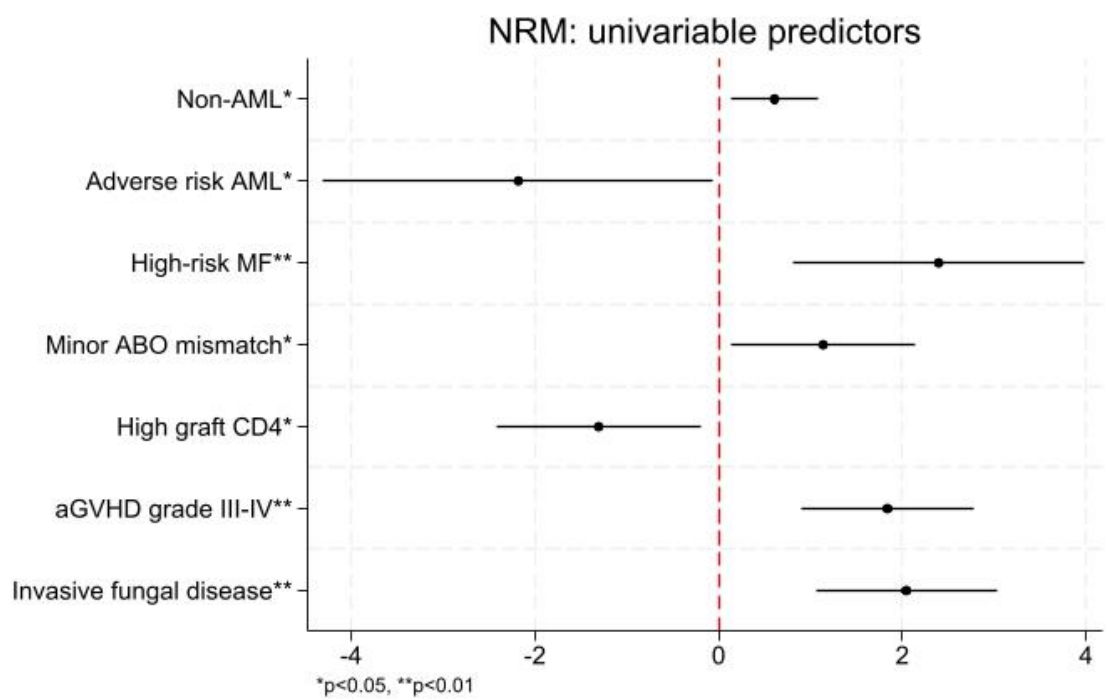


Figure 62: Univariable coefficients for predictors of non-relapse mortality.

Table 7: NRM univariable EASIX Modelling

Variable	Hazard ratio	95% CI	P value
EASIX Pre-transplant	1.16	1.04 – 1.29	0.007
Mean	1.98	0.77 – 5.11	0.15
Mean + 1 SD	2.99	1.06 – 8.44	0.03
Median	1.12	0.43 – 2.90	0.81
25th Centile	1.60	0.47 – 5.45	0.44
75th Centile	2.13	0.82 – 5.55	0.12
EASIX Day 0	0.99	0.98 – 1.01	0.75
Mean	1.37	0.52 – 3.62	0.52
Mean + 1 SD	-	-	-
Median	0.92	0.35 – 2.39	0.86
25th Centile	5.62	0.75 – 41.84	0.09
75th Centile	1.31	0.45 – 3.76	0.62
EASIX at GVHD onset	0.99	0.94 – 1.05	0.85
Mean	3.21	0.81 – 12.61	0.09
Mean + 1 SD	-	-	-
Median	3.18	0.66 – 15.46	0.15
25th Centile	1.01	0.21 – 4.88	0.98
75th Centile	3.21	0.81 – 12.61	0.09

Table 8: NRM univariates: graft T cell content

Variable	Hazard ratio	95% CI	P value
Graft CD3⁺	1.01	0.98 – 1.05	0.97
Mean	<i>0.21</i>	<i>0.49 – 0.93</i>	<i>0.03</i>
Mean + 1 SD	0.74	0.17 – 3.18	0.68
Median	0.48	0.18 – 1.31	0.15
25th Centile	0.59	0.22 – 1.57	0.28
75th Centile	0.33	0.07 – 1.43	0.14
Graft CD4⁺	0.99	0.92 – 1.08	0.88
Mean	<i>0.21</i>	<i>0.04 – 0.92</i>	<i>0.03</i>
Mean + 1 SD	<i>0.24</i>	<i>0.06 – 1.06</i>	<i>0.05</i>
Median	<i>0.27</i>	<i>0.09 – 0.83</i>	<i>0.02</i>
25th Centile	0.75	0.26 – 2.11	0.59
75th Centile	0.34	0.79 – 1.49	0.15
Graft CD8⁺	1.01	0.94 – 1.09	0.75
Mean	0.35	0.10 – 0.19	0.09
Mean + 1 SD	1.02	0.24 – 4.36	0.96
Median	0.85	0.33 – 2.20	0.74
25th Centile	0.44	0.17 – 1.13	0.08
75th Centile	0.56	0.16 – 1.90	0.35
Graft CD4⁺/CD8⁺	0.53	0.06 – 4.28	0.55
Mean	0.31	0.07 – 1.34	0.11
Mean + 1 SD	0.49	0.07 – 3.54	0.48
Median	<i>0.33</i>	<i>0.11 – 0.93</i>	<i>0.03</i>
25th Centile	-	-	
75th Centile	0.32	0.07 – 1.38	0.12

Relapse

The cumulative incidence of relapse at 1- and 2-years was 21.9% and 31.7% respectively (*Fig. 63*). 48 patients relapsed at last follow up, 29 occurred in the first year a further 10 in the second year and the remaining 9 beyond year 2. The latest occurring relapse during the period analysed was 5.9 years post-transplant. Relapse was not affected by sex (*Fig. 64*), age group (*Fig. 65*) or disease class (*Fig. 66*). The 1- and 2-year risk of relapse in the AML cohort was 20.3% and 28.6%. The relapse risk for MDS at 1- and 2-years was 23.5% and 30.3% and for MPNs the risk was 24.3% and 41.2% respectively. Relapse-free survival (RFS) at 1 year was 68.1% and 58.9% at 2 years. A summary of univariable and multivariable predictors of relapse is provided in Table 7.

The CIBMTR-DRI stratification correlated with relapse outcomes, with increased risk in the intermediate group as compared to the low-risk group, and further increased risk in the high-risk cohort (*Fig. 67*). The ELN 2017 risk stratification for AML groupings showed a trend towards correlation with relapse outcomes (*Fig. 68*). The R-IPSS groups correlated with relapse outcomes, with high to very-high risk disease resulting in the highest relapse risk (*Fig. 69*). In the myelofibrosis cohort, relapse risk appeared highest in the intermediate-1 group as compared intermediate-2 and high-risk disease, however this was a trend and not statistically significant (*Fig. 70*).

There was no significant difference in relapse rates between cases with sibling or unrelated donors, and by proxy, ATLG dose intensity (*Fig. 71*). There was no correlation between donor age and relapse risk ($p=0.75$). Single antigen HLA mismatch did not affect relapse risk (*Fig. 72*). An increased risk of relapse was seen in those with an ABO mismatch, primarily in the major mismatch cohort (*Fig. 73*). Graft cryopreservation prior to infusion was associated with an increased risk of relapse (*Fig. 74*). Graft T cell content in total and subset analysis showed a correlation between higher graft T cell content and the risk of future relapse, including CD3⁺, CD4⁺, CD8⁺ and CD4⁺/CD8⁺ levels (*Fig. 75 - 78*). The recipient lymphocyte count pre-conditioning or on the first day of ATLG exposure did not affect subsequent relapse risk (*Fig. 79*). Choice of calcineurin inhibitor was not associated with relapse outcomes. Early volatile calcineurin serum levels in the first 30 days post-transplant were not associated with an increased risk of relapse.

Acute GVHD was not protective against relapse however chronic GVHD was associated with a reduced relapse risk (*Fig. 80 and 81*). Mixed chimerism (total or lymphoid CD3⁺) beyond day 90 was not associated with an increased risk of subsequent relapse (*Fig. 82*). DLI use in those with mixed chimerism at day 90, however, was associated with a significant reduction in the subsequent risk of relapse (*Fig. 83*). The use of DLI in all patients eligible for either pre-emptive DLI or prophylactic DLI was associated with a reduced risk of subsequent relapse as compared to those who did not have a pre-emptive or prophylactic DLI.

The median and mean pre-conditioning EASIX score at a cut-off of 1.89 and 3 did not correlate with subsequent relapse rates (*Fig. 84*). At a median cut-off of 11.54 or mean of 15 at day 0, the EASIX score did not predict subsequent relapse (*Fig. 85*). The EASIX at GVHD onset at a median of 1.97 was not predictive of relapse (*Fig. 86*). At a median of 1.24, the EASIX score at 1-year post-transplant was predictive of subsequent relapse (*Fig. 87*). Univariable predictors of relapse, EASIX score and graft T cell content association with relapse risk are summarized in tables 9-11.

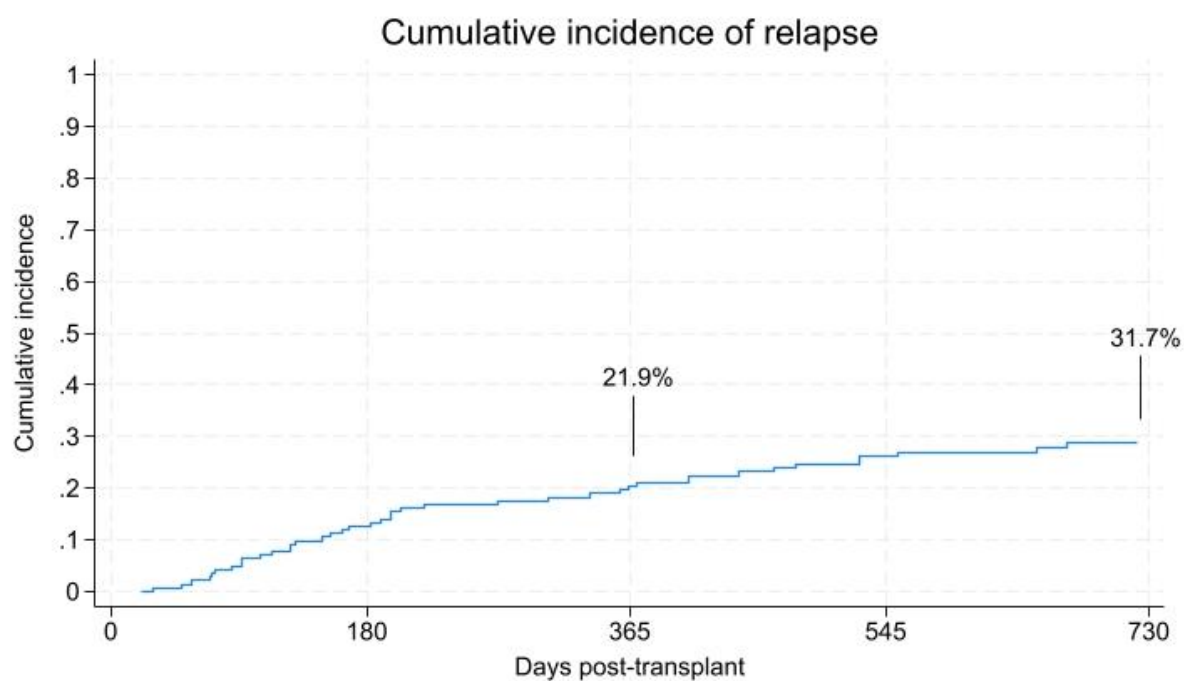


Figure 63: Cumulative incidence of relapse.

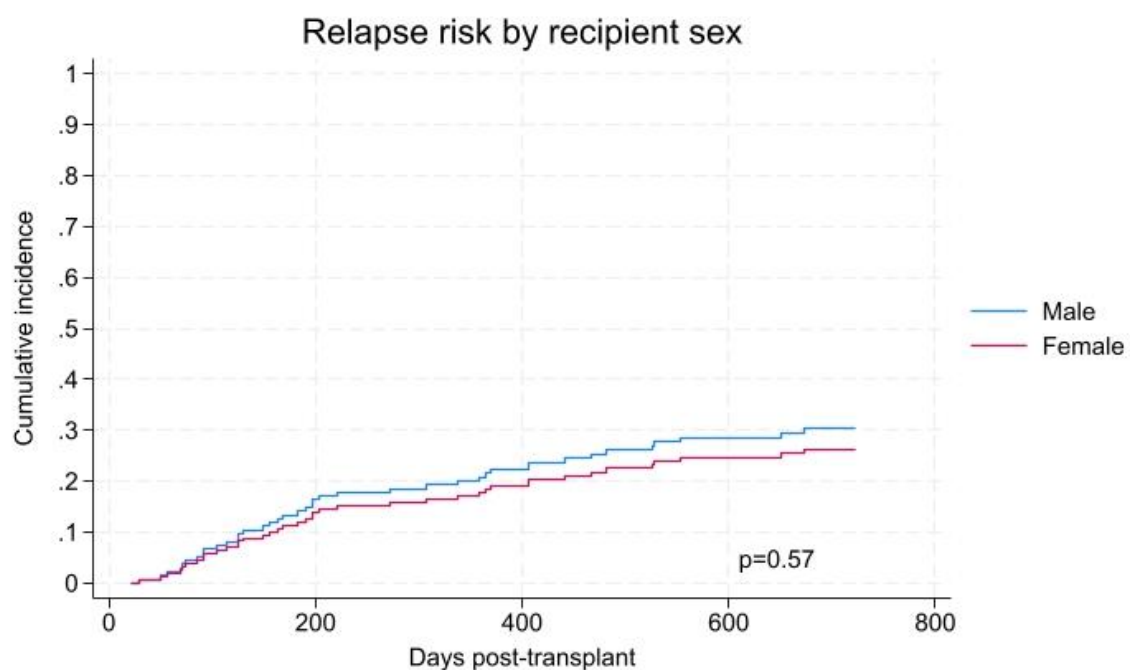


Figure 64: Cumulative incidence of relapse by recipient sex.

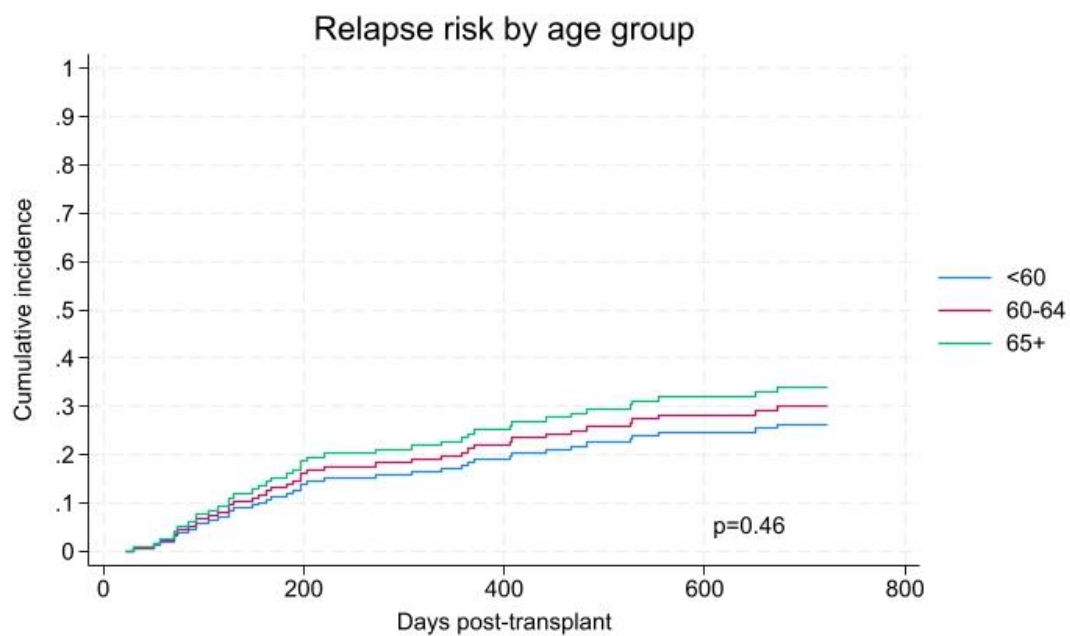


Figure 65: Cumulative incidence of relapse by recipient age group; <60, 60-64 and 65+.

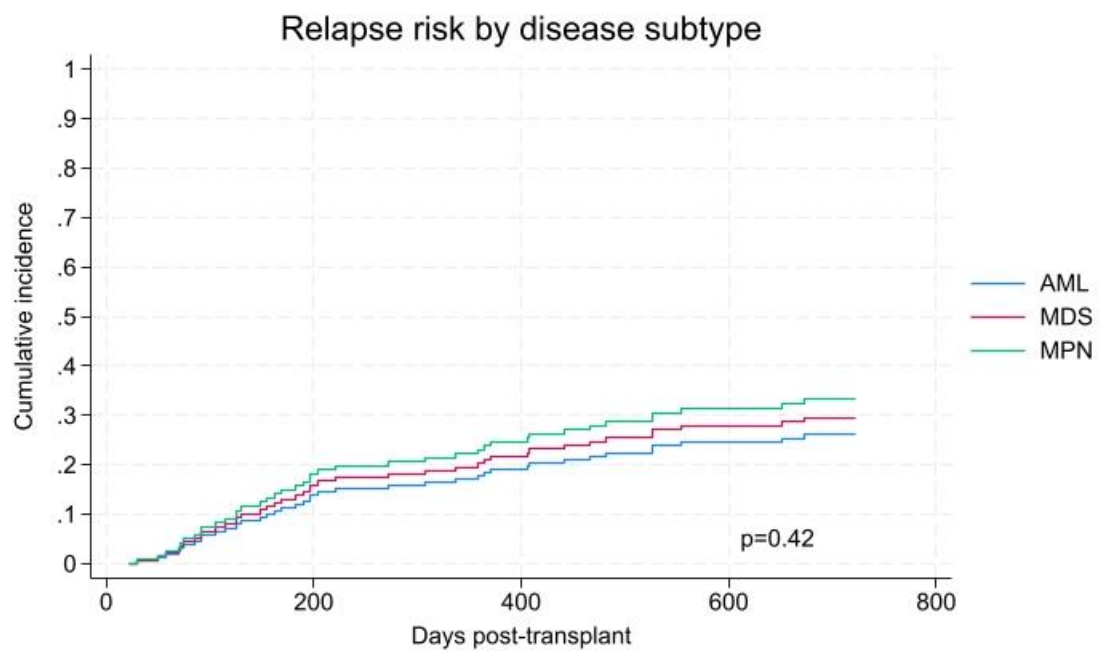


Figure 66: Cumulative incidence of relapse by disease subtype; AML, MDS and MPN.

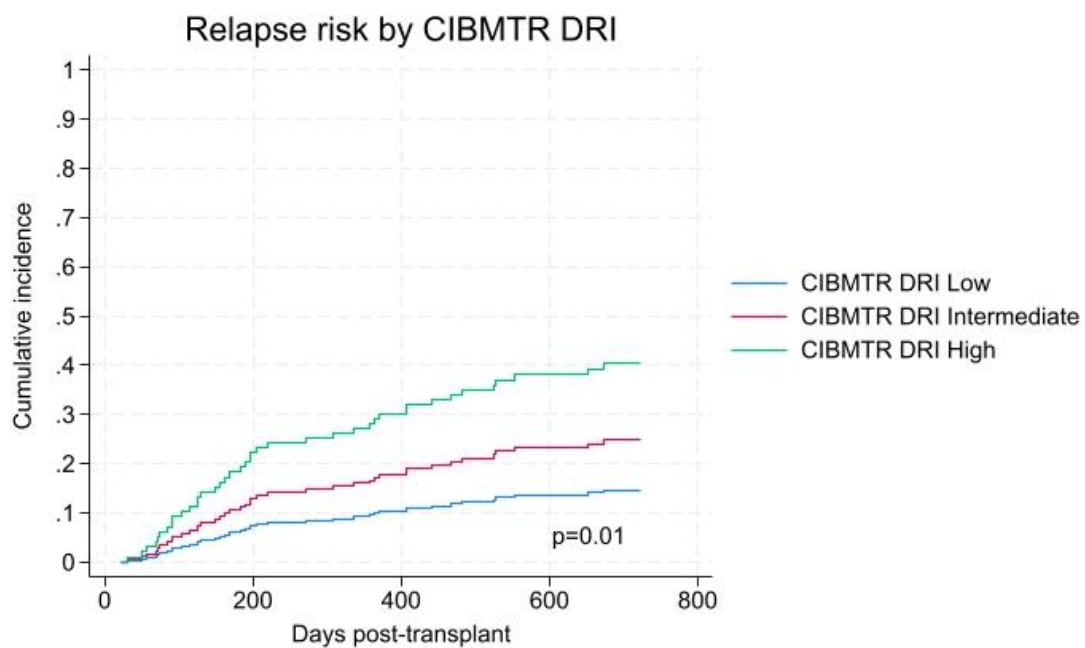


Figure 67: Cumulative incidence of relapse by CIBMTR-DRI group; low, intermediate and high.

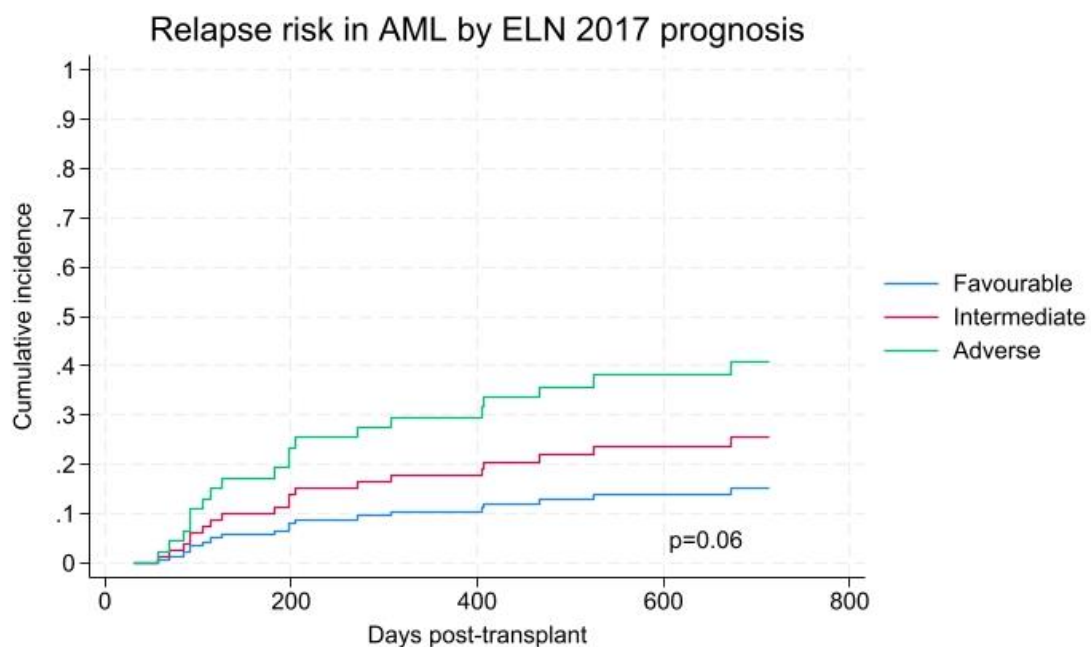


Figure 68: Cumulative incidence of relapse by AML ELN 2017 risk classification; favourable, intermediate and adverse.

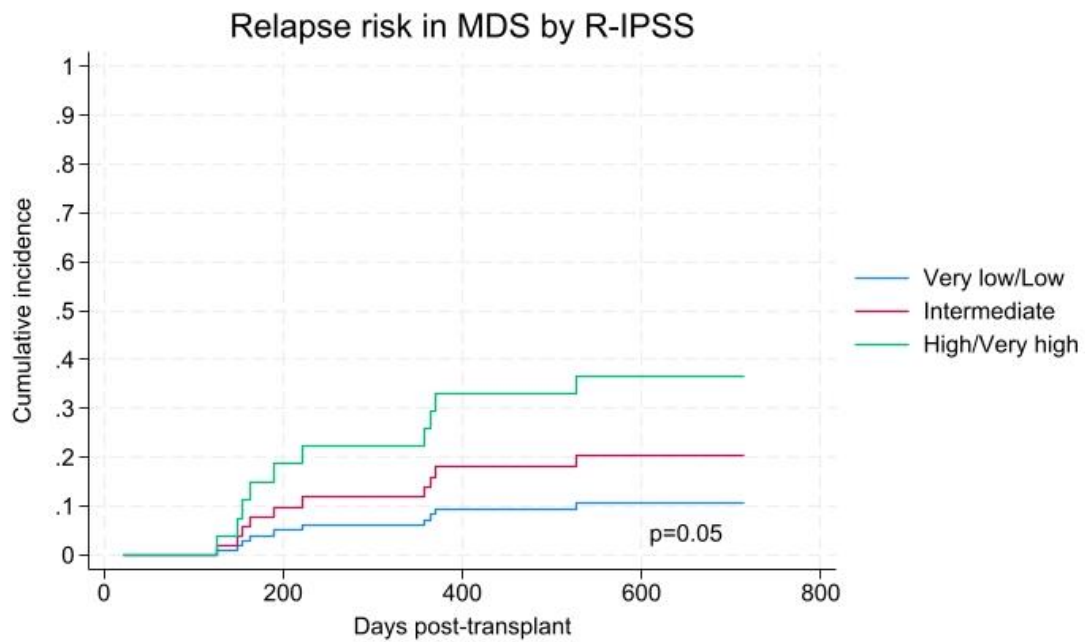


Figure 69: Cumulative incidence of relapse by MDS R-IPSS risk group; very low and low, intermediate and high and very high.

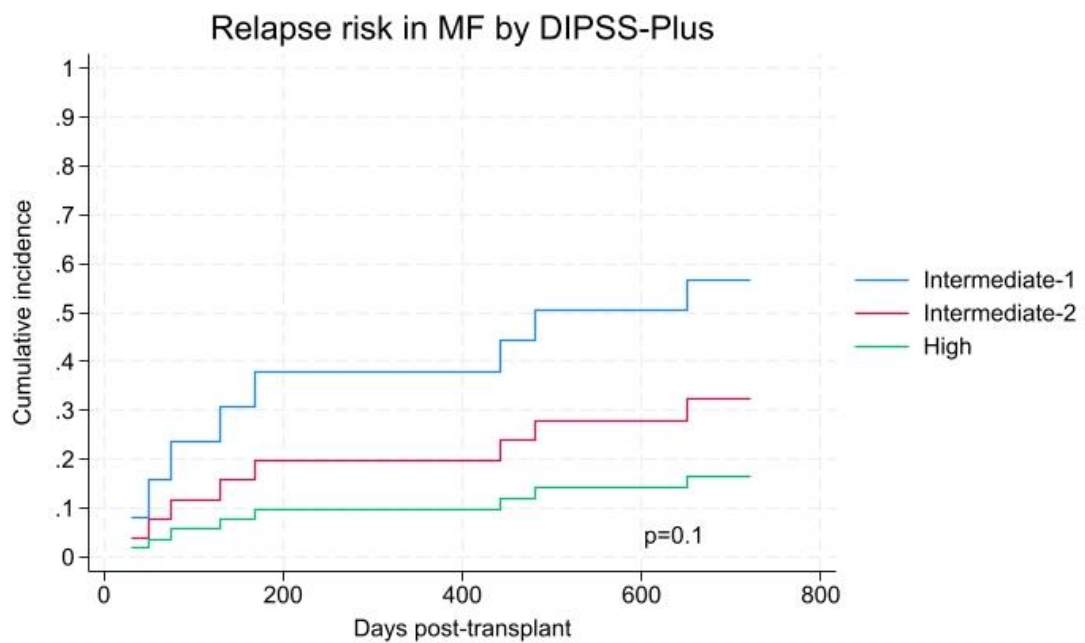


Figure 70: Cumulative incidence of relapse by MF DIPSS-Plus risk group; intermediate-1, intermediate-2 and high.

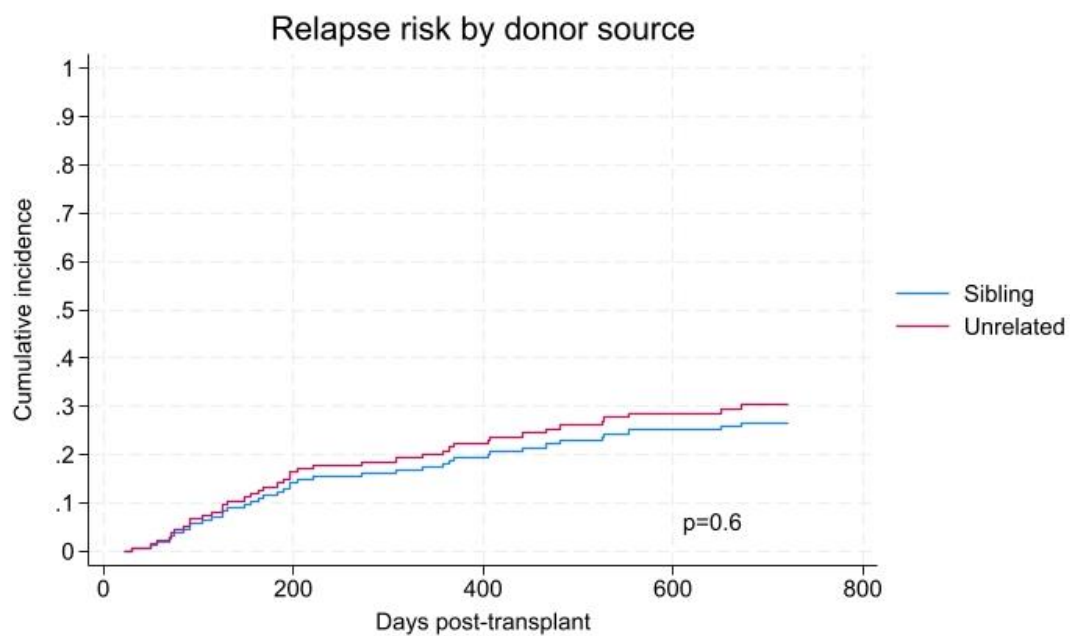


Figure 71: Cumulative incidence of relapse by donor source; sibling vs. unrelated.

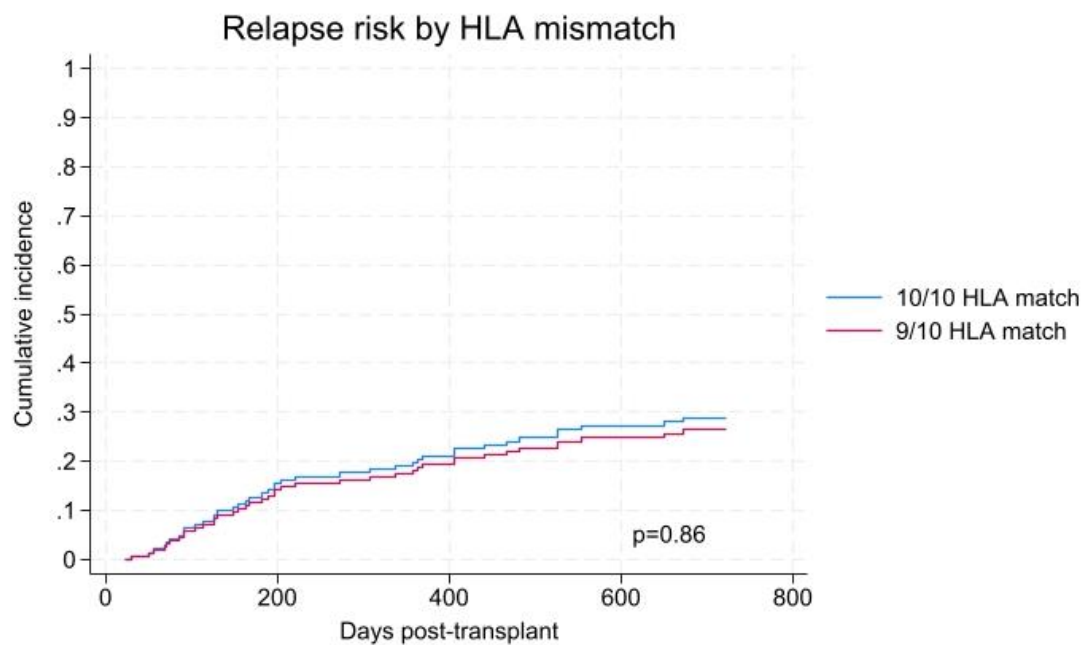


Figure 72: Cumulative incidence of relapse by HLA matching.

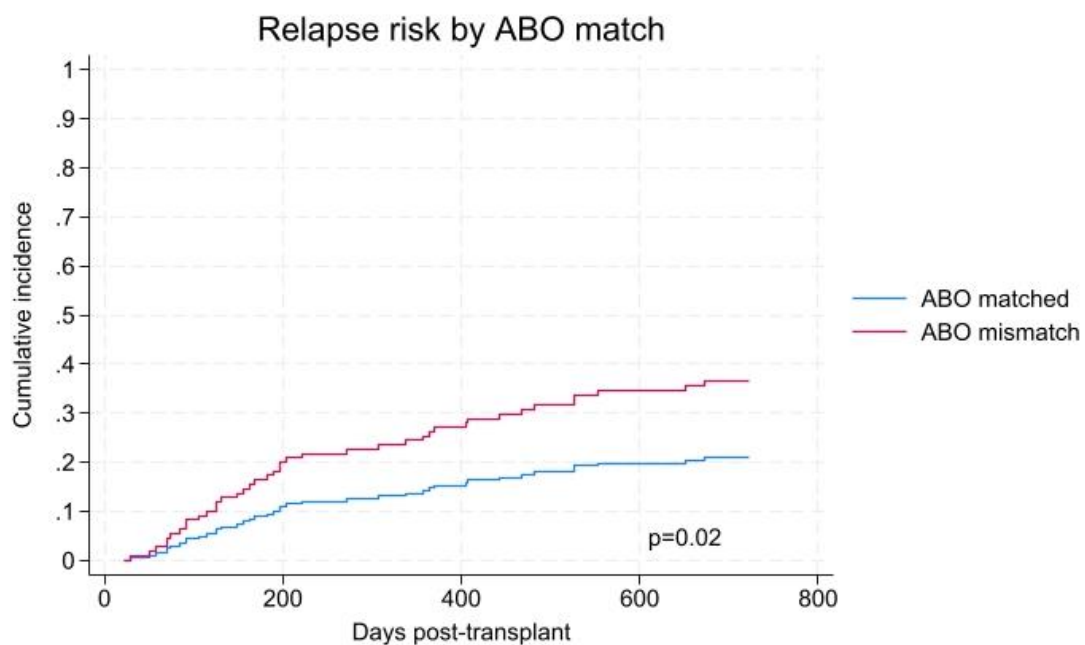


Figure 73: Cumulative incidence of relapse by ABO matching.

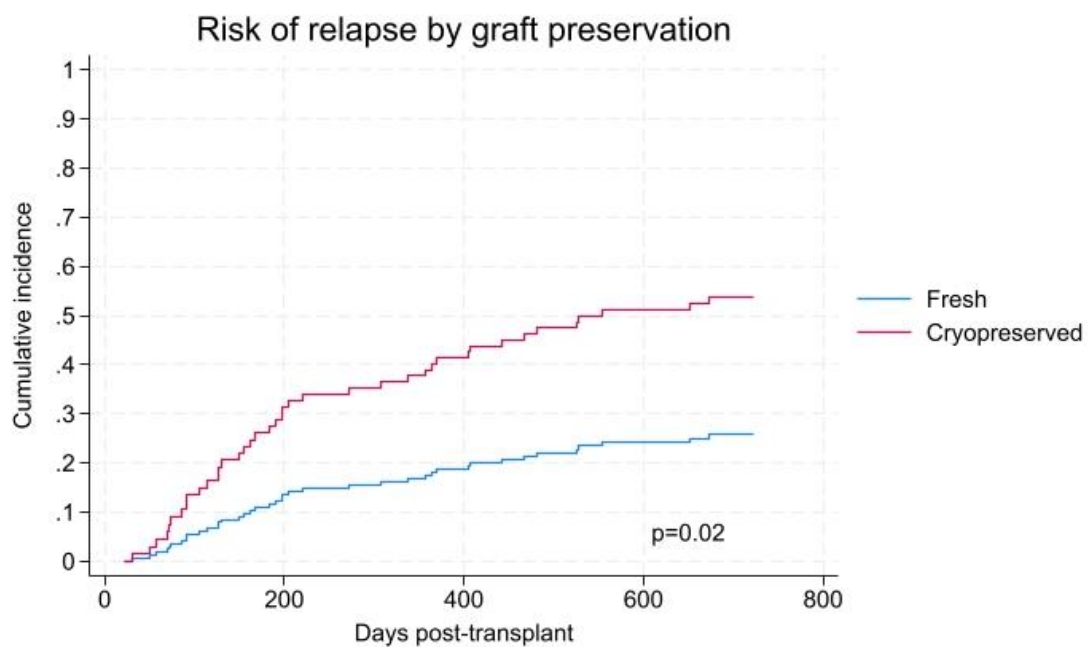


Figure 74: Cumulative incidence of relapse by graft preparation; fresh vs. cryopreserved.

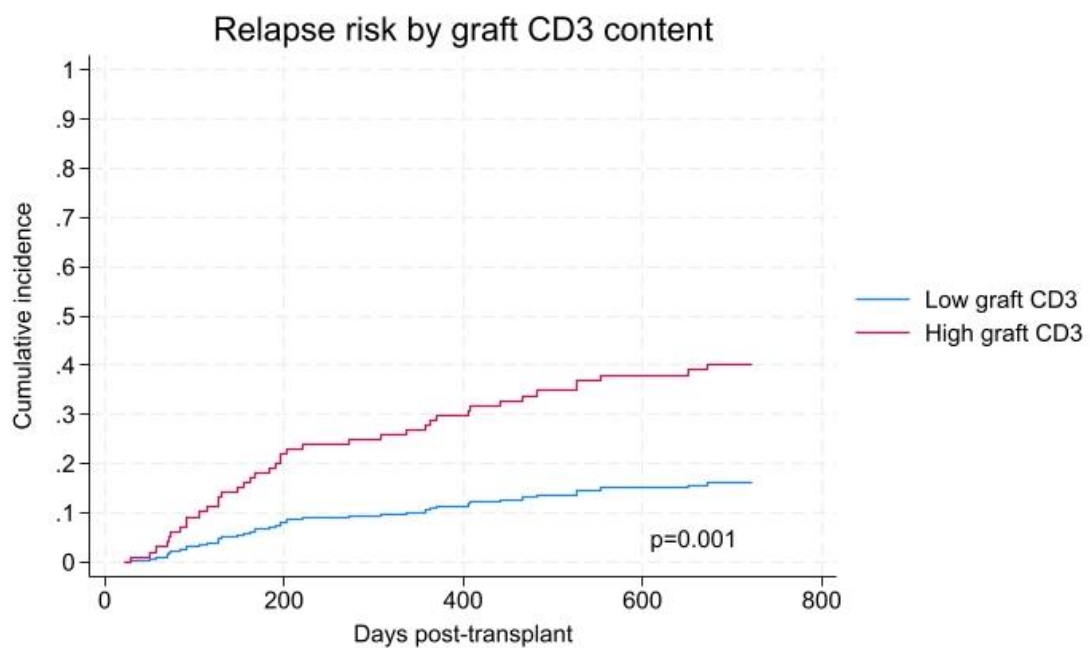


Figure 75: Cumulative incidence of relapse by graft CD3⁺ T cell content above and below median of $17.14 \times 10^7/\text{kg}$.

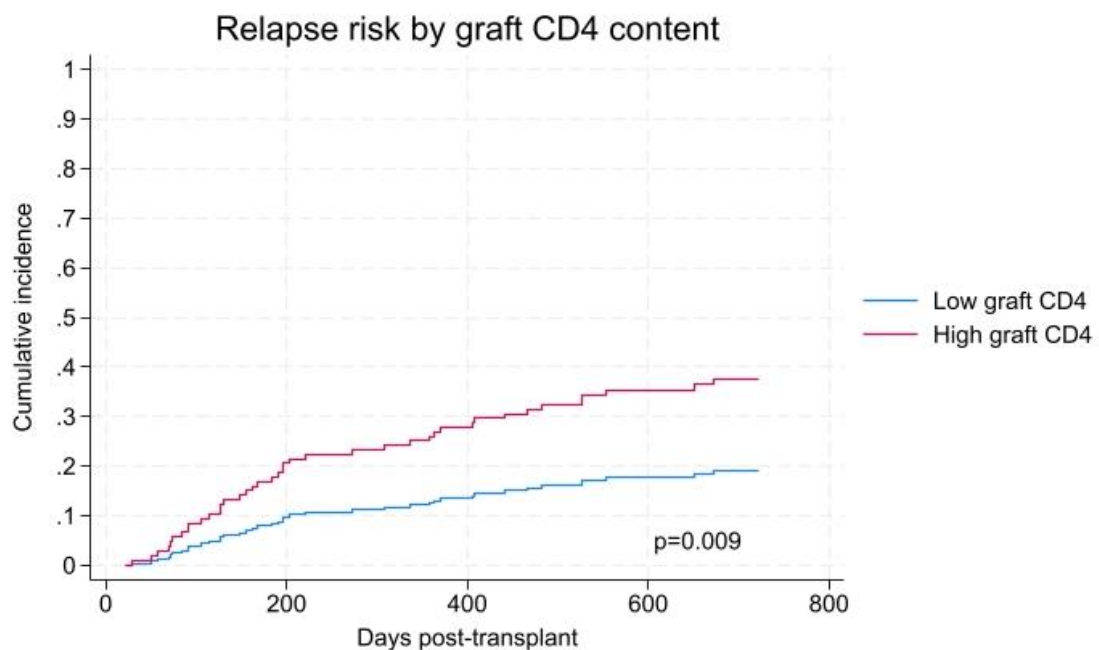


Figure 76: Cumulative incidence of relapse by graft CD4⁺ T cell content above and below median of $11.09 \times 10^7/\text{kg}$.

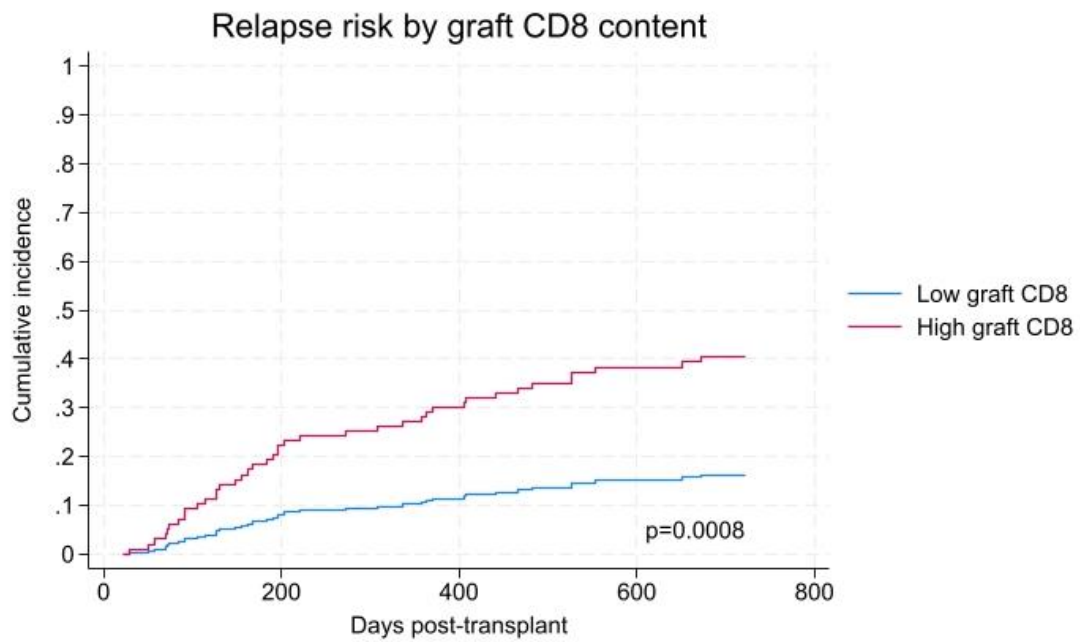


Figure 77: Cumulative incidence of relapse by graft CD8⁺ T cell content above and below median of $6.37 \times 10^7/\text{kg}$.

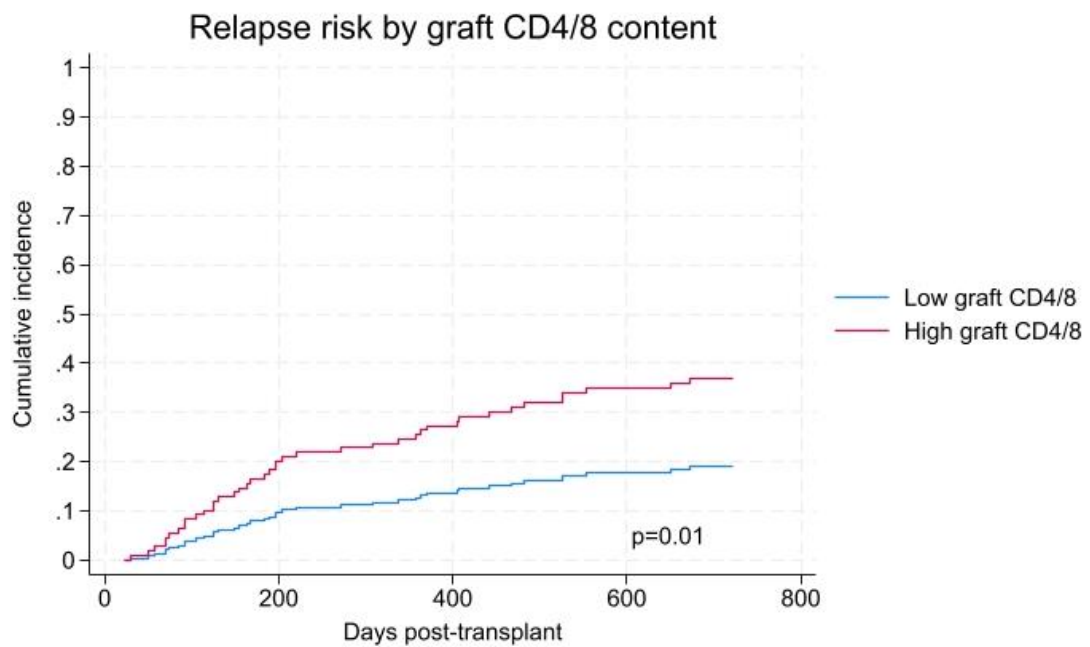


Figure 78: Cumulative incidence of relapse by graft CD4⁺/CD8⁺ T cell content above and below median of $0.24 \times 10^7/\text{kg}$.

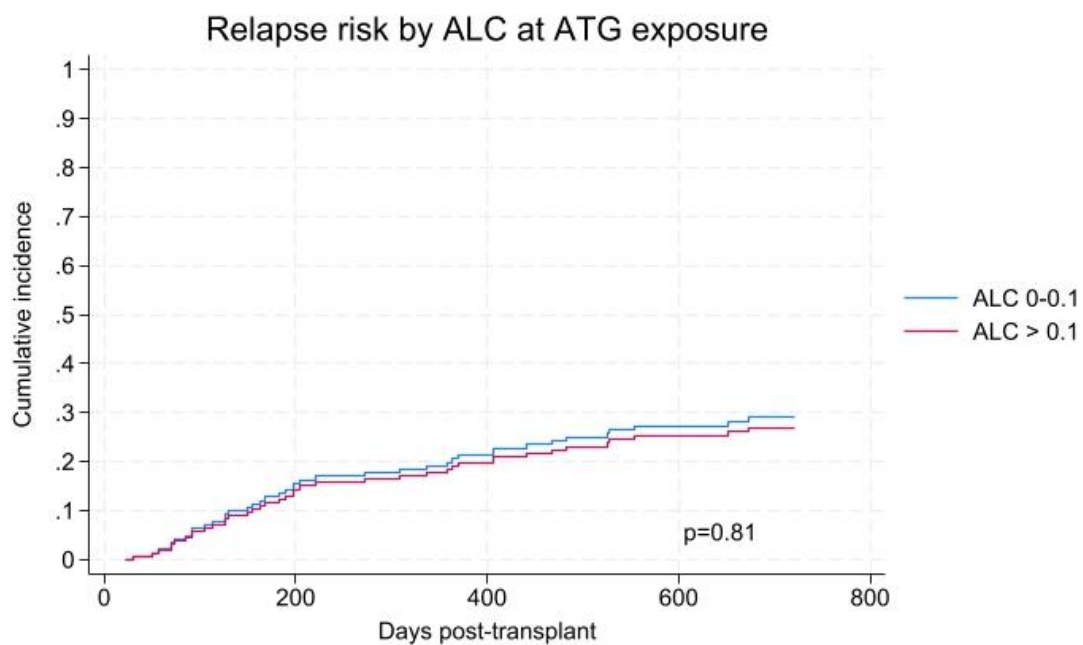


Figure 79: Cumulative incidence of relapse by absolute lymphocyte count on first day of ATLG exposure at cut-off of $0.1 \times 10^9/L$.

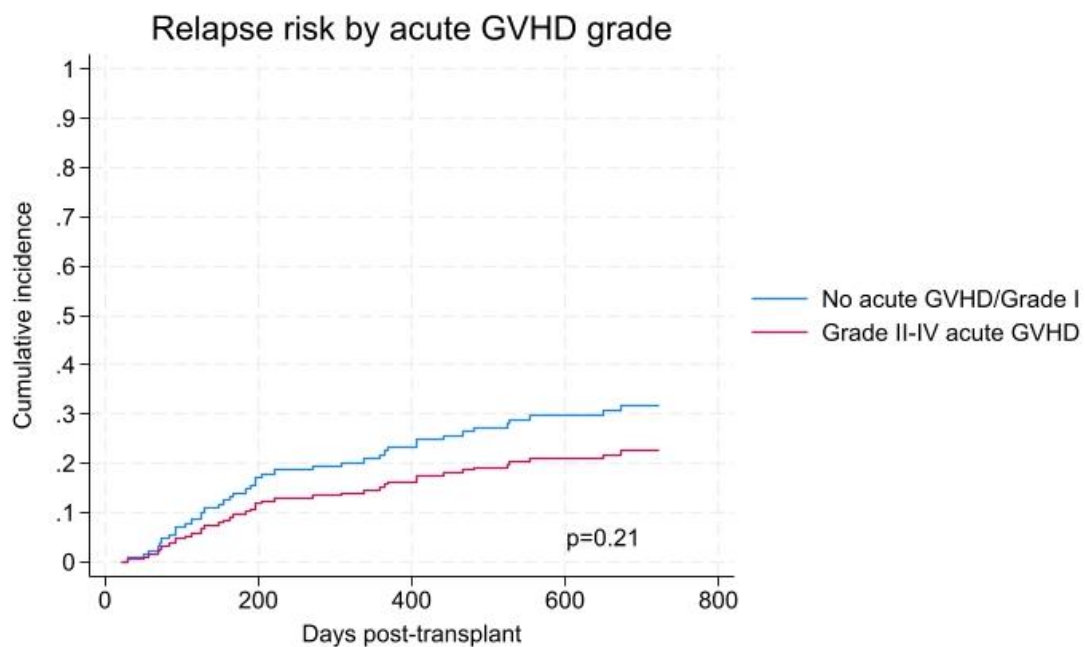


Figure 80: Cumulative incidence of relapse by preceding grade II-IV acute GVHD.

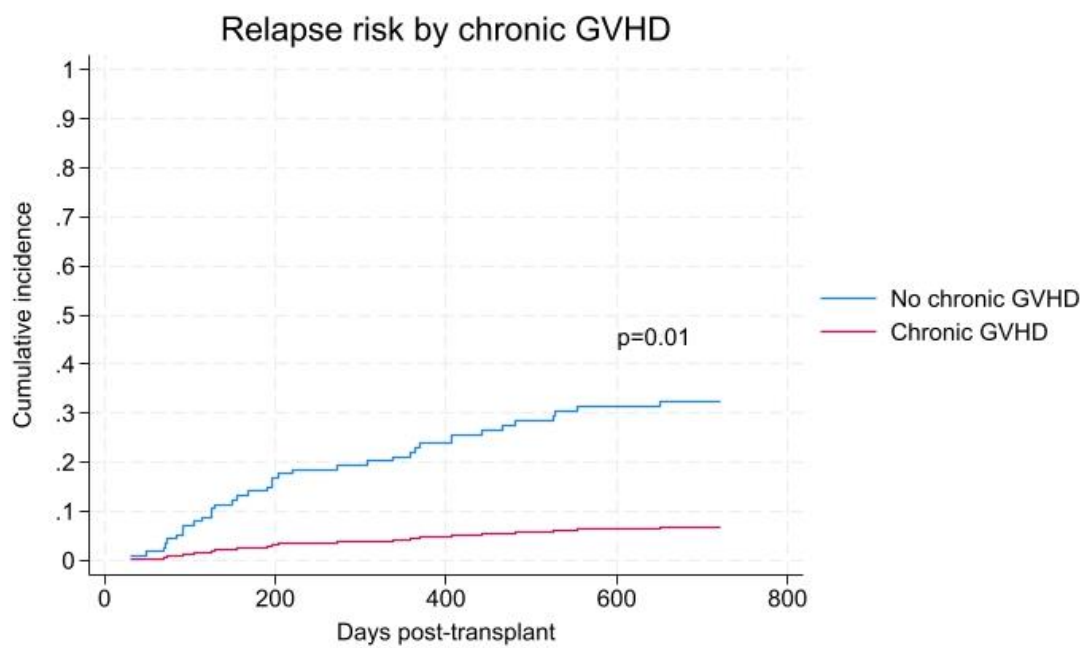


Figure 81: Cumulative incidence of relapse by chronic GVHD.

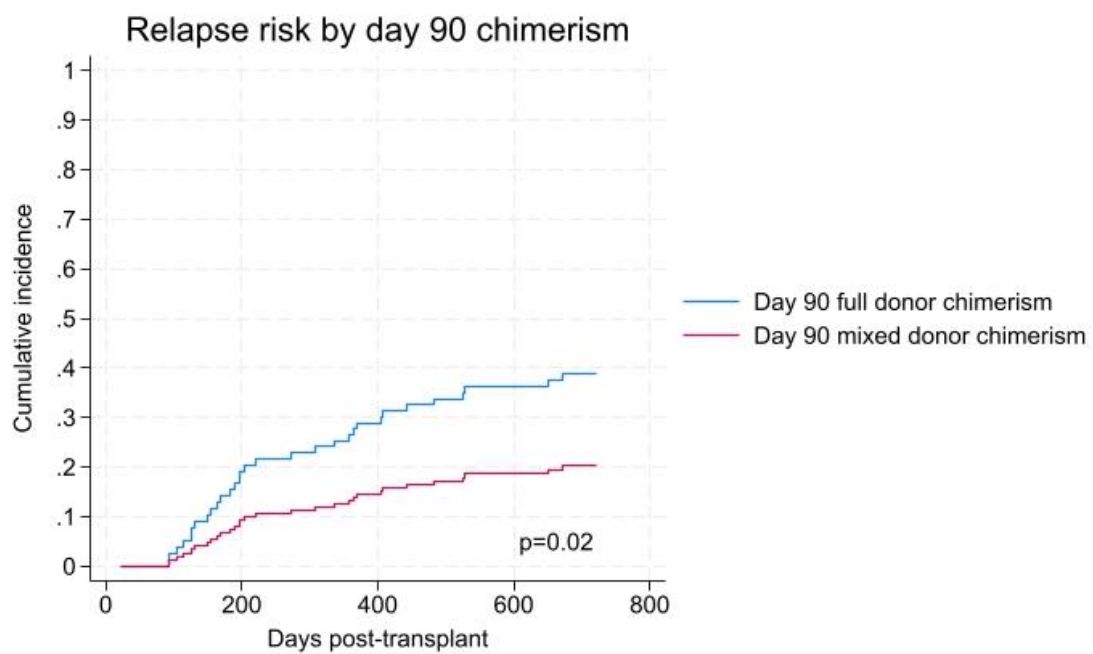


Figure 82: Cumulative incidence of relapse by chimerism status at day 90; mixed vs. full donor chimerism.

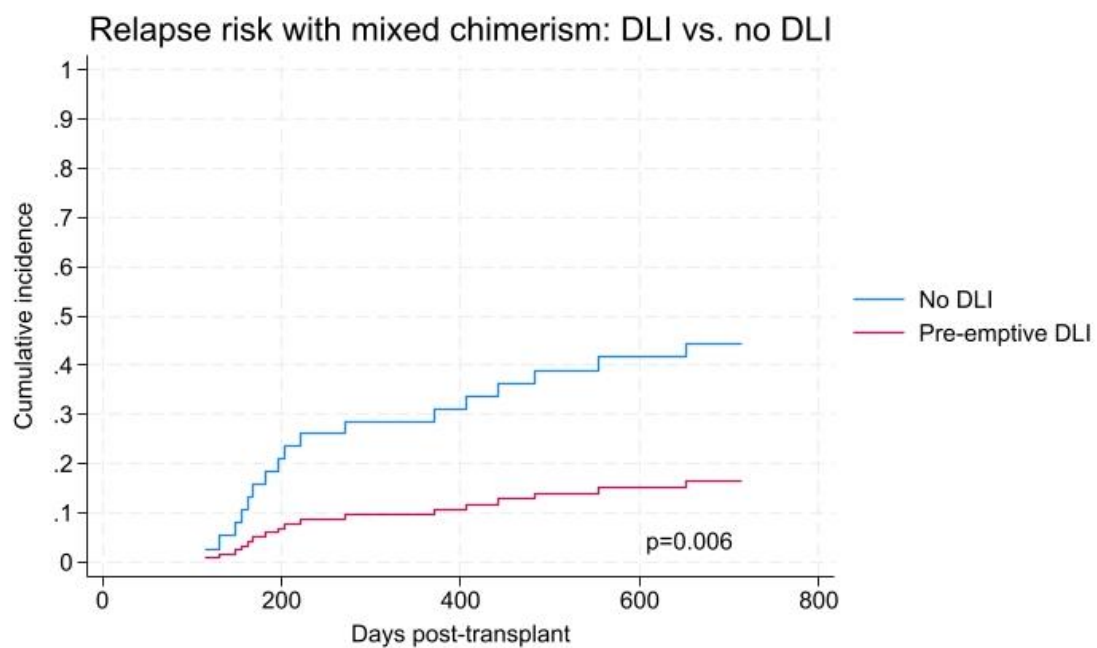


Figure 83: Cumulative incidence of relapse in mixed chimerism beyond day 90; DLI vs. no DLI.

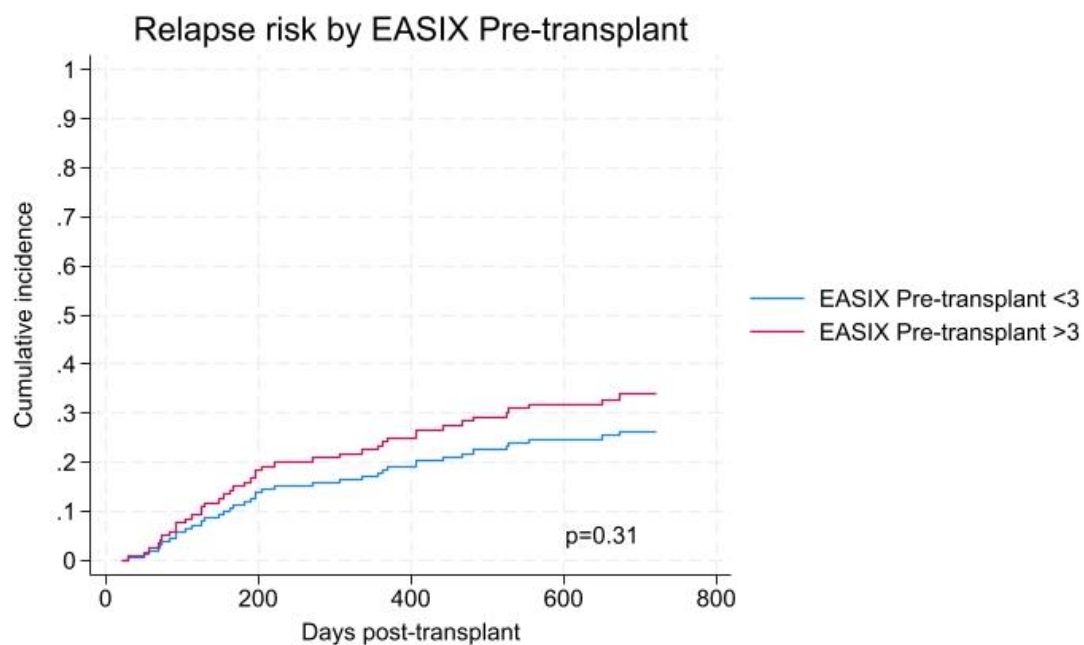


Figure 84: Cumulative incidence of relapse by EASIX at pre-transplant cut-off of 3.

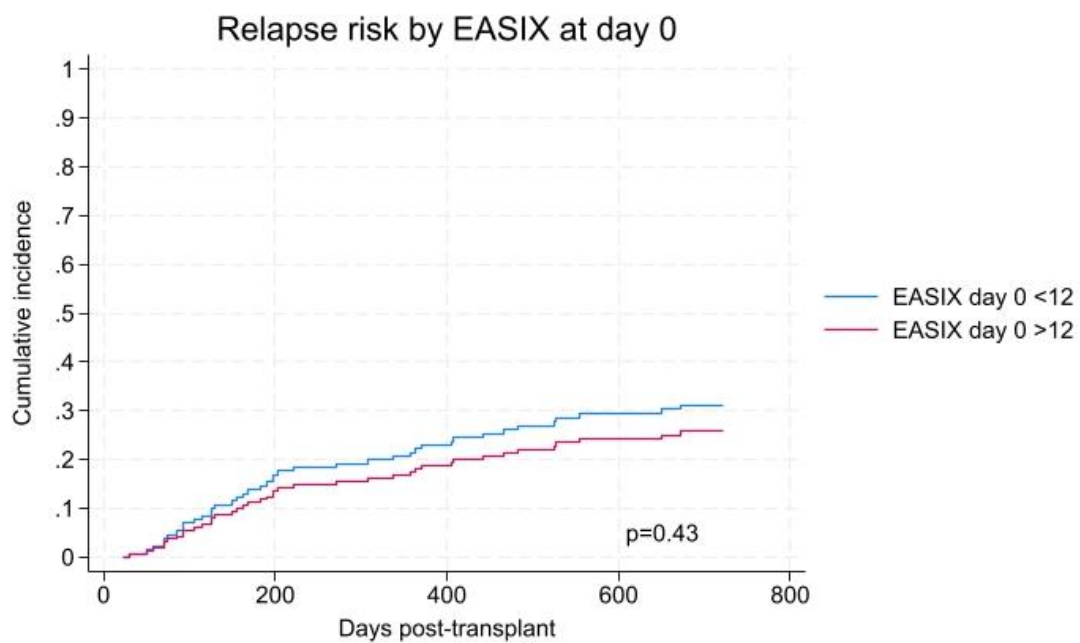


Figure 85: Cumulative incidence of relapse by EASIX at day 0 at cut-off of 12.

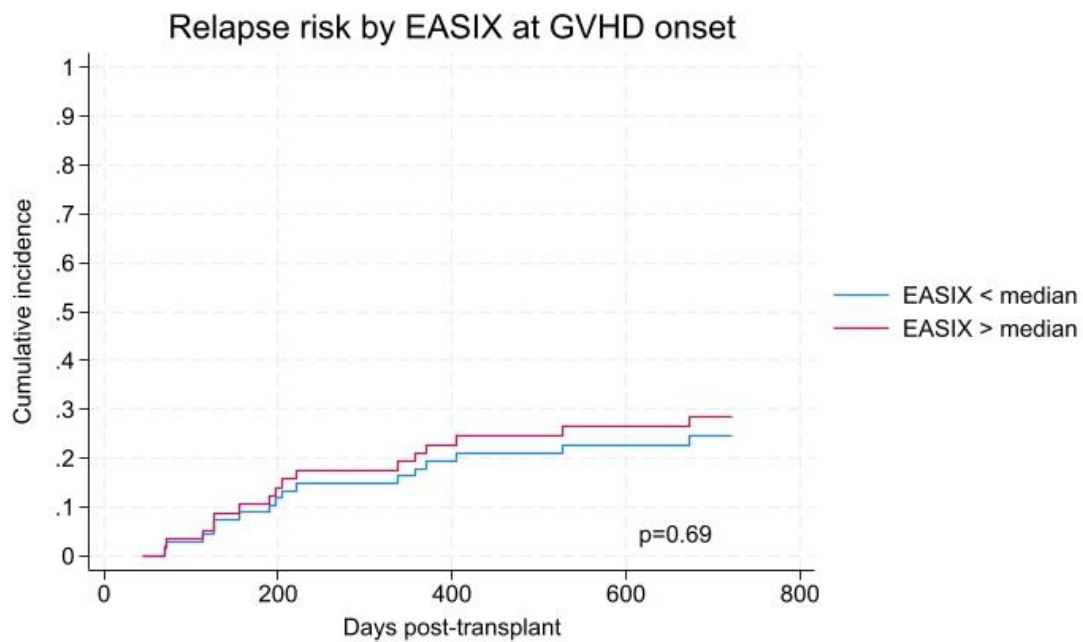


Figure 86: Cumulative incidence of relapse by EASIX at GVHD onset at median of 1.97.

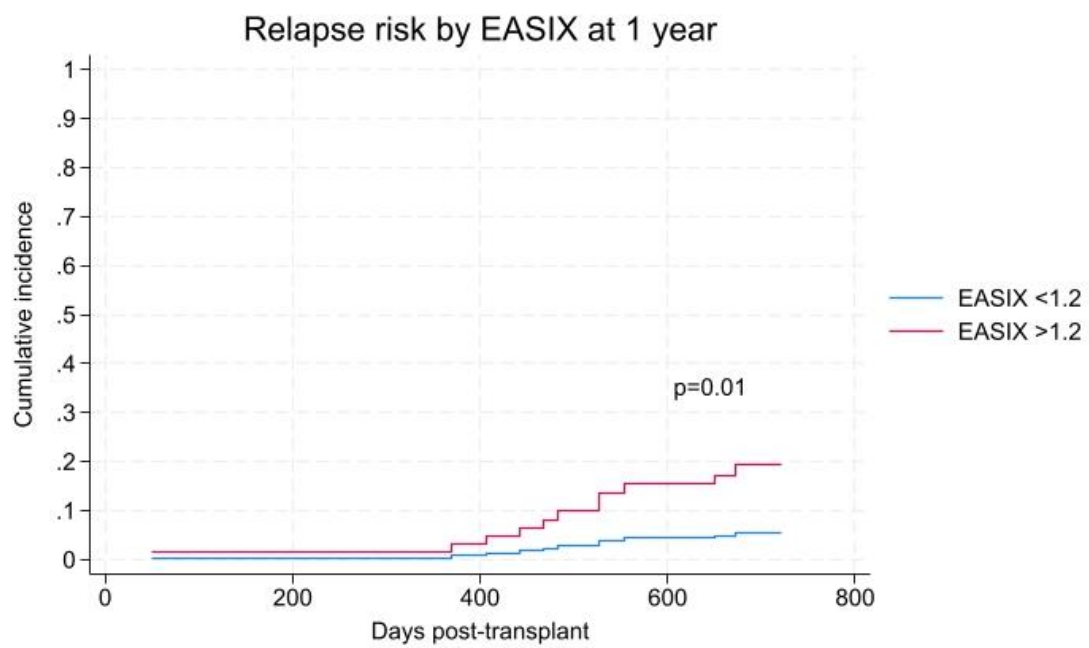


Figure 87: Cumulative incidence of relapse by EASIX at 1-year post-transplant at median cut-off of 1.2.

Table 9: Relapse univariable and multivariable analysis

Variable	Univariable analysis			Multivariable analysis		
	Hazard ratio	95% CI	P value	Hazard ratio	95% CI	P value
Male recipient	0.84	0.46 - 1.52	0.57			
Recipient age	1.17	0.77 – 1.78	0.46			
Disease subtype	1.15	0.81 – 1.64	0.42			
CIBMTR DRI Adverse	1.81	1.12 – 2.91	0.01	0.72	0.27–1.92	0.52
AML ELN 2017 Adverse	1.78	0.97 - 3.28	0.06			
MDS R-IPSS High/Very High	2.00	1.01 – 4.02	0.05			
Myelofibrosis D-IPSS High	0.47	0.19 - 1.16	0.10			
Unrelated donor	1.16	0.66 - 2.04	0.60			
Female donor, male recipient	1.20	0.68 – 2.11	0.51			
Donor < 25	0.74	0.39 – 1.39	0.35			
Donor < 40	0.94	0.53 – 1.66	0.84			
HLA mismatch	0.89	0.26 - 3.07	0.86			
Cryopreserved graft	2.58	1.13 - 5.89	0.02	1.74	0.52–5.83	0.36
High graft T cell content	2.92	1.55 – 5.53	0.001	2.40	0.95–6.04	0.06
ABO mismatch	2.08	1.10 – 3.95	0.02	2.27	0.95–5.45	0.06
ALC at first ATG > 0.1 x 10 ⁹ /L	0.91	0.42 – 1.98	0.81			
ALC pre-conditioning	1.17	0.91 – 1.50	0.22			
Tacrolimus vs. ciclosporin	1.16	0.65 – 2.06	0.61			
Acute GVHD (grade II-IV)	0.67	0.35 – 1.27	0.21			
Acute GVHD (grade III-IV)	0.41	0.13 - 1.24	0.11			
Chronic GVHD	0.18	0.05 - 0.67	0.01	0.32	0.11–0.92	0.03
Mixed chimerism at day 90	0.45	0.23 – 0.89	0.02			
Non-receipt of pre-emptive DLI	3.03	1.39 – 7.75	0.008	5.88	2.38-13.33	<0.001

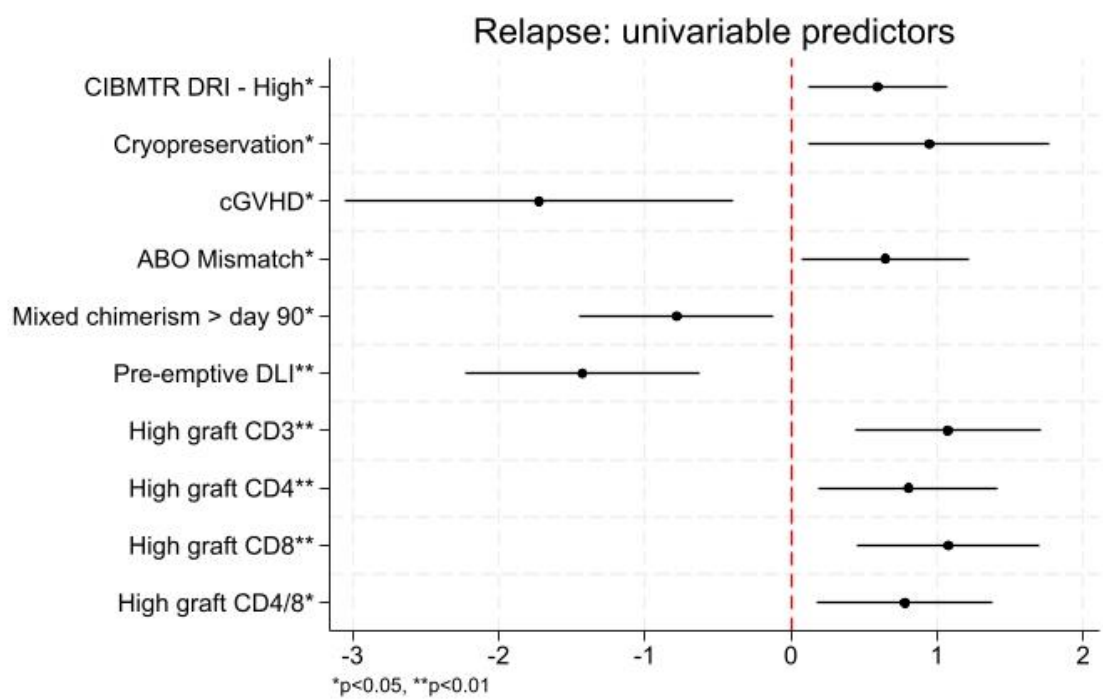


Figure 88: Coefficient plot for univariable predictors of relapse.

Table 10: Relapse EASIX Modelling

Variable	Hazard ratio	95% CI	P value
EASIX Pre-transplant	1.04	0.96 – 1.11	0.34
Mean	1.38	0.77 – 2.48	0.27
Mean + 1 SD	1.13	0.51 – 2.51	0.76
Median	1.17	0.67 – 2.04	0.59
25th Centile	0.94	0.49 – 1.77	0.83
75th Centile	1.46	0.80 – 2.67	0.21
EASIX Day 0	1.01	1.01 – 1.02	0.0004
Mean	0.83	0.45 – 1.54	0.57
Mean + 1 SD	6.44	2.76 – 15.02	<0.001
Median	0.78	0.44 – 1.38	0.40
25th Centile	0.63	0.63 – 0.35	1.13
75th Centile	0.69	0.69 – 2.37	0.43
EASIX at GVHD onset	1.03	1.01 – 1.05	0.002
Mean	1.61	0.68 – 3.82	0.27
Mean + 1 SD	3.86	1.52 – 9.78	0.004
Median	0.96	0.43 – 2.15	0.91
25th Centile	1.22	0.44 – 3.39	0.69
75th Centile	1.41	0.59 – 3.37	0.43
EASIX at 1 year	1.21	1.13 – 1.30	<0.0001
Mean	2.38	1.01 – 5.74	0.05
Mean + 1 SD	0.75	0.09 – 6.25	0.79
Median	3.59	1.29 – 9.99	0.01
25th Centile	1.39	0.46 – 4.22	0.55
75th Centile	1.53	0.57 – 4.08	0.38

Table 11: Relapse Graft T cell content

Variable	Hazard ratio	95% CI	P value
Graft CD3⁺	1.01	0.99 – 1.03	0.09
Mean	2.65	1.49 – 4.72	0.0009
Mean + 1 SD	1.78	0.89 – 3.55	0.10
Median	2.92	1.54 – 5.53	0.001
25th Centile	1.67	0.78 – 3.56	0.18
75th Centile	2.26	1.26 – 4.05	0.0005
Graft CD4⁺	1.02	0.99 – 1.04	0.12
Mean	1.96	1.11 – 3.47	0.01
Mean + 1 SD	2.56	1.45 – 4.52	0.001
Median	2.23	1.21 – 4.12	0.009
25th Centile	2.42	1.05 – 5.57	0.03
75th Centile	2.36	1.32 – 4.21	0.003
Graft CD8⁺	1.03	0.99 – 1.06	0.09
Mean	2.77	1.56 – 4.91	0.0005
Mean + 1 SD	1.32	0.54 – 3.24	0.54
Median	2.93	1.57 – 5.50	0.0008
25th Centile	2.58	1.07 – 6.19	0.03
75th Centile	2.55	1.43 – 4.59	0.001
Graft CD4⁺/CD8⁺	1.28	0.90 – 1.82	0.17
Mean	2.01	1.13 – 3.58	0.01
Mean + 1 SD	1.91	0.84 – 4.32	0.12
Median	2.18	1.19 – 3.99	0.01
25th Centile	-	-	-
75th Centile	2.07	1.16 – 3.71	0.01

Acute GVHD

Acute GVHD occurred in 85/143 patients (59.4%), of which 19 was grade I and 66 was grade II-IV. Within higher grades of GVHD, 48 were grade II, 11 grade III and 5 grade IV. 62 patients (43.3%) developed spontaneous GVHD prior to DLI use, and a further 25 patients who had no evidence of GVHD prior to DLI developed subsequent acute GVHD. Using the MAGIC algorithm for staging and grading of acute GVHD, 27 patients had stage 1 disease, 40 had stage 2 disease, 11 had stage 3 and 5 had stage 4. The median time to *de novo* GVHD onset was 52.5 days. For acute GVHD secondary to DLI use, the median time to onset was 231 days, and the median time to any acute GVHD onset was 94 days.

64 patients experienced acute GVHD of skin, 27 of liver, 27 of the gastrointestinal tract and a further 10 other miscellaneous manifestations including neurological, mucocutaneous and ocular. 66 patients required systemic steroids for management of acute GVHD, of which 44 was for spontaneous acute GVHD, and a further 22 in those who developed acute GVHD post-DLI. Data on steroid-sparing agent use for refractory disease was available for 127 patients, and 10 patients required additional therapy with extra corporeal photopheresis, ruxolitinib, imatinib or mycophenolate mofetil.

The cumulative incidence of grade II-IV acute GVHD was not affected by sex (Fig. 89), age (Fig. 90) or disease subtype (Fig. 91). Grade III-IV disease was more common in those transplanted for MPN (Fig. 92). Considering markers of pre-transplant organ assessment, only a ferritin level of >1,000 ng/ml appeared to correlate with acute GVHD outcomes in univariable analysis (Fig. 93). There was a higher risk of acute GVHD in those with unrelated donors as compared to sibling donors, which was significant in grade III-IV disease (Fig. 94, 95). This trend was not seen in HLA mismatched pairs (Fig. 96). ABO mismatch was not associated with spontaneous acute GVHD outcomes, either as major or minor mismatch (Fig. 97). Cryopreservation of the stem cell graft was not associated with spontaneous acute GVHD outcomes (Fig. 98). Younger donor age of <40 was associated with an increased risk of acute GVHD in univariate analysis (Fig. 99). Graft CD3⁺, CD4⁺, CD8⁺ and CD4⁺/CD8⁺ T cell content did not show an association at meaningful cut-off points (Fig. 100-103).

The absolute lymphocyte count measures at the first day of ATLG exposure did not predict subsequent acute GVHD incidence at any grade (Fig. 104 and 105). The pre-conditioning lymphocyte count was similarly non-predictive at any level (Fig. 106). Ciclosporin use was associated with less spontaneous acute GVHD as compared to those who were treated with tacrolimus for GVHD prophylaxis (Fig. 107). Those who experienced erratic calcineurin inhibitor levels in the first month post-transplant showed an increased risk of subsequent grade II-IV acute GVHD (Fig. 108).

The EASIX score at the pre-conditioning time point was predictive of acute grade III-IV GVHD at higher levels (Fig. 109). At day 0 the EASIX score did not predict acute GVHD of any grade, although a strong trend was noted for the subsequent development of grade III-IV disease in those who had an EASIX score above the mean and 75th centile at day 0 (Fig. 110). Univariable predictors, EASIX correlation and graft T cell correlation with grade II-IV acute GVHD are summarized in Tables 12 - 14. Univariable predictors, EASIX correlation and graft T cell correlation with grade III-IV acute GVHD are summarized in tables 15 - 17.

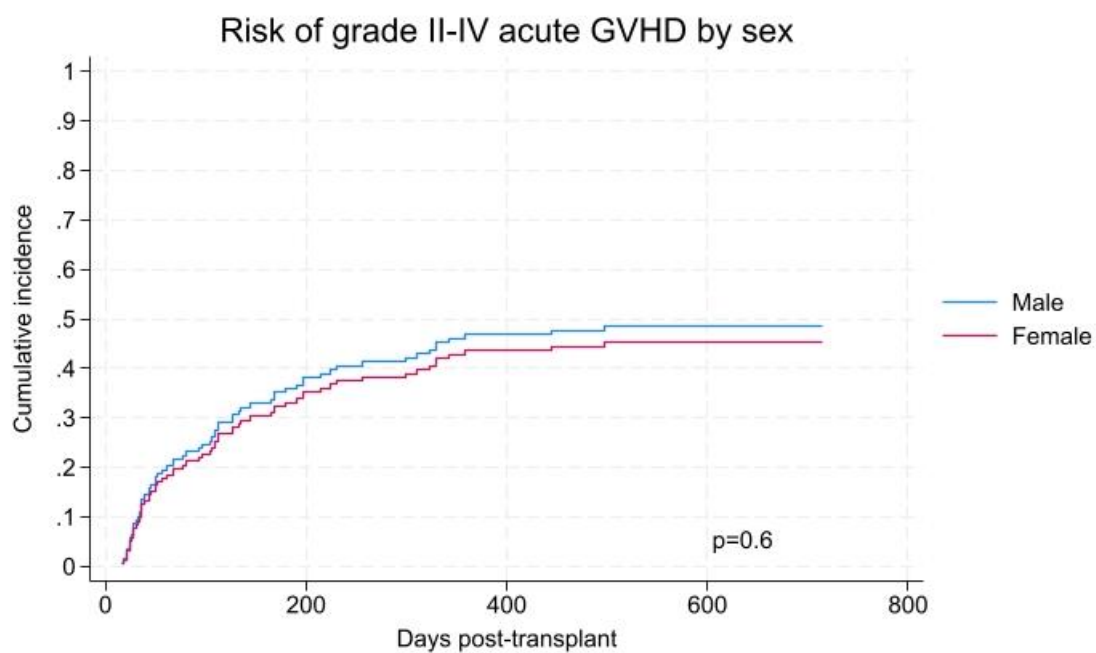


Figure 89: Cumulative incidence of grade II-IV acute GVHD stratified by recipient sex.

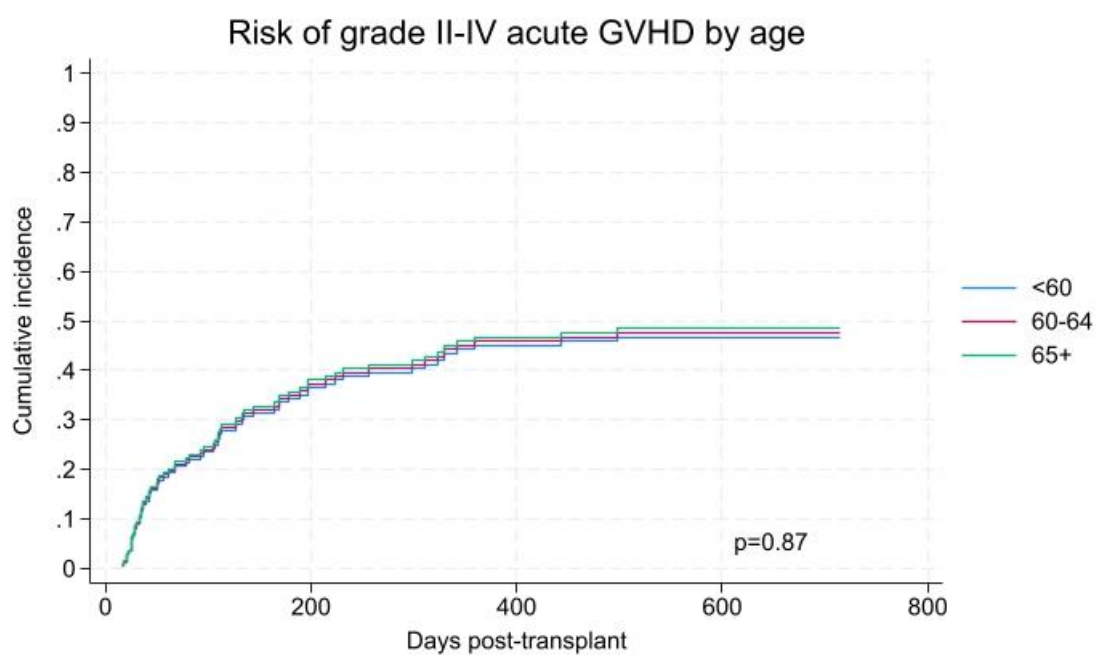


Figure 90: Cumulative incidence of grade II-IV acute GVHD by recipient age <60, 60-64 and 65+.

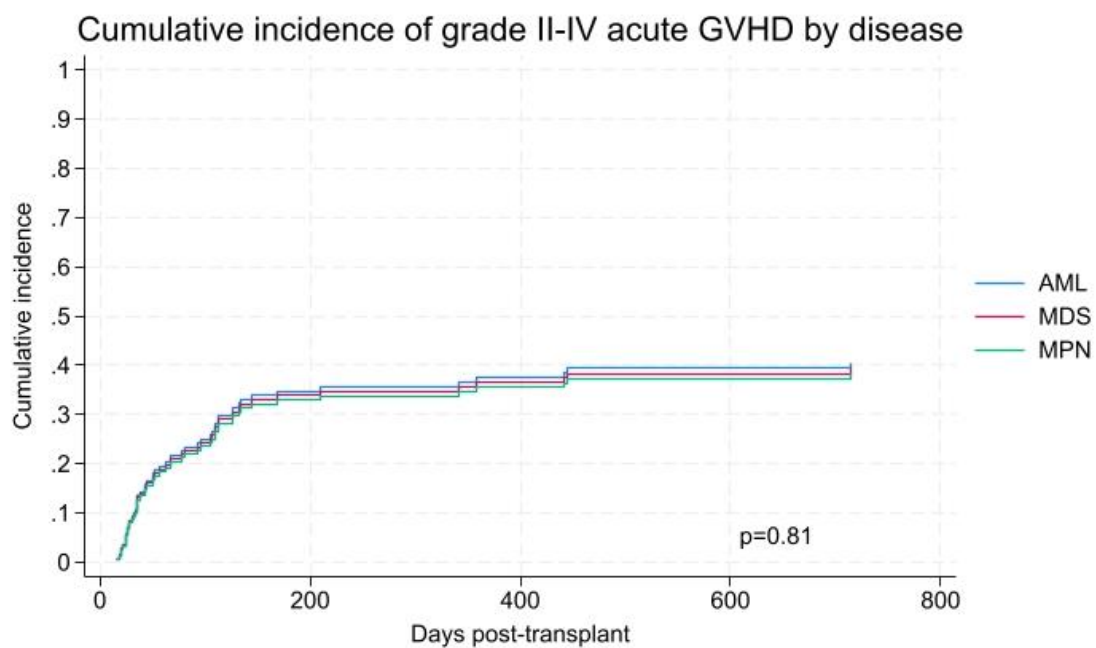


Figure 91: Cumulative incidence of grade II-IV acute GVHD by disease subtype; AML, MDS and MPN.

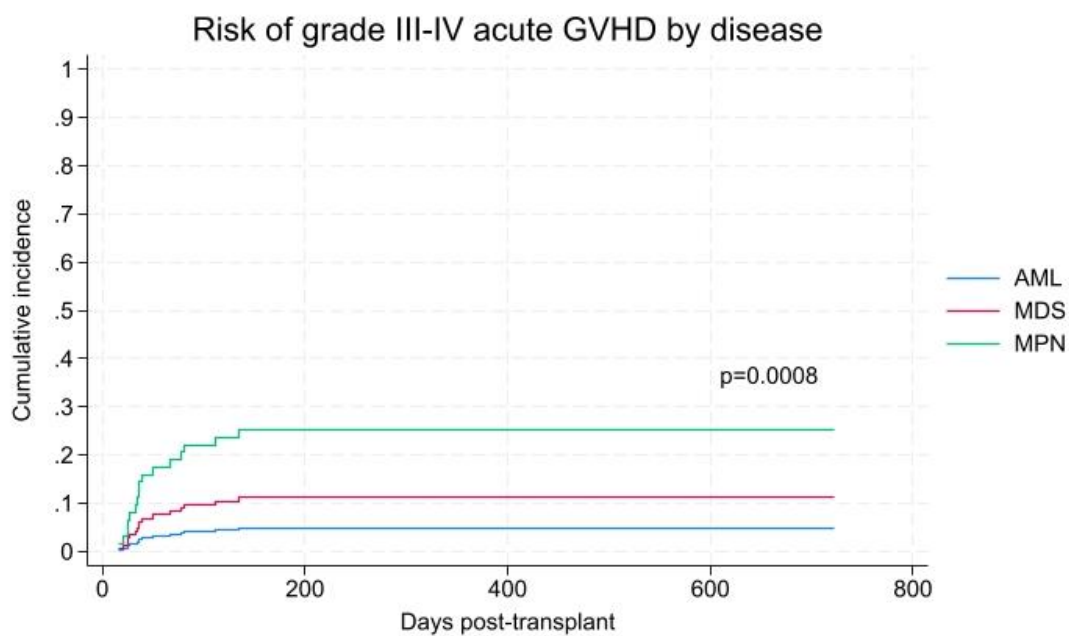


Figure 92: Cumulative incidence of grade III-IV acute GVHD by disease subtype: AML, MDS and MPN.

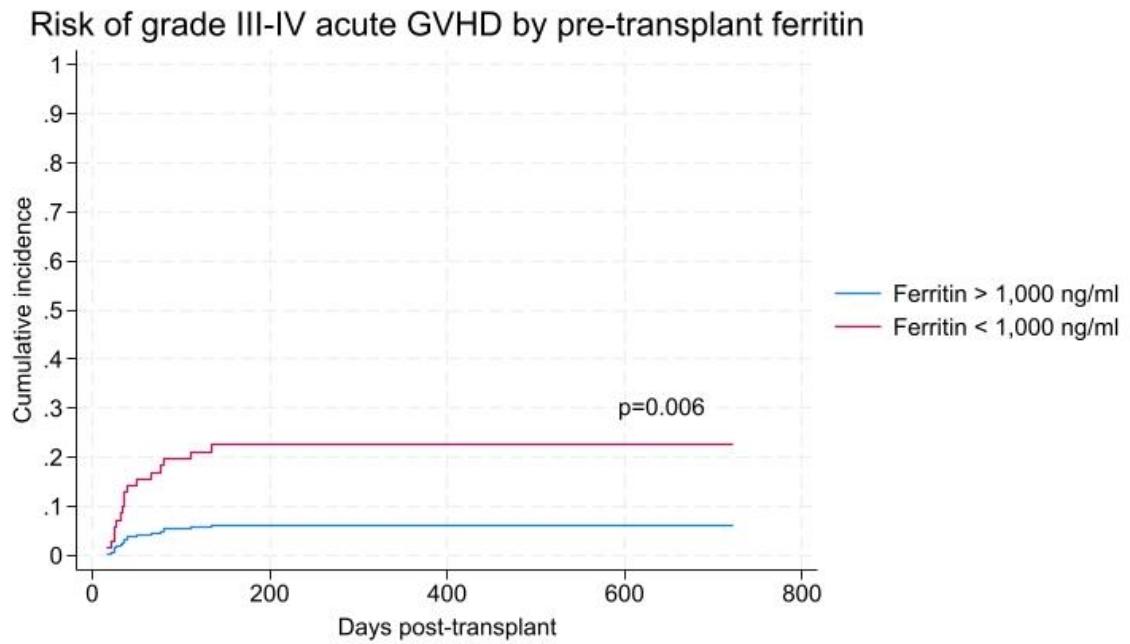


Figure 93: Cumulative incidence of grade III-IV acute GVHD by pre-transplant ferritin at a cut-off of 1,000 ng/ml.

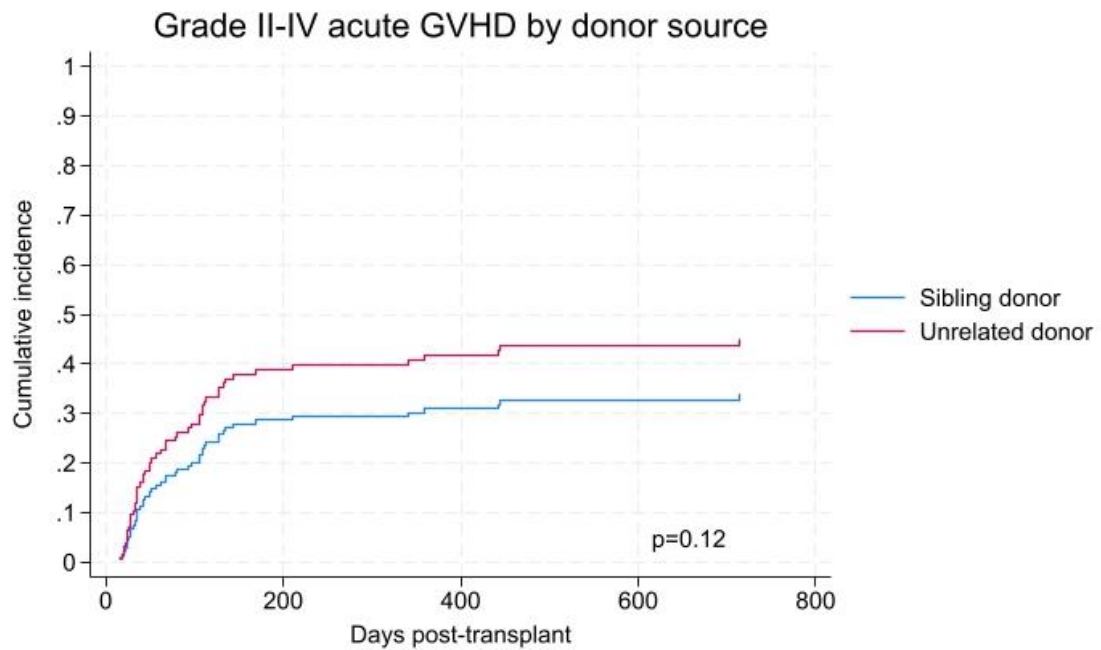


Figure 94: Cumulative incidence of grade II-IV acute GVHD by donor source, sibling vs. unrelated.

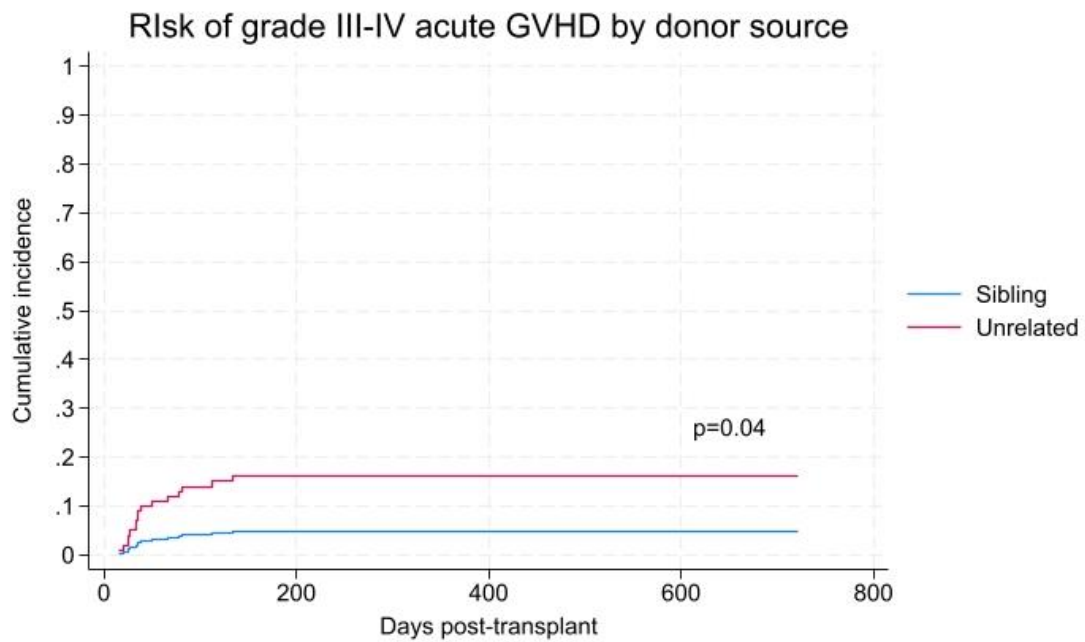


Figure 95: Cumulative incidence of grade III-IV acute GVHD by donor source: sibling vs. unrelated.

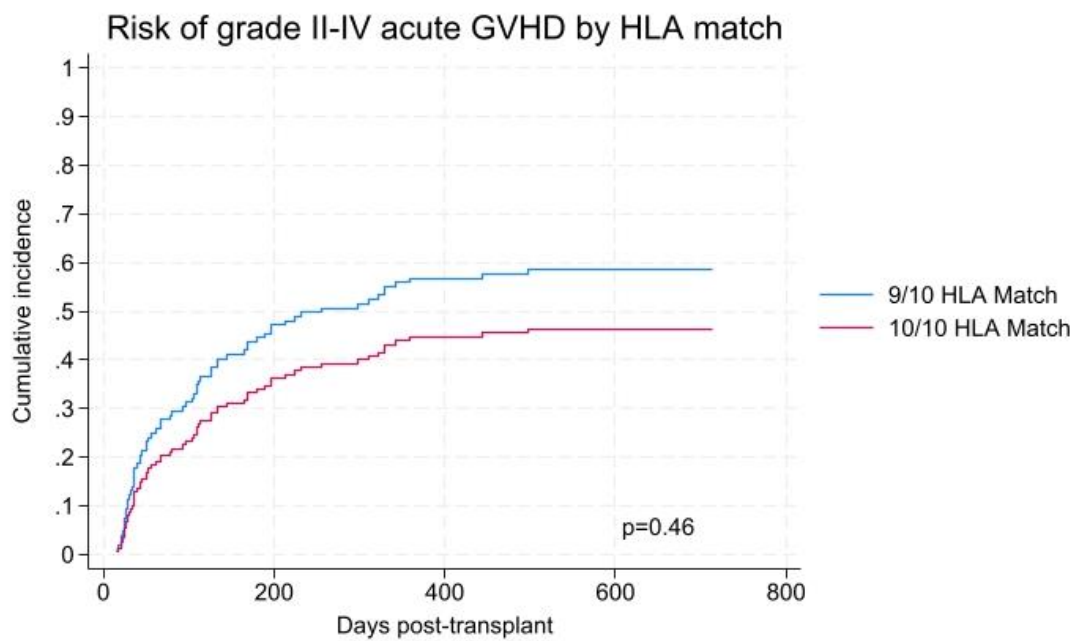


Figure 96: Cumulative incidence of grade II-IV acute GVHD by HLA matching, 9/10 vs. 10/10.

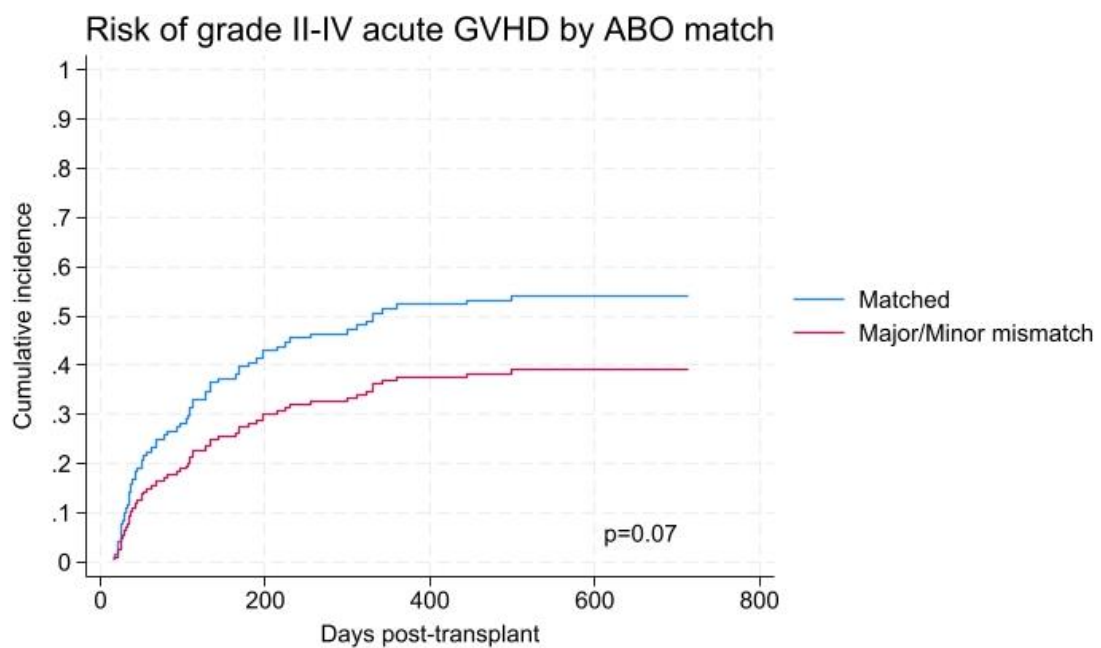


Figure 97: Cumulative incidence of grade II-IV acute GVHD by ABO matching.

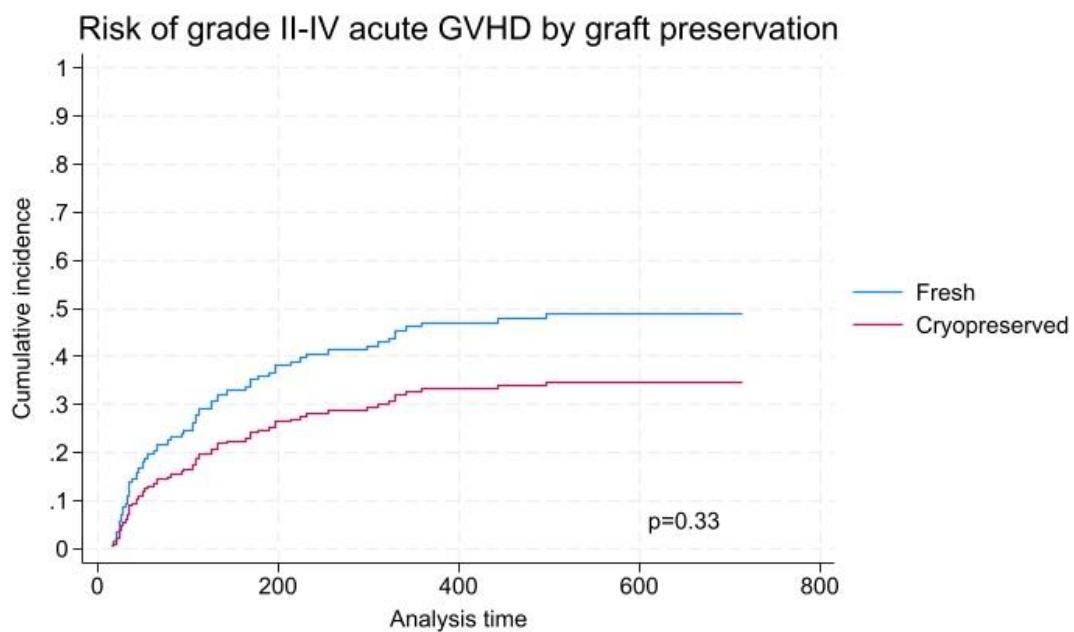


Figure 98: Cumulative incidence of grade II-IV acute GVHD by graft preservation: fresh vs. cryopreserved.

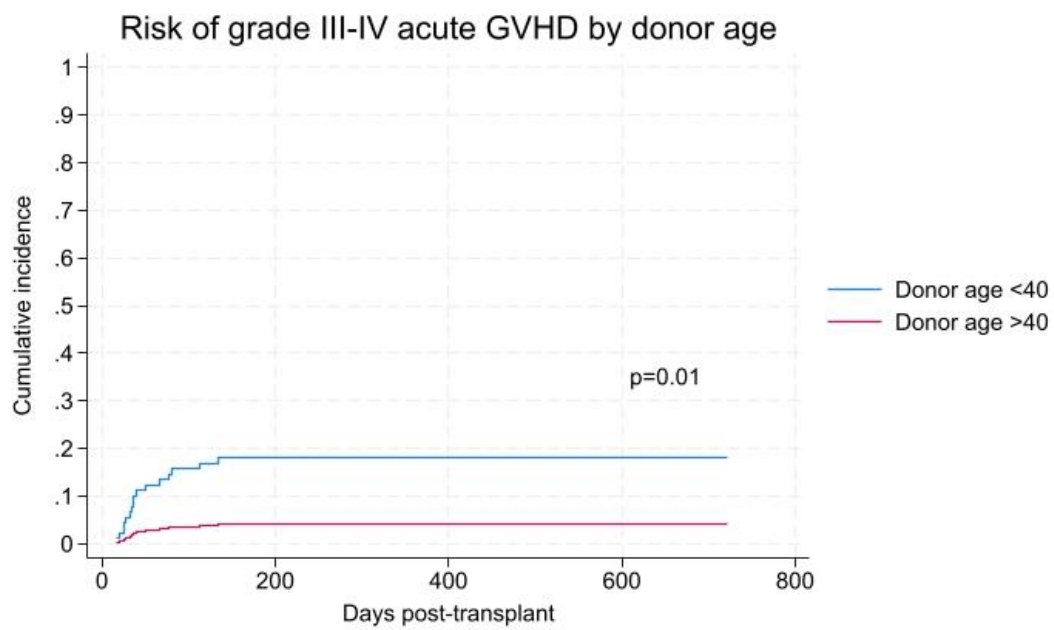


Figure 99: Cumulative incidence of grade III-IV acute GVHD by donor age above and below 40.

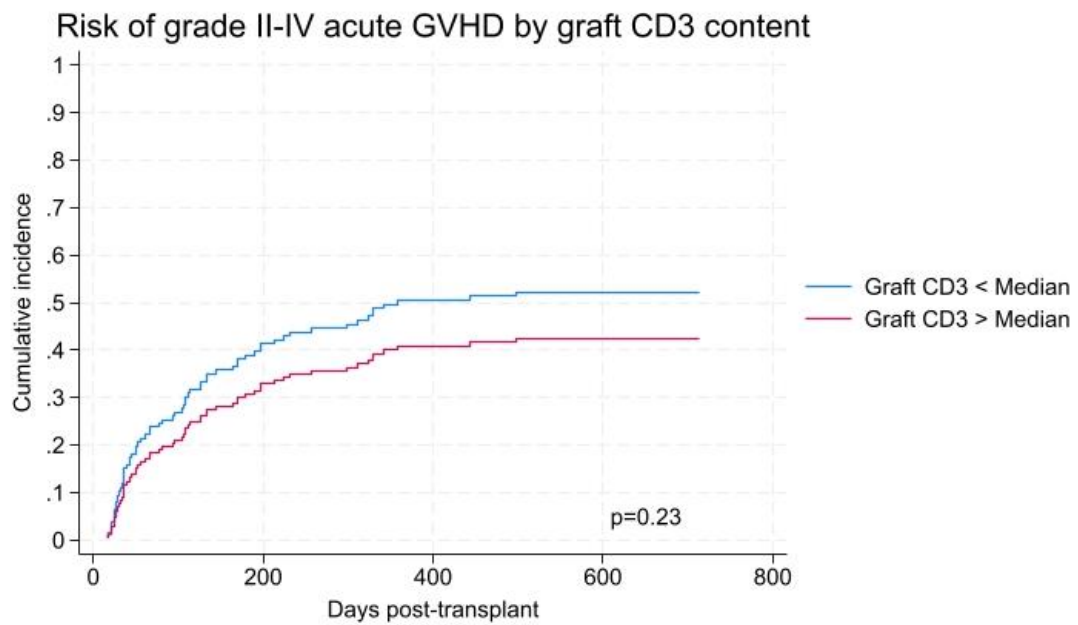


Figure 100: Cumulative incidence of grade II-IV acute GVHD by graft CD3⁺ T cell content above and below median of $17.14 \times 10^7/\text{kg}$.

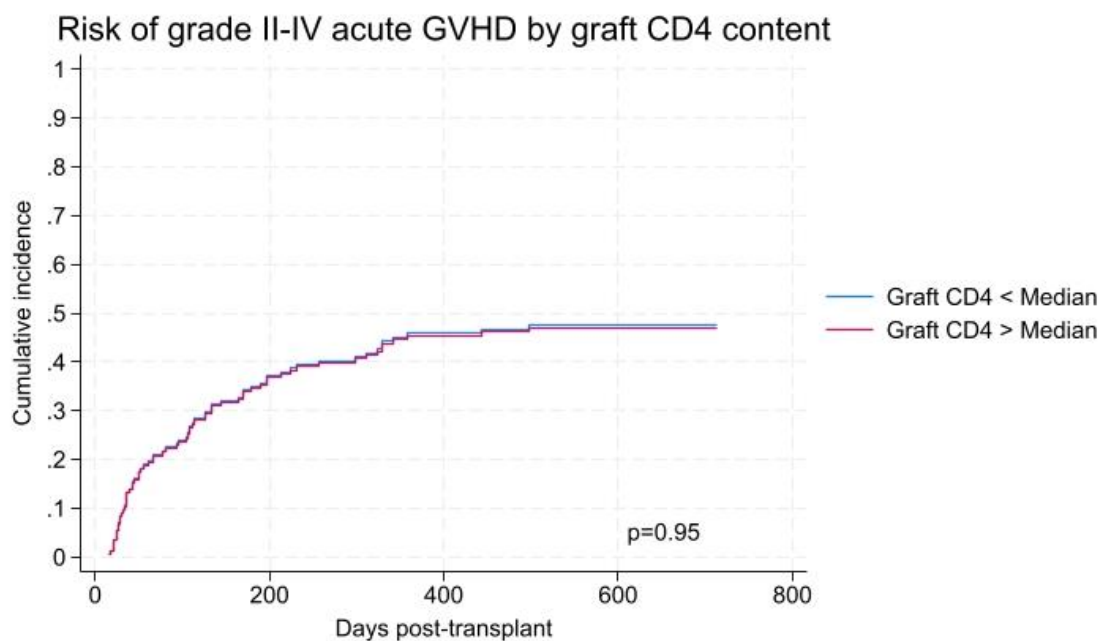


Figure 101: Cumulative incidence of grade II-IV acute GVHD by graft CD4⁺ T cell content above and below median of $11.09 \times 10^7/\text{kg}$.

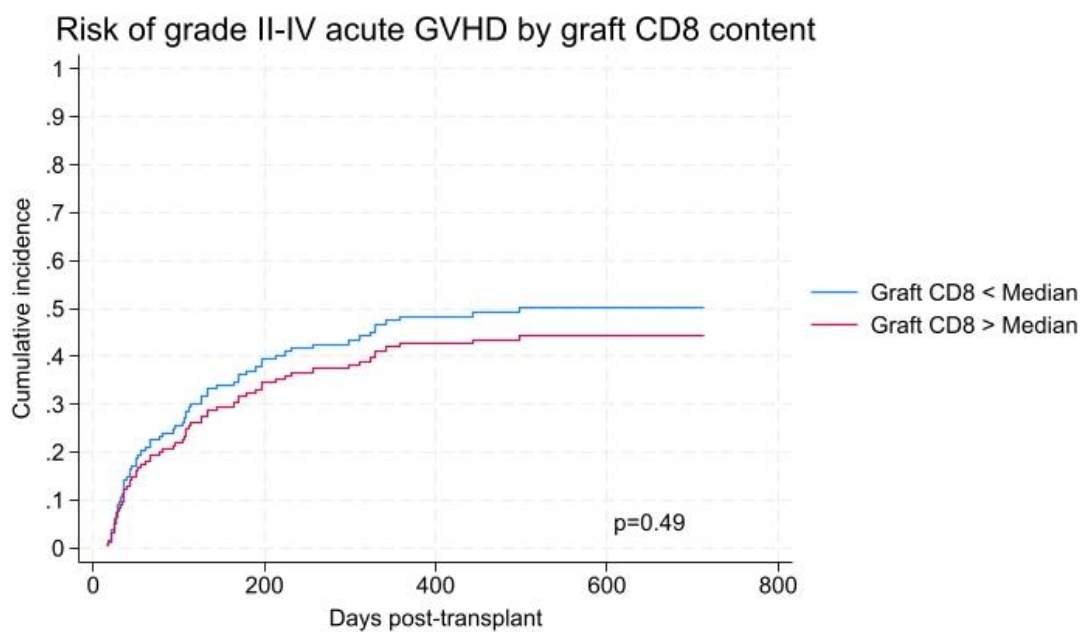


Figure 102: Cumulative incidence of grade II-IV acute GVHD by graft CD8⁺ T cell content above and below median of $6.37 \times 10^7/\text{kg}$.

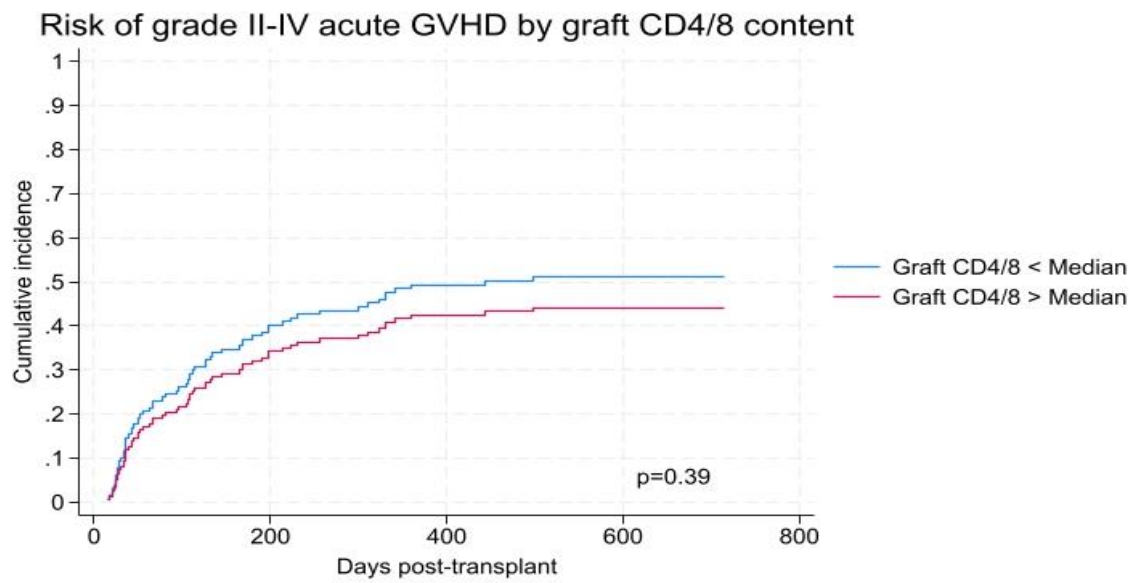


Figure 103: Cumulative incidence of grade II-IV acute GVHD by graft CD4⁺/CD8⁺ T cell content above and below median of $0.24 \times 10^7/\text{kg}$.

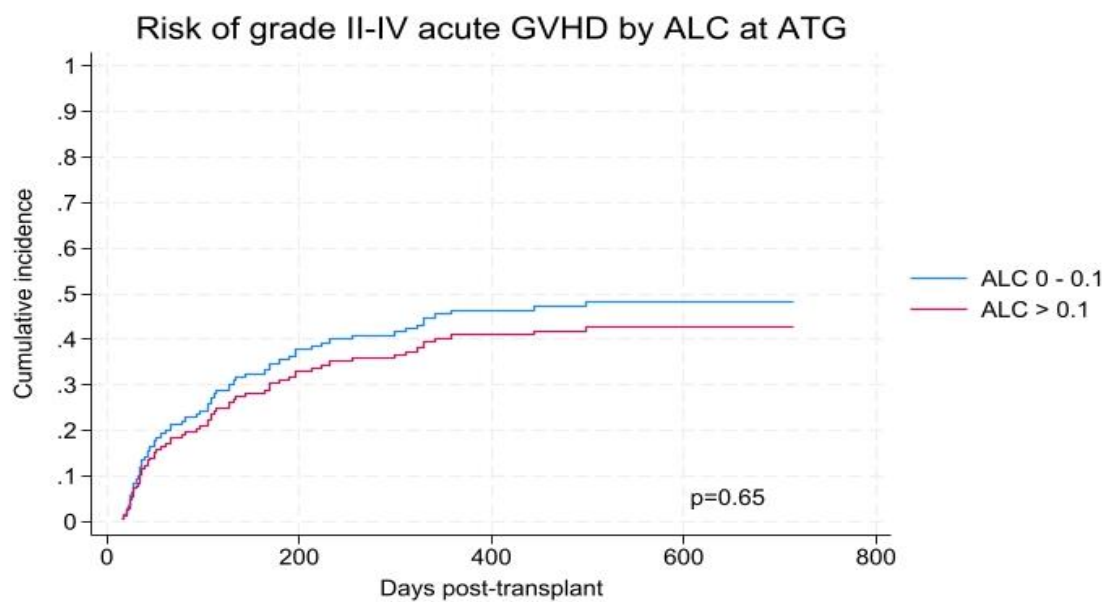


Figure 104: Cumulative incidence of grade II-IV acute GVHD by absolute lymphocyte count on first day of ATG exposure.

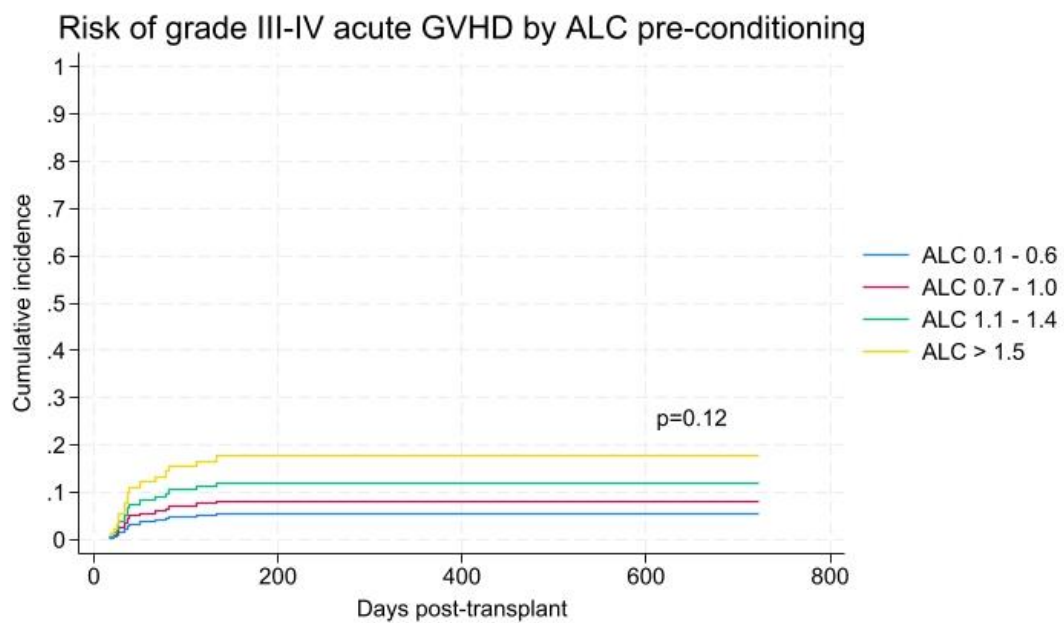


Figure 105: Cumulative incidence of grade III-IV acute GVHD by absolute lymphocyte count pre-conditioning, cut-offs 0.1-0.6, 0.7-1.0, 1.0-1.4 and 1.5+ $\times 10^9/L$.

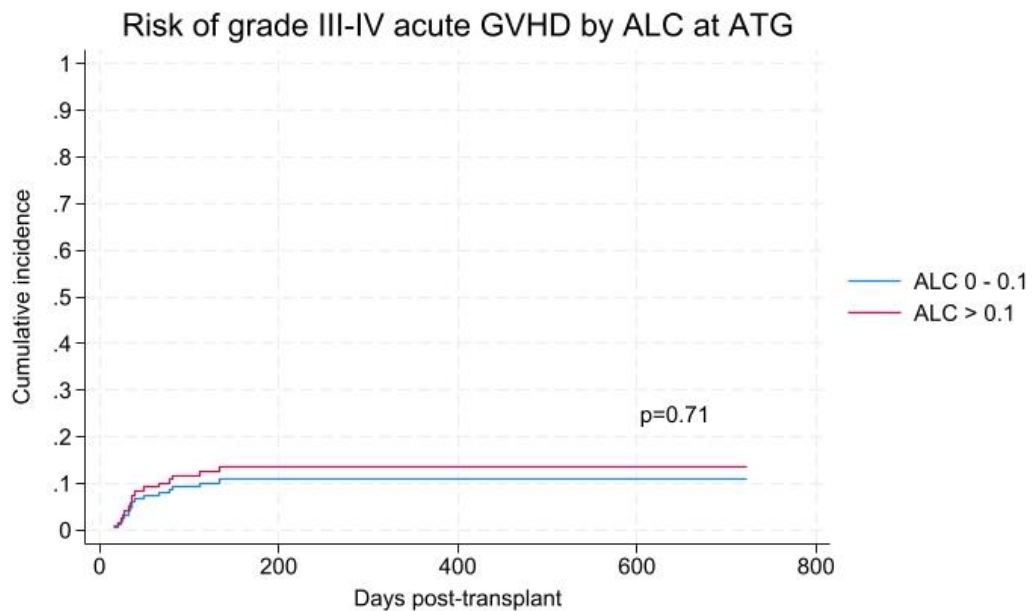


Figure 106: Cumulative incidence of grade III-IV acute GVHD by absolute lymphocyte count on first day of ATG exposure.

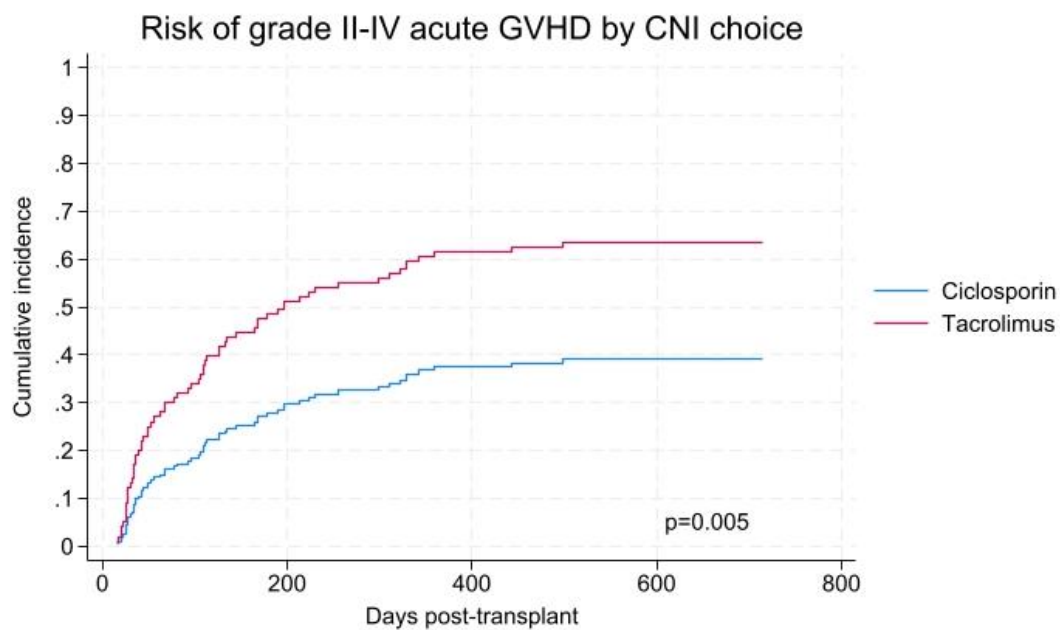


Figure 107: Cumulative incidence of grade II-IV acute GVHD by calcineurin inhibitor choice; ciclosporin vs. tacrolimus.

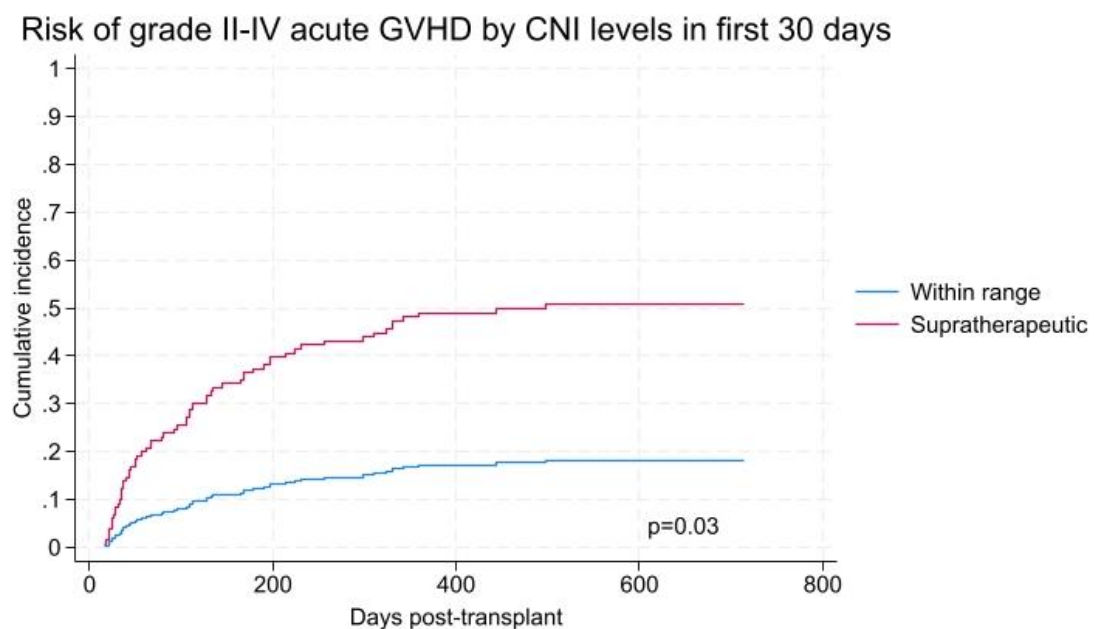


Figure 108: Cumulative incidence of grade II-IV acute GVHD by calcineurin levels in the first 30 days of transplant; on target vs. supratherapeutic.

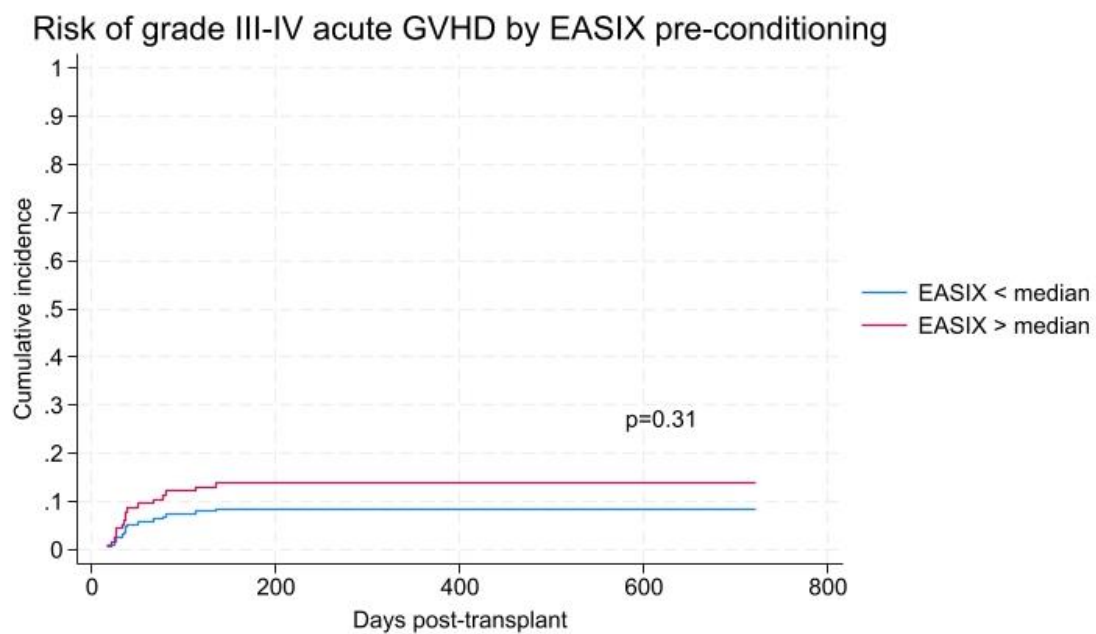


Figure 109: Cumulative incidence of grade III-IV acute GVHD by EASIX score above and below median of 1.89 at pre-transplant time-point.

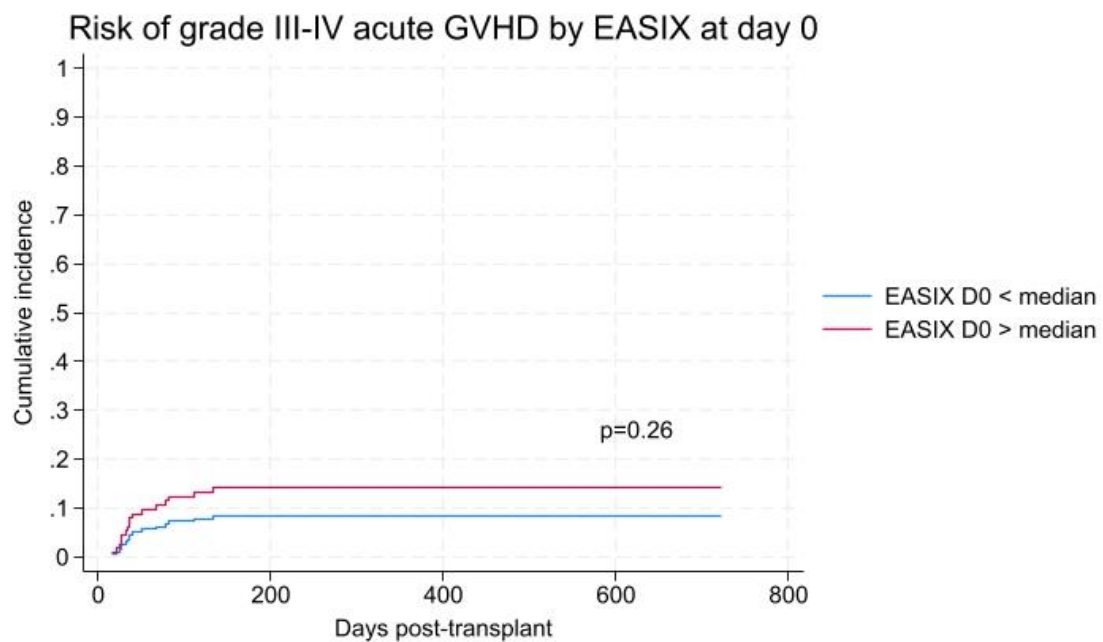


Figure 110: Cumulative incidence of grade III-IV acute GVHD by EASIX score at day 0 time-point above and below median of 11.54.

Table 12: Acute GVHD (Grade II-IV)

Variable	Hazard ratio	95% CI	P value
Female recipient	0.90	0.52 - 1.48	0.68
Recipient age	1.03	0.74 - 1.41	0.49
Recipient age < 60	1.07	0.66 - 1.73	0.78
Recipient age > 65	0.82	0.47 - 1.43	0.49
Disease class	0.95	0.71 - 1.29	0.75
Unrelated donor	1.08	0.67 - 1.75	0.74
Pre-transplant ferritin > 1,000 ng/ml	1.05	0.62 - 1.79	0.84
HLA mismatch	1.96	0.87 - 4.42	0.10
ALC at first ATG > 0.1 x 10⁹/L	0.85	0.41 - 1.74	0.65
ALC pre-conditioning	1.11	0.9 - 1.38	0.33
Tacrolimus (vs. Cyclosporin)	2.02	1.24 - 3.32	0.005
CNI toxicity in first 30 days	3.55	1.07 - 11.7	0.03
Fresh graft vs. cryopreserved	0.64	0.25 – 1.61	0.33
ABO matched	0.63	0.38 – 1.05	0.07
Male donor	1.03	0.63 – 1.7	0.88
Female donor, male recipient	1.04	0.63 – 1.69	0.88
Donor age < 25	1.27	0.69 – 2.32	0.45
Donor age < 40	1.14	0.70 – 1.81	0.61

Table 13: Acute GVHD Grade II-IV EASIX Modelling

Variable	Hazard ratio	95% CI	P value
EASIX Pre-transplant	0.94	0.86 – 1.02	0.19
Mean	0.86	0.49 – 1.47	0.57
Mean + 1 SD	0.53	0.24 – 1.19	0.12
Median	0.79	0.48 – 1.28	0.34
25th Centile	0.71	0.43 – 1.18	0.19
75th Centile	0.93	0.52 – 1.64	0.80
EASIX Day 0	0.99	0.98 – 1.01	0.78
Mean	1.03	0.61 - 1.73	0.92
Mean + 1 SD	1.03	0.27 – 3.97	0.97
Median	0.95	0.58 – 1.54	0.82
25th Centile	0.71	0.43 – 1.18	0.19
75th Centile	1.14	0.64 – 2.03	0.64

Table 14: Acute GVHD Grade II-IV Graft T cell content

Variable	Hazard ratio	95% CI	P value
Graft CD3⁺	0.98	0.96 – 1.01	0.06
Mean	0.81	0.48 – 1.34	0.40
Mean + 1 SD	0.65	0.28 – 1.48	0.30
Median	0.77	0.46 – 1.21	0.23
25th Centile	0.33	0.45 – 1.30	0.33
75th Centile	0.42	0.45 – 1.39	0.42
Graft CD4⁺	0.97	0.95 – 1.01	0.13
Mean	0.87	0.52 – 1.43	0.59
Mean + 1 SD	0.86	0.52 – 1.44	0.58
Median	0.98	0.61 – 1.59	0.95
25th Centile	0.74	0.44 – 1.24	0.25
75th Centile	0.91	0.53 – 1.57	0.75
Graft CD8⁺	0.95	0.90 – 0.99	0.03
Mean	0.71	0.41 – 1.21	0.21
Mean + 1 SD	0.73	0.31 – 1.75	0.49
Median	0.84	0.52 – 1.37	0.49
25th Centile	0.68	0.41 – 1.13	0.14
75th Centile	0.60	0.32 – 1.12	0.10
Graft CD4⁺/CD8⁺	0.83	0.46 – 1.51	0.54
Mean	0.95	0.55 – 1.66	0.86
Mean + 1 SD	0.63	0.25 – 1.60	0.33
Median	0.81	0.50 – 1.31	0.39
25th Centile	-	-	-
75th Centile	1.00	0.57 – 1.74	0.99

Table 15: Acute GVHD (Grade III-IV)

Variable	Hazard ratio	95% CI	P value
Female recipient	0.49	0.16 – 1.53	0.22
Recipient age	1.27	0.70 – 2.32	0.42
Recipient age <60	1.74	0.63 – 4.79	0.27
Recipient age > 65	0.82	0.24 – 2.78	0.75
Disease class (MPN)	2.43	1.45 – 4.1	0.0008
Unrelated donor	3.49	1.01 – 12.13	0.04
Pre-transplant ferritin < 1,000 ng/ml	4.02	1.47 – 10.99	0.006
HLA mismatch	-	-	-
ALC at first ATG > 0.1 x 10 ⁹ /L	1.25	0.37 – 4.31	0.71
ALC pre-conditioning	1.51	0.88- 2.57	0.12
Tacrolimus (vs. Ciclosporin)	2.38	0.89 – 6.36	0.08
CNI toxicity in first 30 days	0.57	0.16 – 1.99	0.38
Fresh graft vs. cryopreserved	-	-	-
ABO matched	1.03	0.39 – 2.74	0.94
Male donor	1.14	0.43 – 3.07	0.78
Female donor, male recipient	1.62	0.48 – 5.43	0.43
Donor age < 25	2.04	0.74 - 5.55	0.16
Donor age < 40	4.54	1.33 – 16.66	0.01

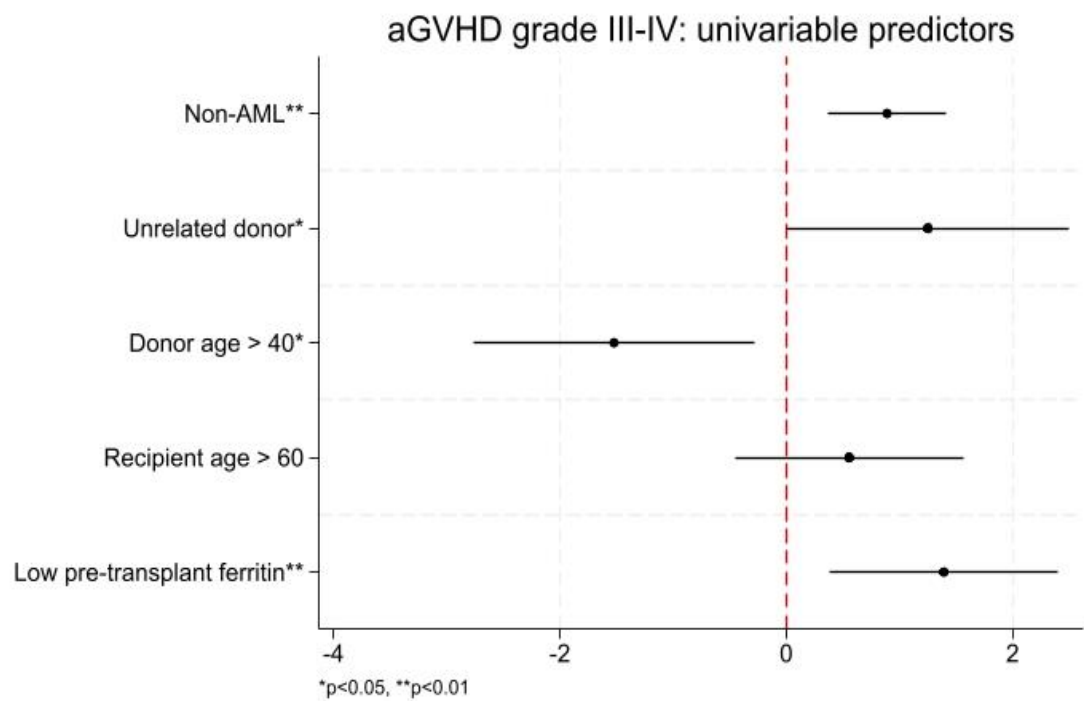


Figure 111: Coefficient plot for univariable predictors of acute grade III-IV GVHD.

Table 16: Acute GVHD Grade III-IV EASIX Modelling

Variable	Hazard ratio	95% CI	P value
EASIX Pre-transplant	1.09	0.98 – 1.21	0.12
Mean	2.26	0.85 – 5.99	0.10
Mean + 1 SD	0.89	0.21 – 3.82	0.89
Median	1.67	0.61 – 4.61	0.31
25th Centile	1.42	0.41 – 5.02	0.57
75th Centile	3.17	1.20 – 8.41	0.02
EASIX Day 0	1.01	0.99 – 1.02	0.09
Mean	2.57	0.97 – 6.86	0.054
Mean + 1 SD	1.36	0.16 - 11.11	0.77
Median	1.76	0.65 – 4.79	0.26
25th Centile	5.27	0.71 – 39.24	0.10
75th Centile	2.51	0.93 – 6.74	0.06

Table 17: Acute GVHD Grade III-IV Graft T cell content

Variable	Hazard ratio	95% CI	P value
Graft CD3⁺	<i>0.94</i>	<i>0.88 – 0.99</i>	<i>0.02</i>
Mean	0.39	0.11 – 1.36	0.13
Mean + 1 SD	-	-	-
Median	0.40	0.14 – 1.15	0.09
25th Centile	0.39	0.14 – 1.05	0.06
75th Centile	0.17	0.02 – 1.24	0.08
Graft CD4⁺	<i>0.91</i>	<i>0.83 – 0.99</i>	<i>0.03</i>
Mean	0.38	0.10 – 1.36	0.13
Mean + 1 SD	0.26	0.06 – 1.19	0.26
Median	0.41	0.14 – 1.16	0.41
25th Centile	0.67	0.23 – 1.93	0.67
75th Centile	0.17	0.02 – 1.29	0.17
Graft CD8⁺	<i>0.87</i>	<i>0.76 – 0.99</i>	<i>0.03</i>
Mean	0.37	0.11 – 1.31	0.12
Mean + 1 SD	-	-	-
Median	0.74	0.27 – 1.98	0.55
25th Centile	0.51	0.18 – 1.38	0.18
75th Centile	0.37	0.08 – 1.64	0.19
Graft CD4⁺/CD8⁺	0.33	0.06 – 1.87	0.21
Mean	0.56	0.15 – 2.01	0.37
Mean + 1 SD	-	-	-
Median	0.65	0.24 – 1.75	0.39
25th Centile	-	-	-
75th Centile	0.58	0.16 – 2.09	0.40

Chronic GVHD

24 patients developed chronic GVHD in this cohort (Fig. 112). 13 patients had previously developed acute GVHD prior to onset of chronic GVHD and most of these patients experienced a continuous evolution from acute into chronic disease. 2 patients developed chronic GVHD without prior diagnosed acute GVHD. 9 patients developed chronic GVHD after a DLI. The median time to chronic GVHD onset was 187 days, range 100 - 1357.

Using the NIH grading criteria, 11 patients had limited and mild disease, 4 had moderate and limited disease and 4 had limited and severe disease. A further 5 had extensive and severe disease. The risk of developing chronic GVHD was not affected by demographic variables, including sex, age, or weight. Similarly, there was no difference in incidence based on disease class, disease risk profile, disease status at transplant or prior treatment.

Graft characteristics did not correlate with the risk of developing chronic GVHD, including donor source (unrelated vs. sibling), donor age, HLA matching (9/10 match vs. 10/10 match), ABO matching (minor or major) and cryopreservation prior to stem cell infusion. Male recipients with female donors were at increased risk of chronic GVHD ($p=0.03$) (Fig. 113). For graft CD3⁺, CD4⁺ and CD4⁺/CD8⁺ T cell content, only the highest graft content levels were associated with chronic GVHD risk, and for CD8⁺ content there was no association.

The dose intensity of ATLG (10mg/kg vs. 20mg/kg) did not affect the risk of developing chronic GVHD. The recipient absolute lymphocyte count on the first day of ATG infusion at day minus 3 did not correlate with this outcome, nor did the ALC at the pre-conditioning time-point. Rates of chronic GVHD were similar in those who received ciclosporin or tacrolimus for GVHD prophylaxis. Volatile calcineurin inhibitor levels in the first month post-transplant did not affect the risk of subsequent chronic GVHD. The EASIX score at the pre-conditioning and day zero time points were not predictive of the risk of subsequent chronic GVHD. The EASIX score at the onset of spontaneous acute GVHD was similarly not predictive of the risk of later developing chronic disease.

There was a trend towards the development of chronic GVHD in those who developed acute GVHD of any grade ($p=0.06$) and a strong association between those who developed grade 2 to 4 acute GVHD ($p=0.004$) and grade 3-4 acute GVHD ($p<0.001$) (Fig. 114 and 115). Chimerism status at day 90 did not affect the risk of developing chronic GVHD, with similar rates in those with mixed and full donor chimerism. Absolute lymphocyte recovery at day 100 or 180 did not predict chronic GVHD, and similarly, T cell subset recovery (CD3⁺, CD4⁺ and CD8⁺) in those with available data ($n=52$) was not predictive. Immunoglobulin recovery by IgG, IgA or IgM levels at these time points was also not predictive of chronic GVHD. Univariable predictors of chronic GVHD incidence, associations with EASIX scores and graft T cell content are summarized in table 18 – 20.

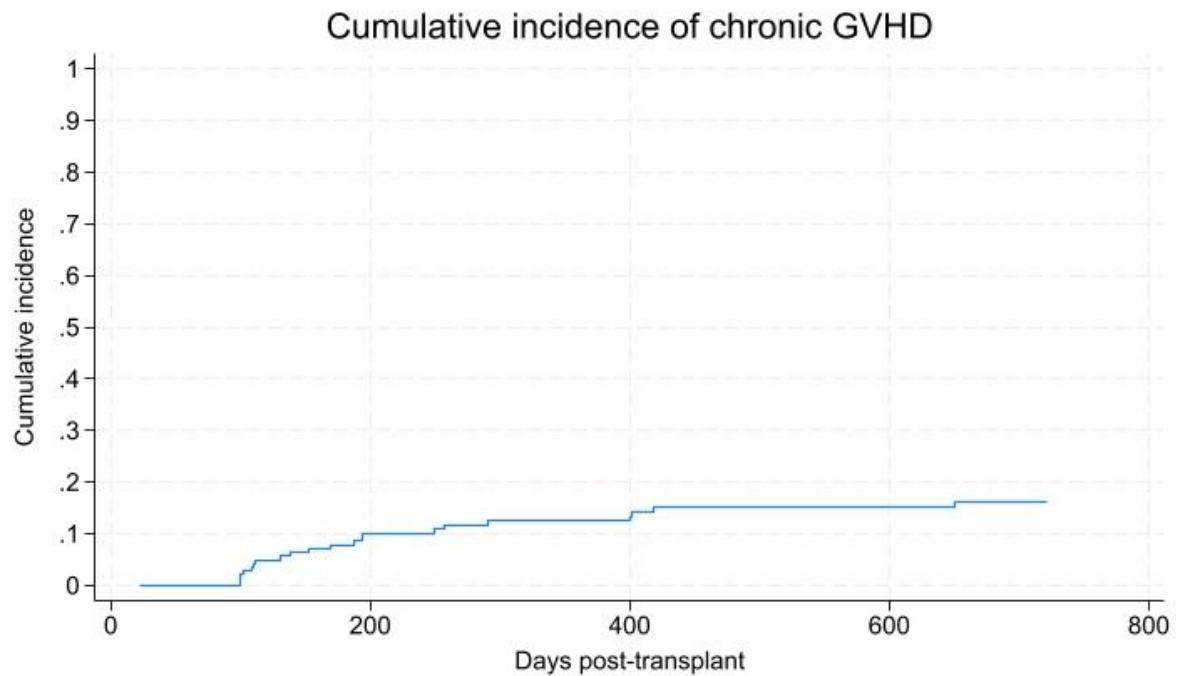


Figure 112: Cumulative incidence of chronic GVHD.

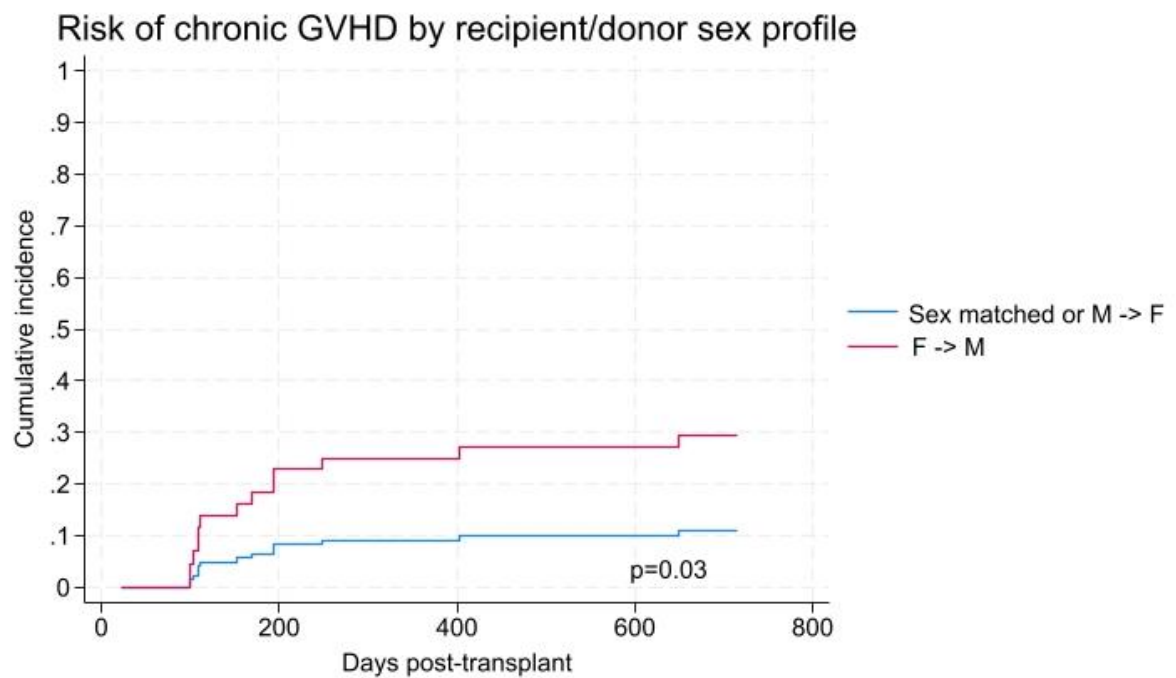


Figure 113: Cumulative incidence of chronic GVHD by donor sex vs. recipient sex; female donor to male recipient.

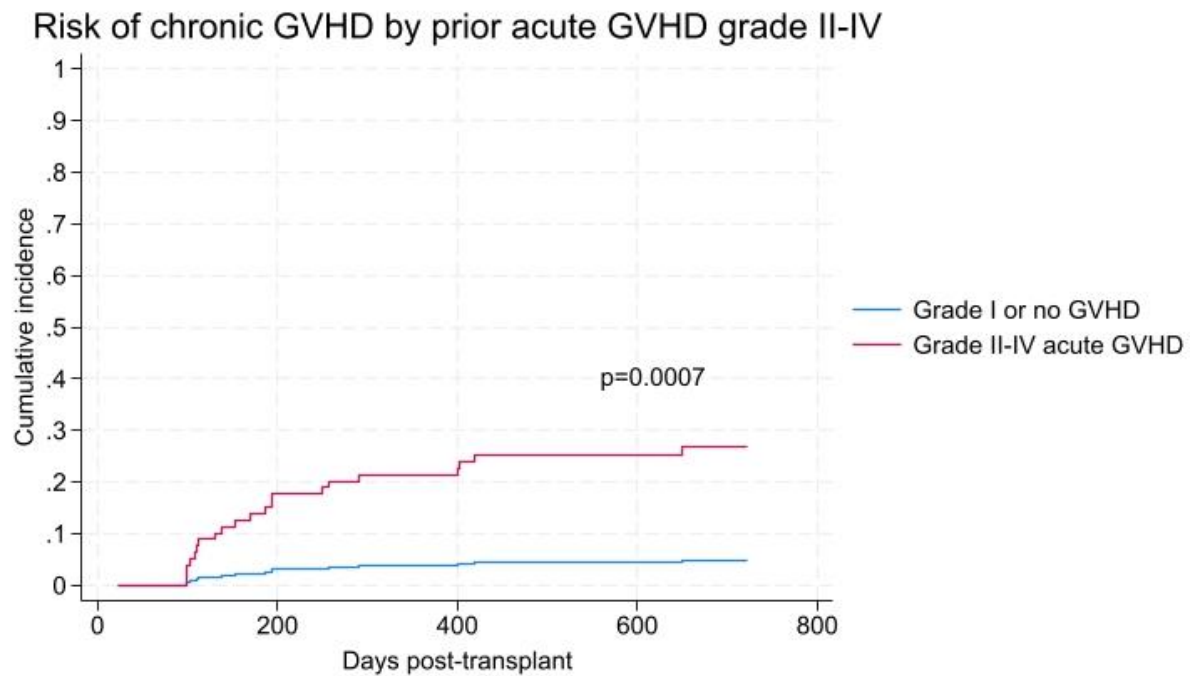


Figure 114: Cumulative incidence of chronic GVHD in those who developed prior acute GVHD grade II-IV.

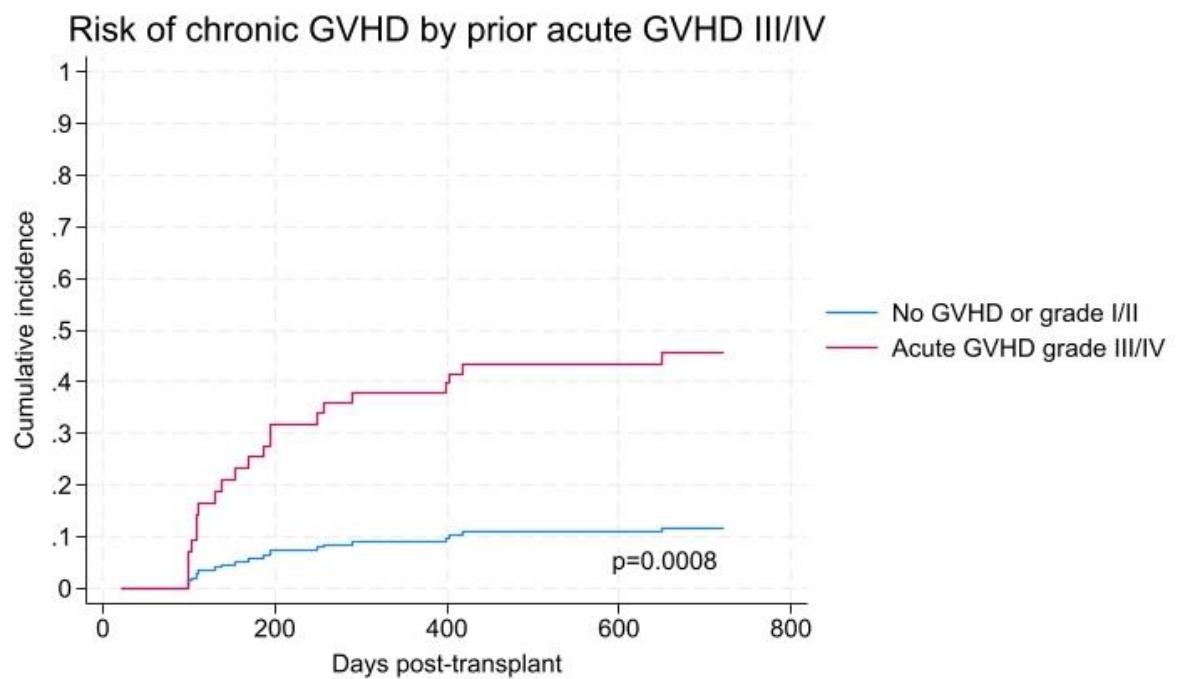


Figure 115: Cumulative incidence of chronic GVHD in those who developed prior acute GVHD grade III-IV.

Table 18: Chronic GVHD

Variable	Hazard ratio	95% CI	P value
Male recipient	0.80	0.34 – 1.87	0.60
Recipient age	1.06	0.62 – 1.81	0.82
Recipient age < 60	1.16	0.52 – 2.60	0.70
Recipient age > 65	0.81	0.30 – 2.13	0.67
Disease subtype	1.19	0.73 – 1.94	0.48
Pre-transplant ferritin < 1,000 ng/ml	1.78	0.78 – 4.04	0.17
Sibling vs. unrelated donor	0.69	0.31 – 1.55	0.36
HLA mismatch	0.57	0.07 - 4.45	0.59
ALC at first ATLG > 0.1 x 10⁹/L	1.57	0.59 – 4.19	0.36
ALC pre-conditioning	1.03	0.72 – 1.50	0.84
Tacrolimus (vs. Ciclosporin)	1.42	0.62 – 3.26	0.40
Fresh graft vs. cryopreserved	0.78	0.18 – 3.22	0.73
ABO matched	0.77	0.34 – 1.73	0.52
Female donor, male recipient	2.99	1.10 – 8.19	0.03
Donor age < 25	1.02	0.37 – 2.82	0.96
Donor age < 40	1.28	0.56 – 2.91	0.55

Table 19: Chronic GVHD EASIX Modelling

Variable	Hazard ratio	95% CI	P value
EASIX Pre-transplant	0.98	0.86 – 1.12	0.86
Mean	1.24	0.52 – 2.94	0.63
Mean + 1 SD	0.27	0.34 – 2.15	0.21
Median	0.94	0.42 – 2.14	0.89
25th Centile	0.91	0.35 – 2.30	0.83
75th Centile	1.44	0.59 – 3.52	0.42
EASIX Day 0	0.97	0.94 – 1.01	0.17
Mean	0.82	0.34 – 1.99	0.66
Mean + 1 SD	-	-	-
Median	0.37	0.15 – 0.93	0.03
25th Centile	0.93	0.36 – 2.35	0.87
75th Centile	0.69	0.30 – 2.22	0.69
EASIX at GVHD onset	0.89	0.70 – 1.14	0.36
Mean	0.45	0.13 – 1.63	0.22
Mean + 1 SD	0.73	0.78 – 6.86	0.78
Median	0.45	0.18 – 1.12	0.08
25th Centile	0.48	0.22 – 1.09	0.08
75th Centile	0.45	0.12 – 1.62	0.22

Table 20: Chronic GVHD Graft T cell content

Variable	Hazard ratio	95% CI	P value
Graft CD3⁺	<i>1.02</i>	<i>1.01 – 1.03</i>	<i>0.03</i>
Mean	1.21	0.52 – 2.79	0.66
Mean + 1 SD	1.83	0.69 – 4.84	0.22
Median	1.42	0.62 – 3.24	0.39
25th Centile	0.99	0.37 – 2.60	0.98
75th Centile	1.01	0.41 – 2.54	0.97
Graft CD4⁺	<i>1.03</i>	<i>1.01 – 1.06</i>	<i>0.03</i>
Mean	1.82	0.81 – 4.09	0.14
Mean + 1 SD	0.92	0.38 – 2.22	0.86
Median	1.99	0.85 – 4.68	0.11
25th Centile	0.82	0.33 – 2.00	0.67
75th Centile	1.06	0.42 – 2.67	0.89
Graft CD8⁺	1.03	0.99 – 1.07	0.11
Mean	1.45	0.63 – 3.30	0.37
Mean + 1 SD	1.76	0.62 – 5.04	0.28
Median	0.97	0.43 – 2.19	0.94
25th Centile	0.68	0.28 – 1.69	0.41
75th Centile	1.36	0.57 – 3.29	0.48
Graft CD4⁺/CD8⁺	<i>1.38</i>	<i>1.15 – 1.70</i>	<i>0.001</i>
Mean	0.96	0.38 – 2.41	0.93
Mean + 1 SD	1.31	0.42 – 4.18	0.63
Median	2.08	0.85 – 5.11	0.10
25th Centile	-	-	-
75th Centile	0.99	0.39 – 2.50	0.99

EASIX

The median EASIX score at the pre-conditioning time point (n=143) was 1.89, with a range of 0.3 to 17.54 (Fig. 12). The median EASIX score at day 0 (n=143) was 11.54, with a range of 1.07 to 180.09. The median EASIX score at the onset of GVHD (n=85) was 1.97, with a range of 0.44 to 51.43. The median EASIX score at 1 year post transplant (n=106) was 1.24, with a range of 0.47 to 19.34.

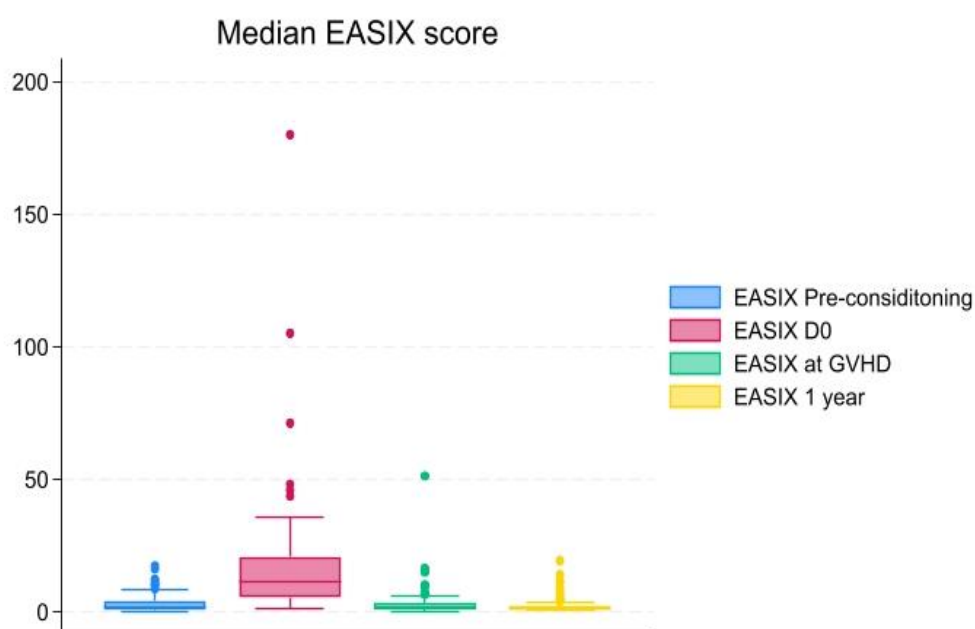


Figure 116. Median EASIX scores at pre-conditioning, day 0, GVHD onset and 1-year post-transplant.

Table 21: EASIX Modelling						
	Number	Mean	+1 SD	Median	25 th cent.	75 th cent.
Pre- transplant	143	3.04	6.14	1.89	1.02	4.14
Day 0	143	15.81	34.99	11.54	5.69	20.68
At GVHD	84	3.35	9.36	1.97	1.15	3.31
At 1 year	106	2.23	5.07	1.24	0.92	2.16

Best response and chimerism

Data on response post-transplant were available on 133 patients. 102 achieved a CR at least once post-transplant, with a further 14 achieving CR with incomplete haematological recovery. 12 patients had refractory disease, 2 had progressive disease, 2 had clinical improvement and 1 had stable disease.

In the AML subcohort, there were 59 CRs, 5 CR with incomplete haematological recovery, 5 refractory disease and 3 not available for assessment. In the MDS subcohort, there were 27 CRs, 4 CR with incomplete haematological recovery, 3 with refractory disease, 2 with progressive disease and 4 not available for assessment. In the MPN cohort there were 16 CRs, 5 CR with incomplete haematological recovery, 4 with refractory disease, 2 clinical improvement, 1 stable disease and 3 not available for assessment.

Chimerism data was recorded at day 45 and day 90 post-transplant in all patients alive at those time-points. Further chimerism analysis was performed based on these results and in post-DLI patients. At the day 45 time-point, 136 patients had a valid result for total chimerism, and 121 for the CD3⁺ lymphoid fraction. 69/136 patients had a 100% total donor chimerism at day 45, with 103/136 having a donor chimerism above 97%, indicating full donor chimerism based on test sensitivity. 27/121 patients had a 100% CD3⁺ lymphoid donor chimerism at day 45, with 71/121 having a donor chimerism above 97%, indicating full donor chimerism.

At day 90, 132 patients and 128 patients had recorded total and CD3⁺ lymphoid chimerism analysis results respectively. 62/132 had a 100% total donor chimerism at day 90, with 93/132 having a donor chimerism above 97%, indicating full donor chimerism. 40/128 patients had a 100% CD3⁺ lymphoid donor chimerism at day 90, with 65/128 having a donor chimerism above 97%, indicating full donor chimerism.

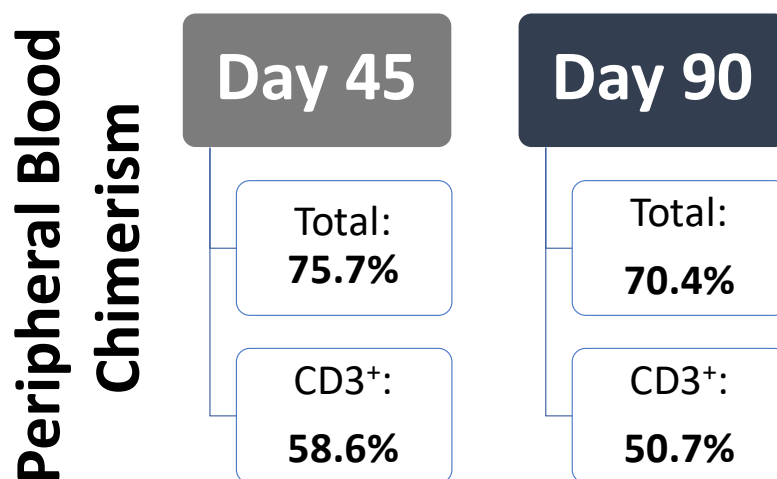


Figure 117: Chimerism status at day 45 and day 90.

Donor lymphocyte infusion

54 patients received a DLI and the total number of DLI doses for this cohort was 97. The median number of doses per patient was 1.5 with a range of 1-5. The median time to first DLI was 190.5 days, with a range of 98 to 1807. 2 patients received a DLI in remission with full donor chimerism (prophylactic). 36 received a DLI in remission with a mixed donor chimerism (pre-emptive). 16 received a DLI as part of a salvage regimen following post-transplant relapse, most often in combination with the hypomethylating agent azacitidine (therapeutic).

The most frequent starting dose was $0.1 \times 10^7/\text{kg}$ in the pre-emptive/prophylactic cohort, and $0.3 \times 10^7/\text{kg}$ in the therapeutic cohort. 28 of 54 patients developed post-DLI acute GVHD, of which 25 had not experienced prior spontaneous GVHD. Of the 36 patients with a mixed donor chimerism who received a pre-emptive DLI, 28 subsequently achieved a full donor chimerism in both total and CD3^+ compartments. 32 of 54 patients demonstrated an increase in donor chimerism percentage after DLI for any indication and 13 demonstrated a refractory reduction. Of the 13 who had a further loss of donor chimerism, 10 did not develop post-DLI GVHD, and 3 did. 9 of these 13 patients were in those who received a therapeutic DLI and 4 in those who received a pre-emptive DLI.

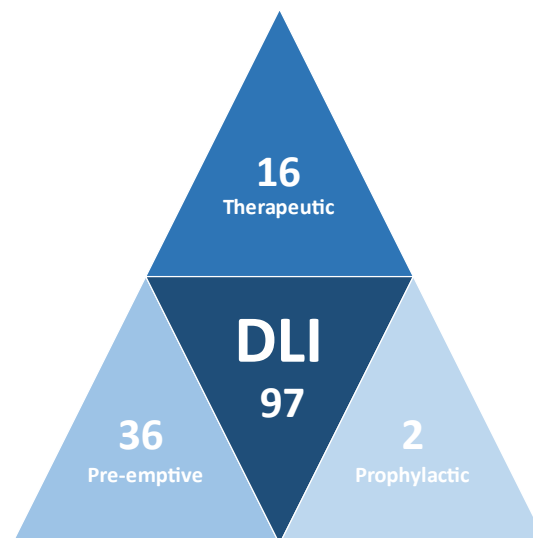


Figure 118: DLI indications.

Lymphocyte and T cell recovery

In the 133 patients alive at day 100, the median absolute lymphocyte count was 0.8, with a range of 0 to 3.7. T cell subset analysis was available on 52 patients at day 100. The median CD3⁺ at day 100 was 408 x 10⁶/L, range 39 - 3932. The median CD4⁺ at day 100 was 85 x 10⁶/L, range 7 – 541. The median CD8⁺ at day 100 was 266 x 10⁶/L, range 31 – 3380.

The median absolute lymphocyte count in 115 patients at day 180 was 1 x 10⁹/L, range 0.1 – 8.7. 86 patients had T cell subset analysis available at day 180 post-transplant. The median CD3⁺ count was 469 x 10⁶/L, range 70 – 6393. The median CD4⁺ count was 118 x 10⁶/L, range 6 – 559. The median CD8⁺ count was 325 x 10⁶/L, range 29 – 6306.

At 1 year, the median absolute lymphocyte count in 103 patients was 1.1 x 10⁹/L, range 0.2 – 7.1. 90 patients had T cell subset analysis available at this time point. The median CD3⁺ count was 563 x 10⁶/L, range 58 – 5080. The median CD4⁺ count was 164 x 10⁶/L, range 27 – 690. The median CD8⁺ count was 340 x 10⁶/L, range 29 – 4601.

The attainment of a CD4 count above 50 or 100 at day 100 post-transplant was no different between ATLG dose groups or in those who developed acute GVHD, however the numbers with available T cell subset results were limited. A trend towards reduced attainment of a CD4⁺ count above 50 by day 180 was seen in those who developed early acute GVHD, but only in those who developed grade II – IV disease and required steroids (p=0.07). Graft T cell content in CD3⁺, CD4⁺, CD8⁺ and CD4⁺/CD8⁺ compartments did not correlate with CD4⁺ recovery by day 100 or day 180.

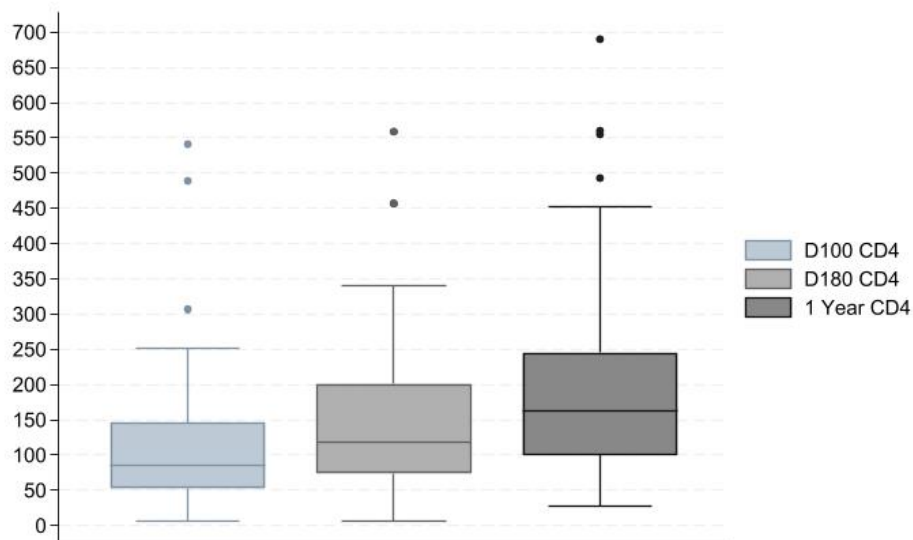


Figure 119: T cell subset analysis at day 100, 180 and 365 post-transplant.

Immunoglobulin recovery and monoclonal gammopathy

Assessment of immunoglobulin levels at day 100 post-transplant was performed in 70 patients. The standard reference range for immunoglobulins in the laboratory was 6.26 – 14.96 g/L for IgG, 0.62 – 2.9 g/L for IgA and 0.47 – 1.82 g/L for IgM. The median IgG level at day 100 was 7.94 g/L, with a range of 0.54 – 15.34. 21 patients had subnormal IgG levels. The median IgA level was 1.33 g/L, range 0.04 – 3.63. 13 patients had subnormal IgA levels. The median IgM level was 0.65 g/L, range 0.04 – 6.18. 22 patients had subnormal IgM levels.

92 patients had immunoglobulin levels assessed at day 180. The median IgG level was 8.24 g/L, range 2.17 – 27.43. 28 patients had subnormal IgG levels at this time-point. The median IgA level was 1.11 g/L, range 0.04 – 4.12. 26 patients had subnormal IgA level at this time-point. The median IgM level was 0.72 g/L, range 0.08 – 5.04. 22 patients had subnormal IgM levels at this time-point.

90 patients had immunoglobulin levels assessed at 1 year post transplant. The median IgG level was 8.67 g/L, range 1.12 – 38.39. 22 patients had subnormal IgG levels at this time-point. The median IgA level was 0.96 g/L, range 0.04 – 3.17. 30 patients had subnormal IgA levels at this time-point. The median IgM level was 0.75 g/L, range 0.04 – 3.18. 26 patients had subnormal IgM levels at this time-point.

Predictors of an incomplete immunoglobulin recovery by day 100 included the dose of ATLG – 10mg/kg vs. 20mg/kg ($p=0.01$), acute GVHD of any grade ($p<0.001$), grade II – IV acute GVHD ($p<0.001$) and tacrolimus use rather than ciclosporin ($p=0.001$).

39 patients developed a monoclonal gammopathy post-transplant, with a median quantified paraprotein of 3.1 g/L in 26 patients and a further 13 patients with paraprotein levels too low to quantify. No patients developed an overt plasma cell dyscrasia and most patients experienced only a transient monoclonal gammopathy which resolved on subsequent analysis.

Secondary malignancy

14 patients developed 15 secondary malignancies at last follow up, including skin squamous cell carcinoma ($n=3$), basal cell carcinoma ($n=3$), prostate adenocarcinoma ($n=2$), lung squamous cell carcinoma ($n=1$), lung carcinoid tumour ($n=1$), lung mesenchymal tumour ($n=1$), spinal ependymoma ($n=1$), pancreatic neuroendocrine tumour ($n=1$), adrenal Hodgkin lymphoma ($n=1$), and pancreatic adenocarcinoma ($n=1$).

Infectious Complications

CMV serostatus and reactivation

There were 89 CMV seropositive transplant recipients by pre-transplant serology and 54 were negative. There were also 89 CMV seropositive stem cell donors by IgG serology, and 54 were

negative. The recipient and donor pairings were 76 negative / negative pairs, 13 negative / positive, 41 positive / positive and 13 positive / negative. CMV re-activation as detected by routine twice-weekly PCR screening was identified in 45 patients. 23 patients had detectable virus loads but did not exceed the clinically significant threshold of 250 IU/ml. 22 patients required anti-viral treatment with ganciclovir or valganciclovir (n = 21) and the addition of foscarnet in 1 patient.

28/45 re-activations were in the positive / positive group (62.2% of all +/+), followed by 12/45 in the positive / negative group (92.3% and all +/-), 4 in the negative / negative group (0.05% of all -/-) and 1 in the negative / positive group (0.07% of all -/+).

39 patients had no complications of re-activation. 3 developed pneumonitis confirmed by PCR of a bronchoalveolar lavage, with one death. 1 patient developed biopsy-confirmed colitis. Routine CMV prophylaxis with letermovir in CMV seropositive recipients was only licensed and available in Ireland from late 2020.

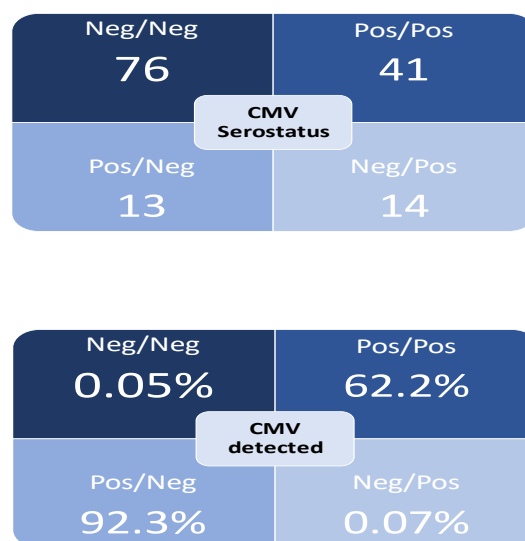


Figure 120: CMV serological pairs (left) and re-activation rates per pairing (right).

EBV serostatus and reactivation

140/143 patients were EBV seropositive pre-transplant. 128 donors were seropositive and 15 were negative. EBV re-activation by routine twice-weekly PCR monitoring was detected in 63 patients. 49 re-activations did not exceed the clinically significant threshold of 800 IU/ml and 14 patients had clinically significant levels on at least one occasion. These patients all received rituximab and 6 had radiological or histological evidence of post-transplant lymphoproliferative disorder (PTLD). No patients required subsequent chemotherapy for PTLD.

Adenovirus reactivation

Adenoviral re-activation as detected by routine post-transplant PCR monitoring was detected in 13 patients. 7 were less than the clinically significant PCR threshold of 800 IU/ml. Of the 6 patients with significant viral loads, 3 had no clinical symptoms, 2 died from pneumonitis and 1 developed diarrhoea and pneumonia but survived.

Opportunistic infection

40 patients had confirmed herpes simplex virus infection during the post-transplant course. No episodes of treatment resistant virus were seen with universal response to aciclovir and valaciclovir. No episodes of toxoplasma re-activation were seen in this cohort of patients. *Pneumocystis jirovecii* infection occurred in 3 patients. All patients were prescribed co-trimoxazole prophylaxis at the time of infection. All were treated with high dose co-trimoxazole. 2 patients recovered and 1 died from pneumonia. BK virus re-activation occurred in 16 patients and was treated with intravenous fluids, cidofovir or intravesical cidofovir. 51 patients developed a range of respiratory viral infections, including influenza A/B, parainfluenza, human metapneumovirus, respiratory syncytial virus, rhinovirus and in the latter phase of the observation period, Sars-CoV-2, with one death from the delta variant.

Invasive fungal infection

Invasive fungal disease prophylaxis was liposomal amphotericin B for 127 patients, caspofungin for 15 and voriconazole for 1. Invasive fungal disease occurred in 9 patients. A fungal species was identified in 3, with *aspergillus fumigatus* and *candida glabrata* seen. The remaining 6 patients had radiological probable disease but did not have microbiological confirmation. 3 patients were on steroids and calcineurin inhibitors at the time of infection, and the remainder were on calcineurin inhibitors alone. 5/9 patients were severely neutropenic at the onset of infection. 3/9 patients died from complications of the invasive fungal infection.

Discussion

Transplant activity and demographics

Transplant activity in the RIC setting for myeloid disease has increased steadily over the observation period of this study, from 18 per annum in 2015, to 34 by the end of the observation in 2021. This is in keeping with European wide trends showing increased overall transplant activity year on year. Our data mirror the parallel increase in the number of reduced intensity transplants undertaken as a proportion of all transplants. The age profile of transplant recipients in our cohort has increased from a median age of 56.5 years in 2015 to 61 in 2021 and continues to rise. The maximum age for transplant candidates in our centre increased to above 70 for the first time in 2021, paving the way for an increasing number of transplants above this age subsequently, which will be addressed in the following chapter.

The disease categories in our cohort broadly match those of general EBMT activity, with slightly less AML cases at 50% of the cohort compared to 58% in the EBMT survey, and 50% having MDS or MPN as compared to 42% (128). In AML, disease status at transplant matches international practice, with two thirds of patients receiving a transplant in first remission, and a third in second remission or beyond. Induction therapy in the AML cohort saw increasing diversification between the earlier phase of the observation study and censor date, with national reimbursement of additional targeted therapies (gemtuzumab ozagamicin: 2020, midostaurin: 2021) and alternative preparations (liposomal daunorubicin/cytarabine: 2021). Venetoclax was also available in combination with non-intensive induction therapy in the last year of the observation but was not yet licensed for combination with intensive therapy.

Disease classification and risk profiling

Disease classification and risk stratification changes occurred in the early part of the observation period and again shortly after the end of observation, with the advent of the 4th and 5th revision of the WHO Classification of Haematolymphoid Malignancies in 2016 and 2022 respectively, the ELN risk classification of AML in 2017 and further revision in 2022 and the ICC consensus classification of AML in 2022 (141, 142, 144, 145, 566). Although retrospective application of the most recent guidance results in some risk category changes within the cohort, all patients had already undergone transplantation, therefore the changes are of academic interest only. There are undoubtedly some contemporary patients who were considered as favourable and intermediate risk and did not receive an alloSCT in first remission, who would now be classified as higher risk primarily on molecular grounds and be considered for transplant in first remission. Although 3 patients were downgraded from high risk to intermediate risk by the change in the 2022 ELN guidelines, all but one patient was treated before the routine availability of *FLT3* inhibitors during induction, which underpins the basis of the revision of the status of this mutation.

Many of the changes to the disease classification systems in the AML cohort are categorical rather than disease defining, and with the information available from this cohort, would not have materially affected disease categorisation. The evolving role of molecular characterisation of disease for classification and stratification purposes is rapidly changing the approach to AML, with ever increasing understanding of the disease biology of these

malignancies and better predictors of response to therapy. Routine access to extended next generation sequencing based molecular panels was standard in the final years of the observation period, with comparably limited PCR-based assays of *FLT3* and *NPM1* and *TP53* only for those from the earlier phase of observation. Additionally, access to sensitive minimal residual disease monitoring by PCR and immunophenotyping may have informed some decisions to transplant in later years in the favourable risk category, where historically these patients arrived at transplant following first overt haematological relapse and salvage therapy. We applied the ELN 2017 risk stratification model for categorisation of our cohort as the model with the most complete information available and as the most reflective of transplant practice during the period of observation.

For MDS, many of the temporal considerations in access to diagnostics and therapy are comparable to the AML cohort and there have been some subsequent changes to disease classification. Changes in the nomenclature of disease classification were significant between the 2008 and 2022 versions of the WHO classification, although for transplant outcomes were broadly transferrable. The ICC 2022 recommendations on classification recategorize several MDS patients (17/40) as MDS/AML. To avoid confounding, our outcomes for this cohort were based on the WHO classification and all patients in this cohort with MDS with a blast count 10 – 19% were classified as MDS (*MDS with excess/increased blasts*). Risk stratification for MDS is similarly in recent evolution, with molecular based algorithms now gaining importance, such as the IPSS-M. Limited molecular data on the earlier cohort of patients preclude application of this tool to our cohort, so the IPSS-R (2012) was the most appropriate stratification tool.

In the MPN cohort, only minor changes in classification have occurred and would not reclassify any of those included. For patients with myelofibrosis and CMML, like those with MDS, there is a move towards disease risk stratification incorporating molecular data, such as the MIPSS-70 (myelofibrosis) and CPSS-Mol (CMML). Insufficient molecular data in the earlier phase of this cohort is available to accurately apply these tools, therefore we analysed outcomes based on the contemporaneously available DIPSS-Plus score (2011) for myelofibrosis and CPSS for CMML (2009).

The CIBMTR DRI (2014) is a particularly useful tool for segregation of patient groups based on 3 risk strata, and when applied to our cohort, outcomes for relapse and survival accurately correlated with the risk category, with the highest risk of relapse in those in the ‘high’ category. The index requires only basic disease-related parameters and overcomes some of the limitations of comparing disease groups that span the evolution of diagnostics and risk guidelines.

Transplant risk categorisation

Pre-transplant prediction of outcome is a difficult task, with limited validated tools to stratify an individual patients’ risk of NRM and survival. The age adjusted HCT-CI is the primary tool available pre-transplant to offer patients an estimation and was in routine throughout the study period. Interestingly, a score of 2 using this model predicts a 2-year overall survival of 70% and the median score for this cohort was 2 with an experienced OS of 70%. The score-predicted 2-year NRM risk of 18% is an overestimation for our cohort, where the 2-year cumulative incidence of NRM was a more modest 13.5%. The higher median aged adjusted HCT-CI score of 3 in the MDS sub-cohort, as compared to 2 in both AML and MPN, reflects the

increased NRM rate and trend towards reduced OS in MDS patients. Despite its utility in estimations for cohort-based outcomes, we could not demonstrate a direct correlation between individual increased scores and inferior survival outcomes, which may limit its use for personalised risk estimation.

The KPS was documented pre-transplant for all patients, however, there was no significant difference in any outcome based on the KPS. We noted older patients tended to have a higher KPS and those less than 50 years old had lower scores, which alludes to the fact that transplant in the oldest age group tends to be the reserve of the fittest patients leading to a degree of selection bias. Similarly, a bias exists in the youngest age group, as traditionally these patients would be offered a myeloablative conditioning regimen, but for eligibility reasons these patients instead proceeded to a RIC alloSCT. In addition to these objective risk scores, we examined the impact of individual organ-related pre-transplant variables. Despite several patients having reduced ejection fraction, diffusion capacity, hyperferritinaemia or reduced creatinine clearance, abnormality of any of these markers individually were not a strong predictor of future outcome.

Donor considerations

In keeping with international trends showing expansion of alloSCT performed with unrelated donors, more donors for this patient cohort were unrelated than were related (128, 130). Stem cell donors were primarily fully HLA matched but there was a small number of 1-antigen mismatches, which is our institutional preference in the absence of a fully matched sibling or unrelated donor. This is particularly pertinent in the RIC setting as suitable sibling availability becomes less likely with advancing age. It is additionally relevant to the Irish situation, where historically larger families resulted in reasonable access to sibling donors. However, family sizes have shrunk significantly since the mid-20th century despite sustained and significant overall population growth (187). The number of mismatched and haploidentical allografts in this age group is likely to grow as the population of Ireland diversifies, including an increasing mixed-race population. In an evolving field of alternative donor transplantation options, including haploidentical donors, proportionally more patients will require a different conditioning approach in the future. Nevertheless, even though PTCY-based platforms have since become a standard in the HLA mismatched setting, the results seen in the small number of mismatches in this group are encouragingly in line with the wider group.

Donor age

Donor age was significantly higher in sibling transplants and was associated with a lower CD34⁺ yield but did not affect engraftment and furthermore did not appear to affect survival, relapse risk or severe GVHD outcomes in this group at any age cut-off. An increased risk of acute GVHD was noted with donors below the age of 40, however this correlates closely with the association of unrelated donors with younger donor age. The oldest patients were the most likely to require an unrelated donor due to advanced sibling ages and on that basis, higher rates of GVHD in older patients might be expected, however, no disparity in GVHD incidence by age was identified. This may reflect the protective benefit of higher ATLG dose intensity when using unrelated donors.

Graft content

We collected detailed technical specifications of the stem cell product including standard measures of viability and stem cell dose, but also additional immune profiling of T cell subsets in the graft. This allowed us to interrogate several questions relating to immune function and recovery post-transplant. One of the primary study hypotheses posited that reconstitution of a robust lymphoid immune system post-transplant is predicated on several variables, of which ATLG exposure is a likely key factor. Successful attenuation of post-transplant relapse risk is predicated on harnessing the T cell-driven GVL effect, which in turn is balanced against the risk of severe GVHD. We identified heterogeneity in graft T cell volume throughout the cohort in both total and subset compartments. By examining the graft T cell content and its relationship to GVHD, relapse and immune reconstitution outcomes, we sought to further validate the hypothesis that increased graft T content neutralizes more of the unbound ATLG, leading to earlier clearance from circulation and leading to swifter immune reconstitution and less relapse, at the expense of increased GVHD rates. Interestingly in univariable analysis we noted an association between an increased risk of relapse in those with high graft T cell content, which was noted for both CD4⁺ and CD8⁺ cells. This finding mirrors that of a similar association between graft cryopreservation and relapse risk as T cell content was notably higher in cryopreserved grafts. However, in multivariable models including both factors, the impact of T cell content retains independent significance. Although there was an increase in transplant delay from diagnosis in those who received a cryopreserved graft, the impact of the T cell content remains independently associated with increased relapse risk. We noted a reduced CD34⁺ viability post thaw in those who had cryopreserved grafts, which is a documented phenomenon (193). However, in this cohort there was no detrimental effect on time to engraftment of platelets or neutrophils. In fact, a one day shortening as compared to fresh grafts was noted. The number of cryopreserved grafts in this cohort is modest, however when we previously interrogated its impact in all-comer patient cohort (myeloid and lymphoid indications) receiving a cryopreserved graft in our centre during a contemporaneous time-period we found no difference in engraftment outcomes, but a replication of the increased risk of early relapse (197).

Universal neutrophil and high platelet engraftment rates were encouraging, particularly in the myelofibrosis cohort, where universal primary neutrophil and platelet engraftment was seen. Although there were some differences in time to engraftment between diseases, with myelofibrosis having a 2-day increase in time to neutrophil engraftment and 5-day increase in time to platelet engraftment, this did not result in any significant clinical complications. However, additional resources are required for patients undergoing transplantation for myelofibrosis due to the added length of hospital stay while awaiting engraftment. With a median CD34⁺ cell dose of $4.91 \times 10^6/\text{kg}$ in those who did not achieve primary platelet engraftment, it is interesting to note failure was not restricted to those with myelofibrosis or low CD34⁺ dose.

GVHD prophylaxis, ATLG, GVHD outcomes and immune reconstitution

The approach to GVHD prophylaxis was uniform in this cohort, with *in vivo* T cell depletion with ATLG, a calcineurin inhibitor and post-transplant low dose methotrexate. We therefore

sought to assess the differences in ATLG dosing schedule as a potential determinant of outcome. The hypothesis that the half-life of ATLG, and thus immune reconstitution, could be partially determined by peripheral blood absolute lymphocyte count of the recipient at the time of ATLG exposure was examined. This was based on studies in the paediatric setting, where this effect was evident, and spurred the desire to develop a dosing nomogram based on patient weight and lymphocyte count to guide individualized ATLG dosing in adults (298). In the paediatric setting ATG was administered at the start of conditioning whereas in this adult cohort, a difference in conditioning schedule means all patients were already treated with 5 days of fludarabine by the time they first received ATLG. This resulted in 84% of patients demonstrating a peripheral absolute lymphocyte count of $0.1 \times 10^9/L$ or less by first exposure. No meaningful stratification of subsequent transplant outcomes could be made on this metric including at a cut-off of 0, 0.1 and higher ALC $\times 10^9/L$. When we further examined the impact of pre-fludarabine lymphocyte counts at stratified levels we similarly could not identify an impact on outcome.

All grade acute GVHD was common at 59.4%, with 48.2% developing grade II or above, and a modest level of significant grade GVHD (III-IV) of 11.1%. Despite a high incidence, only 15% of those with grade II – IV disease developed steroid refractoriness and required additional steroid-sparing agents. An association between increased risk of moderate to severe disease and non-AML disease subtype was evident and those with an unrelated donor experienced more acute GVHD, despite the increased dose of ATLG. HLA mismatch resulted in a trend toward increased risk in mismatched donors, however the numbers were low and there was confounding by a few mismatched sibling donors also. Donor age below 40 was associated with an increased risk of moderate to severe disease but was closely correlated with the donor source. No patient developed acute GVHD higher than grade II following DLI, indicating a low toxicity profile for this treatment approach. No correlation between graft T cell content or the subset T cell populations and subsequent acute GVHD could be seen. Those with a higher volume of donor T cells infused at the time of transplant did not experience a higher rate of acute GVHD, which was independent of the dose of ATLG and donor source.

We noted a higher risk of developing acute GVHD with tacrolimus use for GVHD prophylaxis, which remained significant when accounting for donor source, as tacrolimus was more likely to be prescribed in the unrelated setting. Additionally, some patients with fragile pre-morbid renal function were prescribed tacrolimus and these patients may have experienced more frequent dose interruptions to preserve renal function, thus leading to more erratic drug levels. When we examined the effect of tight calcineurin level control in the first month post-transplant it was noted that those with supratherapeutic levels on more than one occasion were at a higher risk of developing acute GVHD. This may have been due to more frequent dose alterations and labile levels resulting in both supratherapeutic peaks but also rebound subtherapeutic troughs.

The incidence of chronic GVHD in this cohort was significantly lower than expected, with 18% of patients developing all-grade disease at any stage. Documentation of mild and limited disease was less formalised in the earlier phase of the study period and is likely to be underrepresented, however. Nevertheless, severe and/or extensive disease occurred in only 6% of patients. While chronic GVHD is described as occurring more frequently in older patients, no significant difference in incidence was seen in any of the age groups in this older cohort of patients (22). While no increased risk in those with unrelated donors or HLA mismatched grafts was seen, we confirm the strong association between the development of

moderate to severe acute GVHD and the subsequent risk of developing chronic GVHD, as all but 2 patients with documented chronic GVHD had prior acute disease. As seen with acute GVHD, the risk of developing chronic GVHD was not affected by graft T cell content in any of its subsets. Only those with the very highest graft T cell content showed an association, however the cases at this level are too few to correlate. ATLG dose intensity and the lymphocyte count on the day of infusion were agnostic for determining chronic GVHD risk, as seen in acute GVHD. It is possible that this is due to the employment of higher doses of ATLG to attenuate the increased risk associated with unrelated donor grafts. A male recipient with a female donor was at increased risk of chronic GVHD, which is a recognized phenomenon (330).

In a test of potential scope extension for the EASIX score, we examined the association between serial EASIX score and the risk of subsequent acute and chronic GVHD. A small association between higher pre-transplant EASIX score and the risk of developing grade III-IV acute GVHD, and higher scores at day 0 and the risk of subsequent chronic GVHD was seen, findings which will be explored further in the subsequent chapter with additional patient numbers.

NRM

NRM was encouragingly modest, at 13.5% at 2 years, almost 5% lower than predicted by the age-adjusted HCT-CI. While a degree of selection bias occurs in studies of older patients receiving an alloSCT, the validation studies that underpinned the age-adjusted HCT-CI score were subject to these same biases and thus broadly comparable and representative. We could not identify a significant change in NRM risk over the duration of the study, with rates similar in each year from 2015 to 2021, despite the rise in median age at transplant through the same period. An increased risk of NRM in those with MPN and MDS as compared to AML was evident, despite similar median ages in these groups. The difference in time from diagnosis to transplant between each disease group may play a role in increasing the risk of transplant complications. For the MPN category, those with higher risk disease at the time of transplant had an increased risk of NRM, possibly driven by a higher pre-transplant treatment burden. For AML, those with ELN favourable risk were at higher risk of NRM, possibly also indicating pre-transplant treatment burden as these patients arrived at transplant following salvage therapy to achieve CR2. However, in the AML cohort NRM events were low and additionally, increased relapse in the intermediate and adverse risk groups are a competing event for NRM, which likely artificially inflates the apparent risk in the favourable category. The MDS cohort experienced the highest number of NRM events in absolute terms and demonstrated a higher median age-adjusted HCT-CI score as compared to the other groups. However, on a cumulative incidence basis, where the competing risk of relapse is considered, the highest proportional risk of NRM was in the MPN, and specifically the myelofibrosis, group.

An increased risk of NRM was seen in patients with minor ABO mismatched grafts, although not replicated in a multivariate model including additional factors for NRM including grade III – IV acute GVHD and disease subtype. The clear association between high grade acute GVHD and excess NRM is counterbalanced by low rates of severe acute GVHD in this treatment group. The impact of low graft CD4⁺ T cell content on the risk of NRM lost significance in multivariable modelling and may reflect the competing increased relapse risk seen with high graft CD4⁺. A strong association with increased risk in those who developed invasive fungal

disease underpins the high case fatality rate that is classically associated with this opportunistic form of infection. Furthermore, the excess mortality in this group was not confined to death directly from fungal infection, which alludes to additional mortality risks in those who are vulnerable to invasive fungal disease. Failure to recover IgG levels to normal levels by 6 months post-transplant showed a small association with increased NRM risk and is likely driven by prolonged immunosuppression in those with moderate to severe GVHD, particularly extended steroid exposure.

Relapse, chimerism and DLI

A robust initial response following transplant was evident, with a CR or CRi rate of 81.1%. However, relapse and disease progression are the largest obstacle to a successful long term transplant outcome. The cumulative incidence of relapse in this cohort of patients was in line with international experience at 21.9% by year 1 and 31.7% by year 2. 60% of relapses occurred within a year of transplant and a further 20.8% by year 2, however almost 20% of relapses occurred comparatively late. Although MDS was overrepresented in this late relapse group (n=5), late relapses were seen in all disease groups.

The CIBMTR-DRI strongly correlated with relapse risk in this cohort in univariable analysis, confirming the validity of this simple clinical tool. The risk of relapse similarly broadly followed individual disease risk classifications, with a strong trend towards increased relapse in high-risk AML by ELN 2017 criteria and high to very-high risk MDS by IPSS-R. The highest risk of relapse in the myelofibrosis cohort was in the lowest-risk transplanted group, intermediate – 1, although this was likely artificial due to the excess non-relapse mortality experienced by high-risk patients. Nevertheless, patients in this intermediate-1 risk group were more often treated pre-transplant with hydroxyurea alone, as compared to higher use of ruxolitinib, hypomethylating agents and intensive chemotherapy in the higher risk categories, perhaps resulting in higher residual disease burden at the time of transplant and a higher subsequent risk of relapse.

Several graft and donor characteristics correlated with relapse outcomes, the most striking of which was graft cryopreservation, graft T cell content and ABO mismatch. The increased risk of relapse in those who received a cryopreserved graft mirrored findings from a study conducted by our research group on the impact of graft cryopreservation during the COVID-19 pandemic, which identified an increased risk of early relapse in those receiving a cryopreserved graft as compared to fresh on an all-comers basis, which included myeloablative and reduced intensity conditioning for myeloid and lymphoid diseases. It is interesting to see this finding is again identified in this more homogenous population. The association between higher graft T cell content and the subsequent risk of relapse in univariable analysis was surprising, in the context of our initial hypothesis for higher circulating lymphocyte numbers early during ATLG exposure providing additional substrate leading to downstream reduction in immunosuppressive, a more robust GVL effect and hence lower relapse. In fact, the opposite effect was observed. When we applied a multivariable model including disease risk profile, graft T cell content and graft cryopreservation, excess graft T cells continued to show a strong trend towards increased relapse (p=0.06). The role and activity of mature T cell populations in the recipient following infusion alongside the stem cell graft is not well understood and further characterisation of the T helper subclasses, and exhaustion markers is an associated area of study on samples from this patient profile at our

institution. ABO mismatch was a surprising univariable risk factor for relapse, however, published literature on the impact of ABO mismatch is conflicting and this finding should be considered in the context of larger registry-based studies (174, 175, 176).

Acute GVHD development is associated with a trend towards reduced relapse risk in this group and reaches significance in those who develop subsequent chronic GVHD. This finding supports the strategy of liberal use of DLI to boost the GVL effect in those at risk of relapse guided by close monitoring for mixed chimerism and potentially in a prophylactic manner for those with very high-risk disease. We noted a significantly reduced relapse risk in those with mixed chimerism at day 90 or beyond who received a DLI as compared to those who did not receive a preventative DLI. This strategy is likely to account for the lack of an increased relapse risk in those with mixed chimerism at day 90, as over half of these patients received a pre-emptive DLI.

In a further assessment of the predictive capabilities of the EASIX score, relapse risk stratified by score was assessed at serial time-points. Interestingly a strong association between high EASIX scores at 1-year post-transplant and subsequent relapse was seen, which may reflect the association between raised LDH, thrombocytopenia and impending relapse in myeloid disease.

Overall survival

OS on a cohort basis matched the predicted 2-year OS at 70% but as with NRM, it was not possible to delineate OS by individual age-adjusted HCT-CI or KPS scores. Association with relapse by graft and donor variables translated to a survival disadvantage for ABO mismatch and high graft CD8⁺ content, whereas survival was unaffected by donor age, donor source, graft preservation or CD3⁺, CD8⁺, CD4⁺/CD8⁺ graft content. In keeping with the reduced relapse risk in chronic GVHD, improved overall survival was seen in those who developed chronic GVHD, which appeared to overcome the higher NRM that is typically seen in chronic GVHD populations. No survival benefit was seen in those who developed acute GVHD of any grade, both in early spontaneous disease and in acute GVHD secondary to DLI use. Excess mortality in those who developed grade III – IV acute GVHD may account for some of this effect. Enhanced survival in relapsed patients who did not develop acute GVHD but lived longer with post-transplant salvage combination therapies may have further diluted the potential survival benefit in acute GVHD.

The use of DLI resulted in a survival benefit in all recipient groups, and particularly in those who received a pre-emptive DLI for mixed chimerism but had not yet relapsed. This suggests that intervention with early DLI can prevent a proportion of impending relapses and thus improve survival. Longer survival in those who received a therapeutic DLI post-relapse also contributed to the association between survival and DLI use, as survival post-relapse was over a year longer in those who received a DLI.

The EASIX score was confirmed as a predictor of survival outcomes, as has been demonstrated by other groups (227). At the pre-conditioning, onset of GVHD and 1-year post-transplant time-points, higher scores correlated with inferior survival.

Conclusions

Strand 1: Aging and frailty, toxicity and the efficacy of reduced intensity conditioning

This dataset highlights dynamic changes in transplant practice over 6 years of recent activity. We note incremental shifts in demographics and transplant activity within the programme, reflecting trends in the growing and aging general population. With this comes the additional considerations of the role of transplant in older patients, including the resources and tools for pre-transplant optimization and workup, support and care through the acute phase of transplant and longer-term management of survivors. Recent enhanced recognition of the specific needs of teens and young adults during transplant provide lessons for the age-related needs of older patients. Older adults experience many of the same issues as their younger counterparts, but there are clear physiological and psychological differences. The field of older age-related haemato-oncological specialist care is in growth across several domains, and this comes into particularly sharp focus in the transplant setting, where toxicity and effectiveness require careful balancing.

We report encouraging outcomes in all age groups of our cohort, including modest rates of non-relapse mortality and internationally comparable overall survival and relapse risk. Our data endorse the application of this conditioning regimen in this demographic of older patients with myeloid disease and corroborate international experience with its use. A notable strength is applicability to a wide age range and several disease classes. Further strength lies in significant institutional experience and familiarity with the regimen, a metric that is difficult to quantify, but allows for reproducible and longitudinal study of outcomes and ensures robust comparability with neighbour institutions and registry data. Nevertheless, continuous efforts to improve outcomes for patients are worthy of exploration, particularly to optimize relapse-free survival.

Mortality risk is an important consideration in advancing age, but so too are morbidity and the burden of transplant-related toxicities. While the low NRM risk we report alludes to an attractive toxicity profile, there is nevertheless a significant morbidity cost to this curative approach, with 1 in 8 patients dying in remission within 2 years. There is a significant burden of GVHD with this platform in both acute and chronic form, noteworthy levels of a range of infections and a protracted pace of immune reconstitution, leading to exposure to additional treatments and prophylactic measures. The treatment of these side-effects has independent age-related considerations for organ dysfunction, tolerability and quality of life. The cumulative incidence of these complications is notable, however the outcomes for severe acute GVHD, extensive and severe chronic GVHD and life-threatening infection appear modest. Encouragingly, this burden does not appear to have age-specific tropism and the cardinal transplant outcomes NRM, OS, relapse, GVHD, infection and immune reconstitution are broadly age-agnostic. These data suggest alloSCT should be considered in older patients on a case-by-case basis after consideration of global fitness and disease status. In the following chapter we will review the outcomes, resource demands and immunological phenomena of a further subgroup of elderly patients over the age of 70 to determine if these positive findings are reproducible over time and at the extreme of age in transplant practice.

Strand 2: Donor and graft considerations

Evidence supporting a survival benefit in alloSCT performed with younger donors has potential widespread implications (183). In RIC alloSCT donor age is as much a question of pragmatism as it is optimization, and access to a sibling donor provided transplant access for almost half of the patients in this dataset. It is therefore a consolation that older donor age at any cut-off in this study did not have a deleterious impact on recipient survival or relapse risk, and in fact was related to a lower GVHD risk. We noted a reduced CD34⁺ yield from donors over the age of 35 but this was not clinically significant and did not translate to a risk of primary graft failure or late immune reconstitution. Donor gender similarly did not determine outcome except for an increased risk of chronic GVHD in male recipients with female donors, a phenomenon which has been described previously (567, 568). The role of ABO mismatch in survival determination and relapse risk was surprisingly strong in this series. With a large proportion of alloSCTs involving some form of ABO mismatch, optimizing for ABO matching while also retaining HLA matching is likely to be practically challenging. However, this finding may aid in the choice between multiple donors and may also help identify patients at increased risk of adverse transplant outcomes and offer further individualized risk prognostication. HLA mismatch did not result in significantly worse outcomes as compared to full HLA matched alloSCT, but this finding is only supported by a small number of such transplants in this cohort. In subsequent practice, our institutional preference in the HLA mismatched setting has shifted to a PTCY-based platform given the supportive evidence for this approach. We retain our preference for a single antigen mismatch as the alternative donor strategy, although haploidentical alloSCT use is likely to expand in the future.

Graft cryopreservation appeared to increase the risk of relapse in this series and corroborates our previous study in this area. The increased risk of relapse, particularly in the first-year post-transplant in those who received a cryopreserved graft, may be partially driven by increased time to transplant from diagnosis as compared to fresh grafts, but the association is also noted by other groups (194, 195, 199). The further association between increased graft T cell content in all subsets and the risk of relapse has a stronger relationship than cryopreservation. Although T cells are the substrate for ATLG activity, neither graft or recipient T cell volume appeared to determine early transplant milestones including GVHD and early CD4⁺ recovery, and it is unclear if this excess relapse risk is modulated by ATLG or independent thereof. The relationship between graft content and relapse requires further investigation and will be examined in the subsequent chapter in additional patients.

Strand 3: EASIX modelling

The EASIX score has shown efficacy in predicting overall survival and non-relapse mortality in transplant settings (227, 245). We therefore sought to assess its validity in predicting these outcomes for this cohort in addition to testing for correlation with further transplant-related outcomes including acute GVHD and relapse. The score is easily applied retrospectively as the data required is universally available. Our data corroborate the ability of the EASIX score to predict overall survival at the timepoints of pre-conditioning, GVHD onset and at 1-year post-transplant. This offers a simple clinical tool to help in the overall assessment and prognostication of patients at these transplant milestones.

The score is most consistent in OS prediction, where higher scores correlate with inferior survival. NRM was less predictable in this study but was possibly limited by the relatively low number of NRM events. Interestingly, an association with subsequent relapse in those who had higher EASIX scores at 1 year was seen. We believe this is based on the underlying disease biology of myeloid disease, where a rising LDH and falling platelet count are frequently heralds of relapse, both of which lead to higher EASIX scores. The EASIX score at pre-conditioning and day 0 have potential additional utility in predicting the subsequent development of moderate to severe acute GVHD. This finding can broaden the applicability of the score if replicated in larger datasets and encouragingly, a prospective study of this aspect is the subject of an ongoing clinical trial (ClinicalTrials.gov: NCT05718791). We could not assess the score's ability to predict SOS as there were no definitively diagnosed cases in this series. As with any predictive models, there are limitations to use, however in the correct context the EASIX score may offer insights into future risks, particularly for overall survival and late relapse. Application of the score in routine general transplant practice for descriptive purposes is justified by these data and over time further power will be accorded these observations. The score will also be further interrogated in the subsequent chapter and correlated with simultaneous cytokine profiles.

Strand 4: ATLG and immune effects, tests of hypothesis

Despite the encouragement of tailored ATLG dosing proposed in the paediatric and adult settings by Admiraal and colleagues, which limits toxicity and promotes immune reconstitution, we were unable to fit this model in a retrospective adult setting where the conditioning format differs, as ATG was employed from day -8 in that adult study (298, 299). Near universal severe lymphopenia in adults at the time of ATLG exposure may account for this lack of discriminatory effect. When we examined the impact of ATLG dose, lymphocyte count at exposure and patient weight, we could not identify any deterministic effect on transplant outcomes including NRM, relapse, OS or GVHD risk. We therefore examined the role of the pre-conditioning lymphocyte count but were similarly unable to identify an association and the intervening high doses of fludarabine may render variability in pre-conditioning lymphocyte count irrelevant by the time ATLG is administered. Interestingly, post-hoc analysis by Soiffer et al in their RCT identified severe lymphopenia at day -3 in <35% of those who received FluBu MAC conditioning, however those patients received 120mg/m² of fludarabine, as compared to 180mg/m² in this RIC setting (256). We furthermore could not identify an effect on speed or strength of T cell reconstitution and immunoglobulins post-transplant, where such results were available. Replication of the encouraging findings in the paediatric setting will require a rationally designed clinical trial supported by pharmacodynamic studies, specifically to identify difference between the two formulations, ATG and ATLG.

Heterogeneity of outcomes post-transplant involving ATG/ATLG are determined by a range of factors. For immune recovery, single factors may be less predictive than combinations thereof and many of these variables have significant time-sensitivity. We suspect T cell volume in the recipient and graft during peak ATLG exposure to be stochastically significant but is not independently prominent enough in this retrospective study. The static determinants of reconstitution such as the recipient physiology, graft stem cell and mature T cell, NK cell and

monocyte populations and conditioning are further modulated by dynamic outcomes such as GVHD onset, steroid exposure, DLI use, infections, inflammation, and relapse.

With a median CD4⁺ recovery of 85, 118 and 164 x 10⁶/L at days 100-, 180- and 1-year post-transplant respectively a significant number of viral re-activations and opportunistic infections could potentially be avoided by optimizing immune reconstitution. Although mortality from infectious complications was modest, this has cost implications for monitoring and treatment and adds to cumulative patient morbidity. With 1 in 10 patients developing a secondary malignancy within 7 years of alloSCT, poor immune reconstitution may also be responsible for loss of tumour surveillance and enhancing cancer incidence in an age cohort who are already at a higher baseline incidence risk. Potential optimisation of this conditioning platform may best be targeted by study of optimal use of ATLG, and further studies are needed in the adult setting to determine a logical approach to this problem, particularly with minimal sacrifice of the clear benefit of limited toxicity from fulminant acute GVHD and extensive chronic GVHD seen in this study.

Strand 5: Post transplant salvage and DLI

The outcomes demonstrated in patients who received a DLI for any indication are a particular source of encouragement in this data set. Where 52% of patients had a mixed CD3⁺ donor chimerism at day 90 or beyond, we note the striking benefit for those who received a pre-emptive DLI in averting threatened relapse. A liberal and chimerism driven approach to DLI use appears effective, and moreover, in this older age cohort, safe and tolerable. No deaths occurred due to GVHD post-DLI and no severe GVHD cases were induced by DLI. In the small number of prophylactic DLIs employed, outcomes were similarly reassuring and pending further evaluation in larger numbers may have an expanded and formalized role. Noting the considerable risk of relapse following RIC alloSCT, it is encouraging to see that for those who received a therapeutic DLI, outcomes were superior to those who did not receive one, indicating that this adjunct to salvage therapy appears effective in prolonging survival in a setting where further options are frustratingly limited.

Future directions and implications for prospective study

The results of this large retrospective study provide insights into the standard of care, in place for almost 20 years, at this institution. In an era of changing practice, it not only provides a useful standard comparator, but also affirms the validity of continuation of this approach in modern practice. These results inform the basis of the subsequent chapter, where we report outcomes alongside serial biomarkers in a prospective cohort of patients and furthermore interrogate these research strands in the pooled dataset. We will revalidate the encouraging results in older patients by assessing a further subgroup of the oldest transplant recipients at our centre to date. We will examine the effect of frailty peri-transplant, including an assessment of the resources required to support this group through transplant. We will further attempt to identify the proportionate effect of ATLG on outcomes, the predictors of immune reconstitution and the role of DLI. Sequential serum cytokine levels and trends at

relevant time-points will be correlated with the research strands from this retrospective study: frailty, graft characteristics, EASIX modelling, immune reconstitution after ATLG and DLI use.

Chapter 3

Prospective review and cytokine analysis

Abstract

Fludarabine, busulfan and anti-T lymphocyte globulin (FluBu3ATLG) for reduced intensity conditioned (RIC) haematopoietic stem cell transplantation is an established regimen for older patients with myeloid disease. We examined the performance of this combination in modern practice in 175 sequentially treated patients at the National Adult Stem Cell Transplant Programme in Ireland. We furthermore report the clinical correlation and secretion patterns of a 10-cytokine panel measured at serial transplant time-points in a subgroup of 32 patients. 2-year overall survival (OS) was 72.3% and the median follow-up was 23.2 months. Non-relapse mortality was modest at 12.1% by 2-years. The cumulative relapse incidence at 2-years was 29.5% and advancing age was not associated with adverse outcome, including those over the age of 70. The use of ATLG resulted in low levels of severe acute (11.2%) and chronic GVHD (17.3%) and higher doses of ATLG did not associate with excess relapse risk, however it did lead to a protracted T cell immune reconstitution. Predictors of relapse included use of stem cell graft cryopreservation and higher graft T cell content ($p=0.004$) but increasing donor age had no effect on survival or relapse. Relapse was significantly reduced in those with mixed donor chimerism by adopting a liberal pre-emptive donor lymphocyte infusion strategy (DLI) ($p=0.01$) and DLI improved survival length after post-transplant relapse ($p=0.03$).

The EASIX score complements this regimen by predicting OS ($p=0.01$), NRM ($p=0.03$) and moderate-severe GVHD ($p=0.04$) at the pre-transplant time-point, moderate-severe GVHD at day 0 ($p=0.03$), OS from onset of GVHD ($p=0.01$) and OS and relapse at 1-year post-transplant ($p<0.001$, $p=0.01$). Serial cytokine monitoring in a selection of 32 participants identifies trends in cytokine expression and demonstrated a potential role in predicting outcomes including survival and GVHD. Temporal relationships between IFN γ , IL-12p70, IL-17A and survival, IL-12p70 and IL-17A and relapse and IFN γ , IL-2, IL-6 and IL-10 with GVHD offer insight into the inflammatory secretome and its evolution through transplant. Early increased levels of IFN γ , IL-1 β , IL-2, IL-6 and IL-17A, and reduced IL-4 demonstrate associations with acute organ-specific GVHD subtypes and have strong potential as predictive biomarkers. These adjunctive measures offer individualized decision support, and alongside tactical use of DLI allow for an increasingly personalised approach to alloSCT. FluBu3ATLG supported by modern supportive care is well tolerated by older patients, with modest toxicity and favourable long-term outcomes, and most importantly demonstrates the potential for further optimization supported by these data and guided by rationally designed clinical trials.

Introduction

The results of the retrospective analysis presented in chapter 2 confirm the FluBu3ATLG conditioning platform is efficacious and boasts a modest toxicity profile. This chapter will now examine its performance in a contemporaneous cohort of patients recruited immediately after that observation period, who were treated with the same conditioning protocol. The five principal research themes discussed previously are again interrogated in this group and furthermore pooled with the larger retrospective dataset to provide robust appraisals of these central questions. A sixth supplementary area of interest is explored by sequential and

longitudinal analysis of serum cytokine levels to identify the underlying biological processes that underpin the dynamic nature of reduced intensity alloSCT.

Eligible patients over the age of 70 with myeloid malignancies are offered this conditioning regimen based on historical experience in those aged up to the age of 70. The results in those aged 65-70 are encouraging and consistent with those of a younger age, with no additional mortality risk or increased toxicity burden. As only one patient aged above 70 was included in the previous analysis, the following review seeks to confirm those positive trends in a larger group of representative patients. The age-related needs of this patient group drive the need for formalized peri-transplant specialist care. If positive long-term outcomes can be demonstrated, access to the additional resources needed to safely perform alloSCT for older patients is supported and justified. The pressing question for this age-group stands: is FluBu3ATLG an appropriate conditioning approach for elderly patients?

Optimal donor choice is informed by an evidence base in continuous evolution, with the weighted importance of various characteristics varying by context, changing over time, and ultimately subject to the pragmatic constraints of donor access. Recent evidence pointing to the impact of donor age on survival outcome has significant clinical implications and adapting local donor choice algorithms in response to these data should be supported by the testing of this finding in local patient cohorts. We examine the relative deterministic effects of various donor and graft variables on future outcome including survival, GVHD, relapse and immune reconstitution. Following signals of the deleterious effect of graft cryopreservation and the surprising association between relapse and graft mature T cell content in Chapter 2, those associations are again interrogated in this additional group of patients.

In the face of a mounting challenge from PTCY, the justification for continued use of ATLG is currently supported by a lack of direct comparator trials in the matched donor setting. There are potential avenues for tailoring ATLG based on models for ATG, and thereby its role may be preserved in haematopoietic stem cell transplant for time to come. Further exploration of the factors that drive variability in outcome after ATLG exposure is needed to identify areas in need of future optimization. Homogenous severe lymphopenia at ATLG exposure in the retrospective review limited the appraisal of the potential role of absolute lymphocyte count as an effector of ATLG lymphotoxicity, and this hypothesis is evaluated further in this chapter. Globally protracted immune reconstitution of T and, to a lesser extent, B cells was experienced in all ATLG dose intensities in the previous review and exposes patients to cumulative toxicity from infection and diminished tumour surveillance. The factors that drive timely immune reconstitution are myriad, with ATLG playing an early role. However, this direct toxicity is carefully balanced by the risk of moderate to severe acute GVHD and chronic GVHD, both of which result in additional immunosuppression exposure and the attendant delays in immune recovery. The observed trends reported in these chapters may help guide bespoke interventions to maximise both facets of this dichotomy.

The important twofold role of DLI was manifest in the previous chapter. A clear protective effect against relapse is seen in those patients who had mixed donor chimerism at 3 months or beyond. Further demonstrable survival benefit in relapsed patients is encouraging and accompanied by a low toxicity profile, which supports safe application in an older patient group. Replication of these benefits in larger patient numbers will support this approach.

The EASIX score proved a useful descriptive risk tool in this conditioning setting and has demonstrated potential for prediction of important transplant outcomes including mortality,

high grade acute GVHD and relapse. The score is hereby applied at the same time-points and correlated with subsequent clinical outcomes to add further strength to these associations and endorse its routine use within the transplant programme.

As outlined in chapter 1, cytokine secretion through transplant is variable over time and influenced by transplant complications. The interplay and patterns of secretion by both the recipient immune system pre-conditioning and that of the engrafted donor population offers insights into the pro and counter-inflammatory forces driving the underlying myeloid disease processes, engraftment, host/donor interactions and relapse. Cytokine levels at time-specific stages of the transplant journey can describe the nature of temporally associated clinical outcomes, but also importantly, may herald the future development of complications, and thereby allow for intervention to modulate those risks. In addition to a potential predictive role, some cytokines may also offer clinically meaningful targets for directed therapy as mediators of inflammation and immune phenomena. We hereby explore a broad panel of cytokines in the context of older patients treated with the FluBu3ATLG RIC platform with a combined goal of describing the real-world *in vivo* inflammatory biological processes that underpin the dynamic nature of this alloSCT model, and furthermore assess early feasibility for clinical application of these assays for decision support.

The FluBu3ATLG platform, with occasional modifications, has been a core conditioning regimen in long service at the National Adult Stem Cell Transplant Programme in Ireland over years with favourable results. With increasing choice in modern transplant practice, its continued use must be measured against advances in the field. Longitudinal outcome assessment and incremental refinements will ensure older patients with myeloid diseases receive optimal and internationally comparable therapy.

Methods

Study Design

An observational study of 32 patients was conducted over 1 year, comprising sequential RIC alloSCT transplant recipients from 2021 to 2022 in the National Adult Stem Cell Programme at St. James’ Hospital in Dublin, Ireland. Clinical and demographic details were collected using electronic patient records, the National Integrated Medical Imaging System (NIMIS) and the laboratory information system. A database was created cataloguing 260 variables spanning pre-transplant to last follow up, with a censor date of 31st July 2023. Following pooling of patients from 2015 onwards, 175 patients were included for analysis of global outcomes. In the latter 32 patients, serum samples were collected at pre-defined time-points and stored in aliquots at -80° centigrade. Sampling time-points were synchronised with significant transplant time-points and in line with routine post-transplant monitoring schedules. The testing schedule is summarised in Table 22.

Table 22: Sampling schedule	
Time-point	Testing
Pre-transplant	Serum cytokine sample
Day 0	Serum cytokine sample

	Graft characteristics sample (CD34 ⁺ , BFUE, CFU-GM, CD3 ⁺ , CD4 ⁺ , CD8 ⁺ , CD4 ⁺ /CD8 ⁺)
Day 7	Serum cytokine sample
Day 14	Serum cytokine sample
Day 28	Serum cytokine sample
Day 45-50	Peripheral blood total/CD3 ⁺ chimerism Peripheral blood T cell subsets and immunoglobulins
Day 60	Serum cytokine sample
Day 90-100	Serum cytokine sample Peripheral blood total/CD3 ⁺ chimerism Peripheral blood T cell subsets and immunoglobulins
Day 180	Serum cytokine sample Peripheral blood total/CD3 ⁺ chimerism Peripheral blood T cell subsets and immunoglobulins
Day 365	Peripheral blood total/CD3 ⁺ chimerism Peripheral blood T cell subsets and immunoglobulins

Patients and donors

Patients with WHO-defined AML, MDS or MPN undergoing the institutional default RIC allogeneic stem cell transplantation with fludarabine, busulfan and ATLG during the observational period were invited to participate in the study. The study was approved by the Joint St. James' Hospital and Tallaght University Hospital Research Ethics Committee and conducted in accordance with the Declaration of Helsinki. All participants provided explicit consent for involvement, including data analysis and serial blood sampling. All 32 patients screened for enrolment provided and maintained consent and were thus included in follow-up data. Participants over the age of 18 were considered eligible. Donor characteristics, graft composition and graft processing data were collected in all available cases and included donor age and gender, graft cryopreservation status, CD34⁺ dose and viability, progenitor, and T cell content (CD3⁺, CD4⁺, CD8⁺ and CD4⁺/CD8⁺). Data on previous patients as presented in chapter 2 were pooled in the analysis of clinical outcomes and cytokine analysis and correlation was limited to the most recent 32 patients.

Conditioning regimen and GVHD prophylaxis

The conditioning regimen was fludarabine (180mg/m² over 6 days from day -9), busulfan (8mg/kg intravenously over 3 days from day -5) and *in vivo* T cell depletion with rabbit ATLG (30mg/kg for sibling donor, 60mg/kg for unrelated donor from day -3 to -1). GVHD prophylaxis was methotrexate (total dose 30mg/m²) and a calcineurin inhibitor (ciclosporin or tacrolimus starting day -1). The conditioning regimen is available in Appendix 2. Stem cell source was fresh or cryopreserved unmanipulated peripheral blood from sibling or unrelated donors with $\geq 9/10$ HLA matching.

Outcome measures

Primary outcomes assessed included overall survival, non-relapse mortality, cumulative relapse incidence, acute GVHD incidence, chronic GVHD incidence, CD4⁺ and immunoglobulin

reconstitution at day 100 and day 180, DLI use and viral re-activation incidence. Additional outcome measures included were engraftment rates, graft failure rates, sinusoidal obstructive syndrome incidence and secondary malignancy. Acute GVHD was graded according to the MAGIC criteria or Glucksberg scale. Chronic GVHD was graded according to the NIH criteria. The first day of acute GVHD was used to calculate cumulative incidence curves for that grade. Donor chimerism was assessed using short tandem repeat (STR) PCR at set time-points of 45 and 90 days, and furthermore as required and was categorised as full (>97%), mixed (3-97%) or graft rejection (<3%).

Cytokine analysis

Cytokine analysis was conducted as a batch process following completion of 6 month follow up of the last recruited patient. 12 x 96-well plates were tested with all samples analysed in duplicate alongside 8 serially diluted calibrator controls in duplicate per plate. Serum was processed according to the manufacturer's instructions (Meso Scale Diagnostics, USA). To assess pro-inflammatory and anti-inflammatory secretions from serum, a custom 10-plex electrochemiluminescence ELISA kit separated across 2 discrete assays was used (Meso Scale Diagnostics, USA). The multiplex kit was used to quantify the levels of IFN γ , IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A, IL-33 and TNF α . All assays were run as per manufacturer's instructions, with an overnight supernatant incubation protocol. All technical quality measures were met as per the manufacturing guidelines and there were no control failures. The stepwise laboratory process is available in the Appendix 3. Analyte concentrations were calculated using Discovery Workbench software (Version 4.0).

Statistical methods

Demographic and categorical characteristics were described using frequencies, medians and ranges for continuous variables and proportions for categorical variables. Survival outcomes were calculated using Kaplan-Meier estimates. Cumulative incidence of NRM (with relapse as competing risk), relapse (with death in remission as a competing risk), acute and chronic GVHD (with death as a competing risk) and immune reconstitution of CD4⁺ T cells and immunoglobulins (with death as a competing risk) was calculated using Fine and Gray's test (competing risks model). Univariable analyses were performed using a Cox model for OS and Gray's test for relapse, NRM and GVHD. A Cox proportional-hazards model was used for multivariable regression. A backward-stepwise approach was used to determine independent variables considered significant and variables recognized as potential prognostic factors were screened using LASSO regression and included in the Cox model. Selection of variables was proportionate to number of recorded events to avoid overfitting of the model. Proportional hazard ratios and 95% confidence limits are provided as a summary measure in the absence of binary outcomes. Comparison of categorical variables with binary outcomes was conducted using the chi-squared test and for means using the t-test (Mann-Whitney U for unpaired non-parametric data and Wilcoxon matched-pairs signed rank test for paired data). Comparison of medians was conducted using the Kruskal-Wallis H test for non-parametric data and one-way ANOVA for comparing categorical and continuous variables. For serial variance a two-way ANOVA with Tukey's HSD post-hoc was used. Correlation of quantitative variables for non-

normally distributed data was conducted using Spearman's rank correlation coefficient (ρ) and the Holm-Bonferroni correction was applied to account for multiple comparison testing. *P* values are two sided and 0.05 was considered statistically significant. Statistical analysis was conducted using STATA software (version 18.0 MP-Parallel edition, StataCorp, Texas, USA), GraphPad Prism (version 10.0.1, GraphPad Software LLC, Boston, USA) and R/RStudio (Build 4.3.2, R Foundation, Vienna, Austria / Build 463, Posit Software, Boston, USA).

Results (observation period 2021-2022)

Clinical outcomes

Demographics, disease status and co-morbidities

The median age at transplant was 60 with a range 47 – 74. 25% of patients were over the age of 65. There were 20 male transplant recipients and 12 female and the disease indications for transplant were AML (17/32), MDS (6/32) and MPN (9/32). Disease subclassification and the molecular profiles are presented in Table 23. Risk stratification was performed using the ELN 2022 guidelines for AML, the IPSS-M for MDS and the CPSS-Mol and MIPSS-70 for CMML and MF. Within the AML subgroup, 82.3% had intermediate or high-risk disease. In the MDS group, 80% had intermediate, high or very-high risk disease. In the myelofibrosis group 60% had intermediate-2 or high-risk disease. The CIBMTR DRI risk groups were predominantly high and intermediate risk (93.75%). 17 patients were transplanted in CR1, 5 in CR2, 2 with improved disease, 7 with stable disease and 1 in a morphologic leukaemia-free state (MLFS).

The median KPS was 80 with a range of 50-100. The median age-adjusted HCT-CI score was 2, range 1 – 6. 2 patients had an ejection fraction of <50% pre-transplant, 5 had a DLCO/VA of <80, 10 had a creatinine clearance of <90 mls/minute and 16 had a ferritin of > 1,000 ng/ml.

Table 23: Diagnostic classification

Disease	Frequency
AML, myelodysplasia-related	6
AML NOS	6
AML with NPM1 mutation	3
AML with DEK::NUP214 fusion	1
AML with CEBPA mutation	1
MDS with increased blasts 1	2
MDS with increased blasts 2	2
MDS post-cytotoxic chemotherapy	2
Chronic myelomonocytic leukaemia	3
Myelofibrosis	5
Myeloproliferative neoplasm, NOS	1

AML Mutations	Frequency	MDS Mutations	Frequency
<i>FLT3</i>	6	<i>DNMT3A</i>	3
<i>ASXL1</i>	4	<i>BCOR</i>	2
<i>DNMT3A</i>	4	<i>RUNX1</i>	2
<i>TET2</i>	3	<i>ASXL1</i>	2
<i>NPM1</i>	3	<i>GATA2</i>	1
<i>IDH2</i>	3	<i>ZRSR2</i>	1
<i>WT1</i>	2	<i>TET2</i>	1
<i>NRAS</i>	2	<i>PHF6</i>	1
<i>DDX41</i>	2	<i>TP53</i>	1
<i>KRAS</i>	1	<i>IDH2</i>	1
<i>STAT5B</i>	1	<i>ETV6</i>	1
<i>CUX1</i>	1	<i>STAG 2</i>	1
<i>EZH2</i>	1	<i>U2AF1</i>	1
<i>U2AF1</i>	1		
<i>TP53</i>	1		
<i>CEBPA</i>	1		
<i>SRSF2</i>	1		
<i>RUNX1</i>	1		
CMML Mutations	Frequency	MF Mutations	Frequency
<i>SRSF2</i>	2	<i>ASXL1</i>	3
<i>ASXL1</i>	2	<i>U2AF1</i>	2
<i>RUNX1</i>	2	<i>CALR</i>	2
<i>NRAS</i>	2	<i>MPL</i>	2
<i>EZH2</i>	1	<i>JAK2</i>	1
<i>KRAS</i>	1	<i>CBL</i>	1
<i>TET2</i>	1	<i>TP53</i>	1
<i>CSF3R</i>	1		



CMMML: Word cloud of somatic mutations



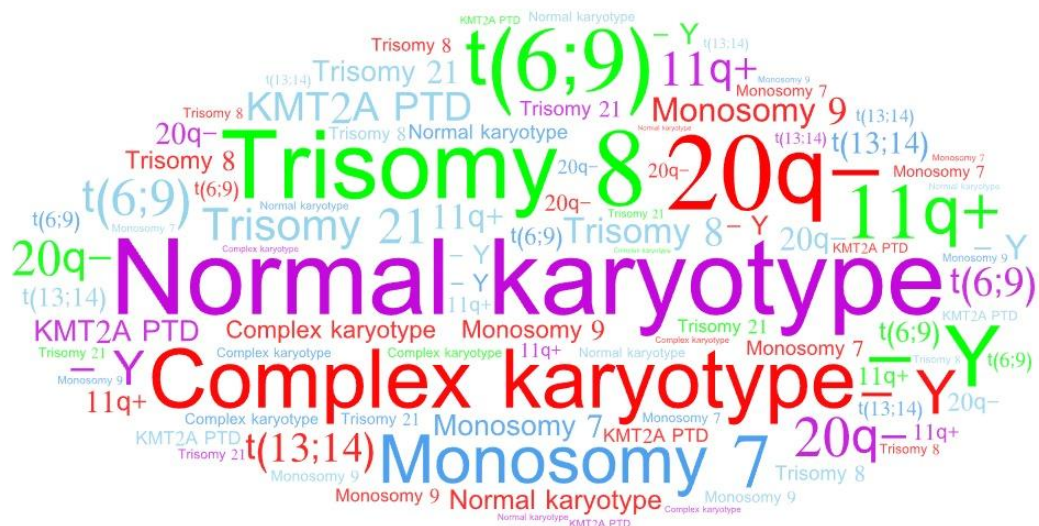
MPN: Word cloud of somatic mutations



MDS: Word cloud of somatic mutations



AML: Word cloud of somatic mutations



Cytogenetic abnormalities across all disease types, word cloud representation

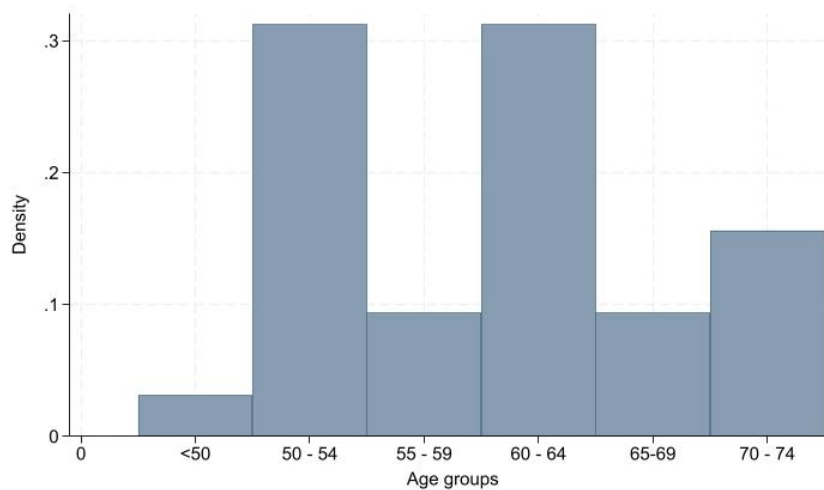


Figure 121: Age profile of transplant recipients.

Conditioning, GVHD prophylaxis and engraftment

26 patients developed severe lymphopenia of $0.1 \times 10^9/L$ or less by the first day of ATLG exposure at day – 3. Pre-fludarabine absolute lymphocyte counts were 0-0.6 (9/32), 0.7-1.0 (10/32), 1.1-1.4 (6/32) and $> 1.5 \times 10^9/L$ (7/32). Tacrolimus was the CNI of choice in 20/32 and ciclosporin in 12. All patients received the full course of low-dose post-transplant methotrexate. Adjustments of CNI based on off-target levels were recorded in 28/32 on at least one occasion in the first 30 days post-transplant, and in 24/32 on more than one occasion.

Neutrophil engraftment occurred in 31/32 at a median of 18 days and range 12 – 37 days (Fig. 122). Platelet engraftment occurred in 30/32 at a median of 19.5 days, with one patient never experiencing severe thrombocytopenia, and an upper limit of 64 days (Fig. 123).

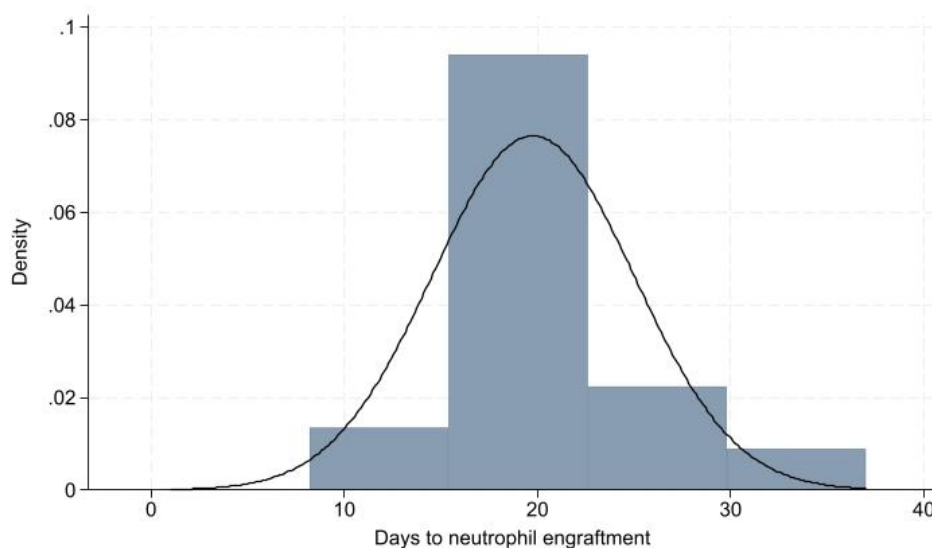


Figure 122: Days to neutrophil engraftment.

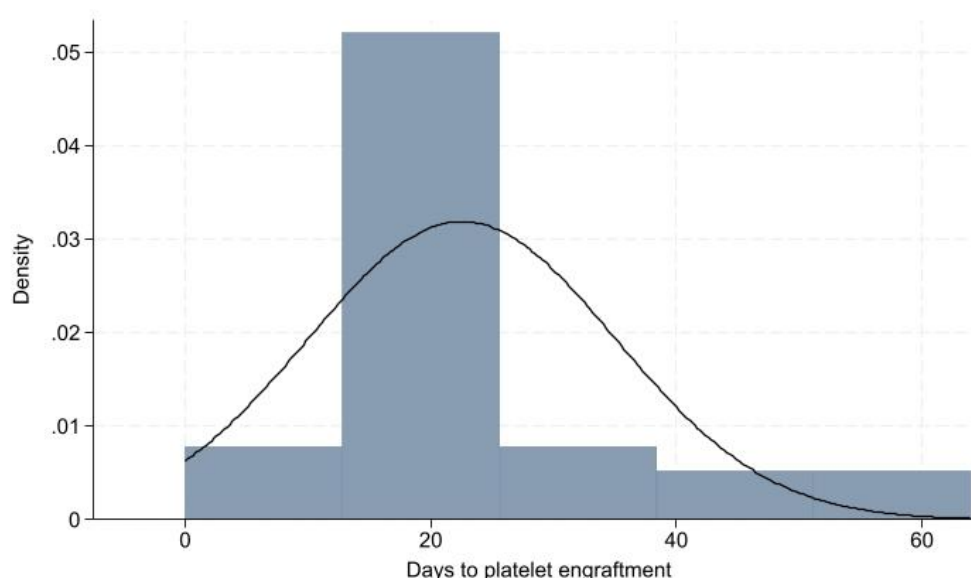


Figure 123: Days to platelet engraftment.

Donor and graft

50% of stem cell donors were siblings and there were no HLA mismatches in this group. The median donor age was 38 with a range of 20 – 67. The median unrelated donor age was 27, range 20-38. For sibling donors, the median age was 54, range 43-67. ABO mismatch was present in 10 pairs, with 2 minor and 8 major or bidirectional. 4/32 stem cell grafts were pre-harvested and cryopreserved. Time to transplant from diagnosis was 327 days, range 151 – 2900. In the cryopreserved group the median time to transplant was 278 days and 356 days in the fresh group.

The graft T cell content characteristics are summarized in Table 24. There was no significant difference in content between fresh and cryopreserved grafts (CD3⁺: p=0.28, CD4⁺: p=0.07, CD8⁺: 0.86, CD4⁺/CD8⁺: p=0.14), nor was there a difference in T cell content between sibling and unrelated donors. The CD34⁺ count was similar in both types of stem cell graft (median 5.03 x 10⁶/kg fresh, 5.09 x 10⁶/kg cryopreserved).

Table 24: Graft T cell content

	Mean	Mean + 1 SD	Median	25 th Centile	75 th Centile
CD3 ⁺	18.16	26.28	15.98	11.69	21.92
CD4 ⁺	11.17	16.00	10.37	7.57	13.19
CD8 ⁺	6.34	10.04	4.84	3.57	9.19
CD4 ⁺ /CD8 ⁺	0.25	0.41	0.21	0.12	0.38

OS, NRM and Relapse

At a median of 502 days of follow-up, the overall survival was 87.2%, with a 6-month survival of 93.7%. 4 deaths occurred at a median of 173 days. 2 deaths were non-relapse mortality events, one due to cerebral toxoplasmosis and one due to bacterial sepsis and haemorrhage. 2 deaths were due to relapsed or refractory disease. All deaths occurred in the AML subgroup. The relapse-related deaths occurred in the setting of intermediate and adverse risk AML respectively.

The cumulative incidence of relapse was 6.6% at 6-months and 16.6% at 1-year. There were 5 cases of relapse, occurring at a median of 197 days, range 85 – 233. 3 relapses occurred in AML, 1 in MDS and 1 in MF. The IPSS-M risk group was high in the MDS-related relapse and the DIPSS-Plus was intermediate-2, but MIPSS-70 high, in the MF-related relapse. The median age in relapsed patients was 58, as compared to 60 in patients who did not relapse. Overall RFS at 6-months and 1-year respectively was 87.5% and 78.1% respectively. Best response by CIBMTR criteria was CR in 25, CR with incomplete haematological recovery in 1, improvement in 1, refractory disease in 3 and not assessable in 2.

GVHD

Acute GVHD of any grade occurred in 20/32 patients (64.5%) at a median of 52 days, range 29-190. Grade II-IV disease occurred in 15 and grade III-IV in 3. 12 patients developed skin GVHD, 12 gastrointestinal and 6 liver. The median duration of steroid treatment was 116 days. 5 patients exhibited steroid—refractory or sub-optimally responsive disease and required the addition of ruxolitinib. Chronic GVHD developed in 4 patients by last follow up with onset at a median of 280 days. Of those 4 patients, 2 had moderate and limited disease, 1 moderate and extensive and 1 severe and extensive. No GVHD-related deaths occurred.

EASIX

The EASIX score was recorded at the pre-conditioning, day 0, GVHD onset and 1-year post-transplant time-points and is summarized in Table 25. Relapse and survival outcomes were not calculated for the 1-year timepoint due to follow-up duration. The EASIX score was not statistically predictive of transplant outcomes in this smaller sub-cohort of patients due to number of events and therefore pooling of data with the historical group described in Chapter 1 will be addressed subsequently.

Table 25: EASIX

	Mean	Mean + 1 SD	Median	25 th centile	75 th centile
Pre-conditioning	2.9	5.63	1.7	0.98	4.45
Day 0	15	29.64	11.25	5.84	20.8
GVHD onset	3.99	9.86	2.44	1.27	4.32
1-year	1.53	2.3	1.47	0.84	2.19

Chimerism and DLI

Complete myeloid donor chimerism was seen in 26/31 patients at day 45, and 8/31 had a complete T cell donor chimerism. At day 90 full myeloid donor chimerism was seen in 25/30 patients and 11/26 had a full T cell fraction donor chimerism.

A total of 16 DLI doses were delivered to 11 patients at a median of 183 days, range 121 – 435 days. 7 patients received a pre-emptive DLI for mixed chimerism beyond day 90. 3 received a therapeutic DLI as part of a salvage strategy post-relapse. 1 patient received a prophylactic DLI based on disease risk-profile in the presence of a full donor chimerism. 2 patients who did not experience acute GVHD pre-DLI developed GVHD subsequently. 5 patients had a rise in donor chimerism post-DLI, 1 was unchanged and 2 had a further decrease.

Immune reconstitution

The reconstitution of absolute lymphocytes, T cell subsets and immunoglobulins is summarized in Table 26. The median CD4⁺ count at day 100 was 58 x 10⁶/kg, range 3 - 272. 18/28 patients recovered CD4⁺ counts to > 50 and 6/28 had a CD4⁺ count of over 100 at this time-point. By day 180, the median CD4⁺ count was 95, with a range of 19-318, and by 1 year this was 128, range 26-503. The median IgG level at days 100, 180 and 365 was 8.46, 7.69 and 8.27 g/L respectively. Transient monoclonal gammopathy was detected in 6/30 patients.

Table 26: T cell Immune recovery

	Median	Range
ALC x 10⁹/L		
ALC day 100	0.65	0.2 – 1.4
ALC day 180	0.85	0.3 – 3.7
ALC day 365	0.95	0.2 – 3.4
CD4⁺ x 10⁶/kg		
CD4⁺ day 100	58	3 – 272
CD4⁺ day 180	95	19 – 318
CD4⁺ day 365	128	26 – 503
IgG g/L		
IgG day 100	8.46	2.21 – 16.0
IgG day 180	7.69	1.52 – 18.71
IgG day 365	8.27	3.1 – 20.72

Infections

21/32 patients were CMV donor/recipient serostatus negative/negative, 8 positive/positive, 2 positive/negative and 1 negative/positive. All CMV seropositive recipients received letermovir prophylaxis until a minimum of day 100. CMV re-activation was detected in 3/32 patients. All cases occurred in positive/positive pairs. EBV was detected in 10/32 with no cases of PTLD. ADV was detected in 5 patients. 5 patients developed clinical HSV infection, 1 developed *pneumocystis jirovecii* pneumonia, 2 developed BK cystitis, 10 developed Sars-Cov-2 and no patient developed invasive fungal disease.

Results - Pooled clinical data

Demographics

In the pooled dataset including the retrospective and contemporaneous treatment groups there were 175 patients from 14 national and cross-border centres (Fig. 124). Summary characteristics are listed in Table 27. Both groups were treated using the same protocol and supportive care algorithm except for universal adoption of letermovir prophylaxis for CMV seropositive recipients in the latter group. No HLA mismatched alloSCTs occurred in the latter group as institutional preference in this setting switched to a PTCY-based platform in the intervening period. The median donor age in the pooled dataset was 38 and 98/175 donors were unrelated. Disease classifications for the latter group of patients were grouped as per the 2016 WHO classification when comparing to the historical cohort.

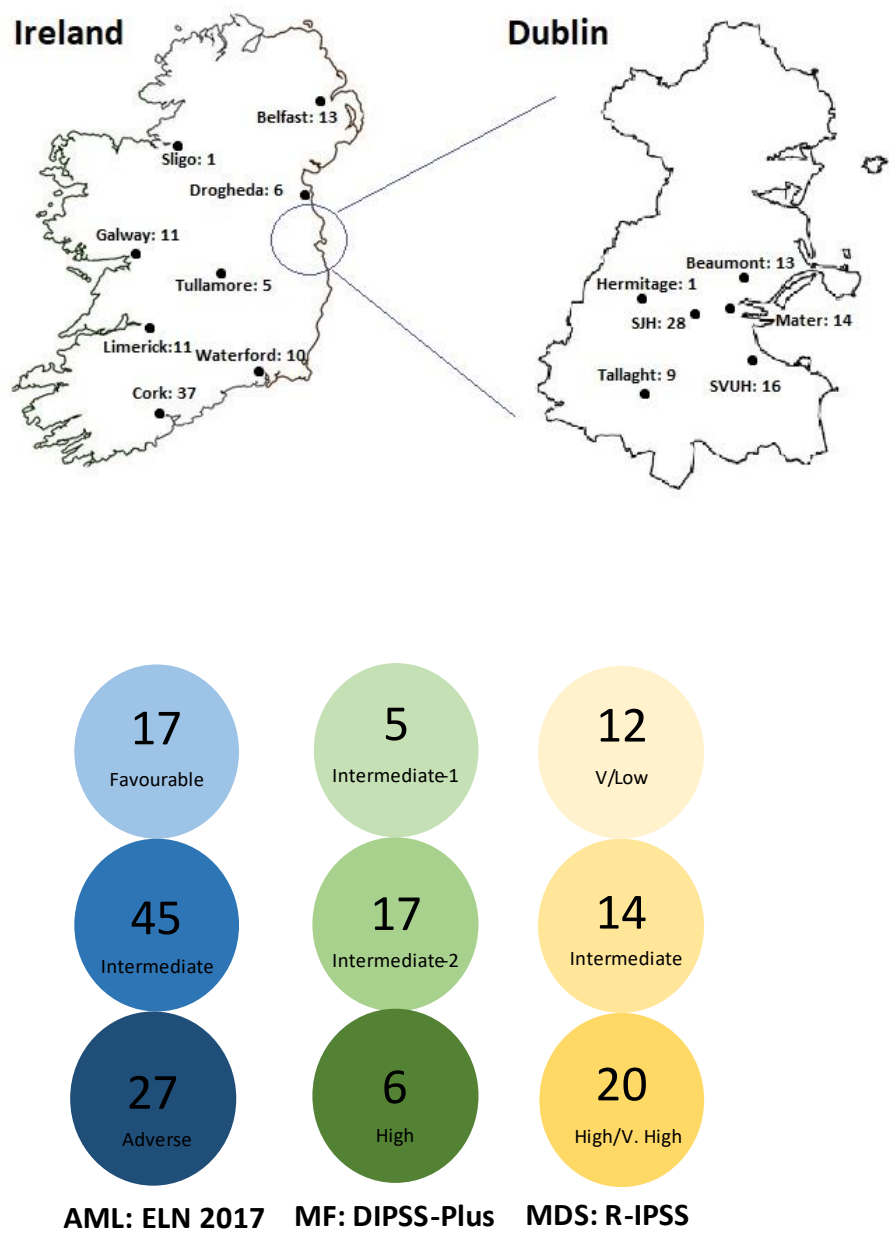


Figure 124: Referral sources and disease risk categories.

Table 27: Pooled demographics	
Age at transplant	Median: 60, range 27 – 74
Age above 65	27/175
Male: Female	106 : 69
AML	89
MDS	46
MPN	40
CIBMTR-DRI High	54/175
KPS	Median 80, range 50 – 100
Age-adjusted HCT-CI	Median, range 1 – 8
Donor source	Sibling 77, Unrelated 98
Graft cryopreservation	20/175
HLA mismatch	11/175
ABO mismatch	81/175
Donor age	Median 38, range 18 - 67
	Sibling 54, Unrelated 26
Neutrophil engraftment	174/175, median 20 days
Platelet engraftment	169/175, median 18 days

Overall survival

The median overall survival has not been reached at a median follow up of 652 days, range 1 – 2839 (*Fig. 125*). 6-month OS was 89.05%, 80.34% at 1 year, 76.55% at 18 months and 72.35% at 2 years. In AML OS was 90.87%, 81.66%, 77.84% and 76.41% at 6 months, 1 year, 18 months and 2 years respectively. In MDS, OS was 86.96%, 73.54%, 71.09% and 64.63% at the same time-points. In MPN the OS for these time-points was 87.5%, 85%, 79.52% and 72.29% respectively. Univariable predictors of mortality include CIBMTR-DRI high risk category (HR 1.74, 95% CI 1.04 – 2.90, $p=0.03$) (*Fig. 126*), high-risk myelofibrosis (HR 4.36, 95% CI 1.34 – 14.17, $p=0.01$) (*Fig. 127*), high graft CD8⁺ content (HR 1.92, 95% CI 1.07 – 3.45, $p=0.02$), chronic GVHD (HR 0.35, 95% CI 0.12 – 0.98, $p=0.02$) (*Fig. 128*), ABO mismatch (HR 2.34, 95% CI 1.30 – 4.15, $p=0.003$) (*Fig. 129*), and pre-emptive or salvage DLI (HR 0.37, 95% CI 0.19 – 0.73, $p=0.002$) (*Fig. 130, 131*) (Table 26, *Fig. 132 and 133*). Survival was similar across both pre-conditioning and day of ATLG absolute lymphocyte counts (Unquantifiable vs. quantifiable ALC pre-ATLG: HR 0.91, 95% CI 0.50 – 1.68, $p=0.78$).

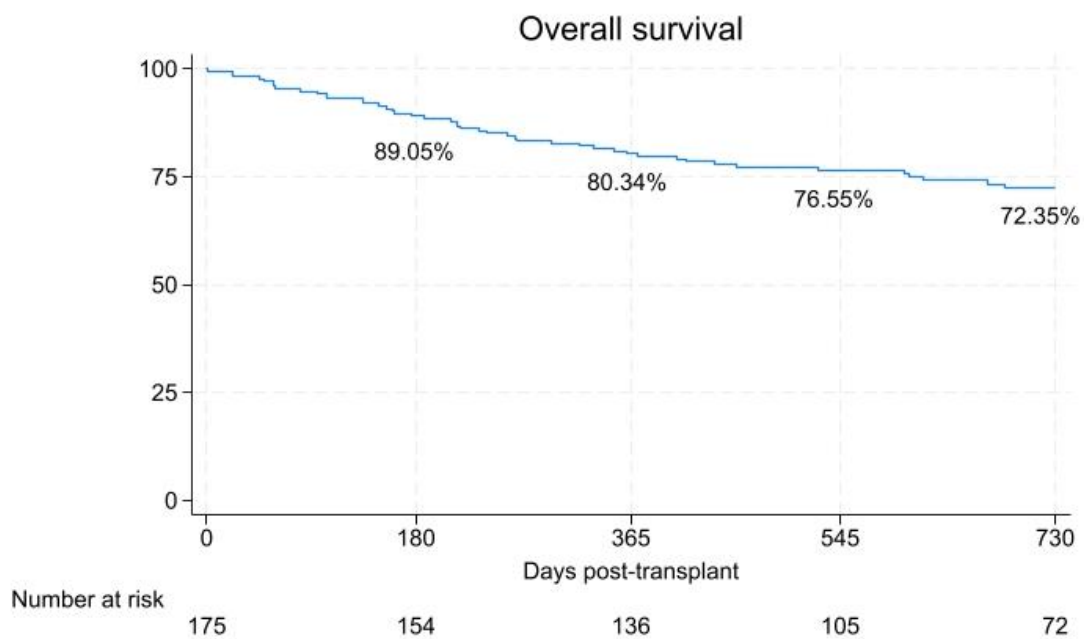


Figure 125: Overall survival.

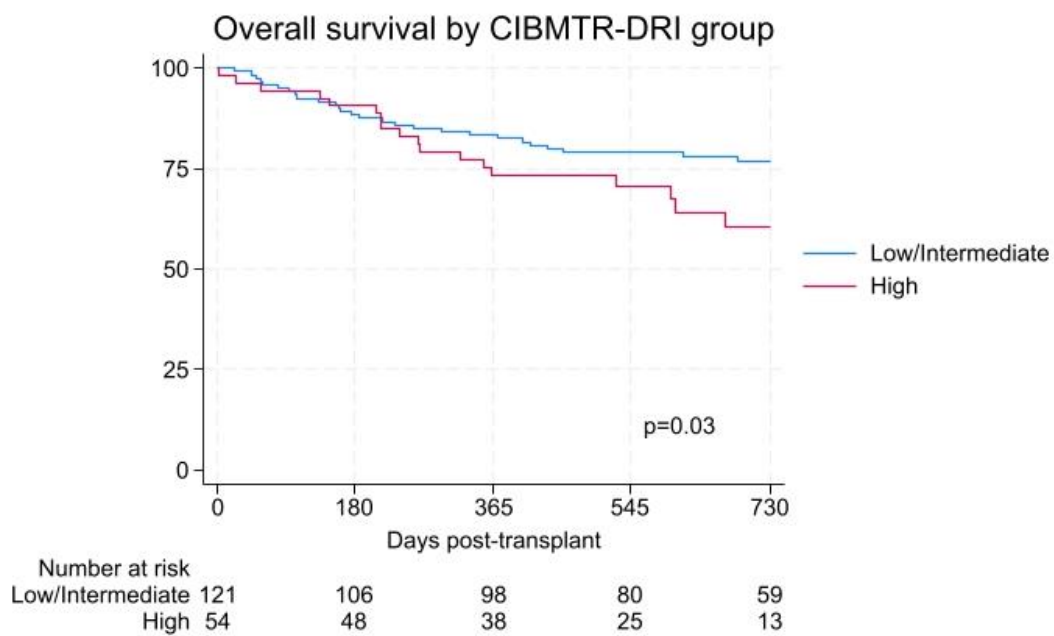


Figure 126: Overall survival by CIBMTR-DRI risk group; low/intermediate vs. high.

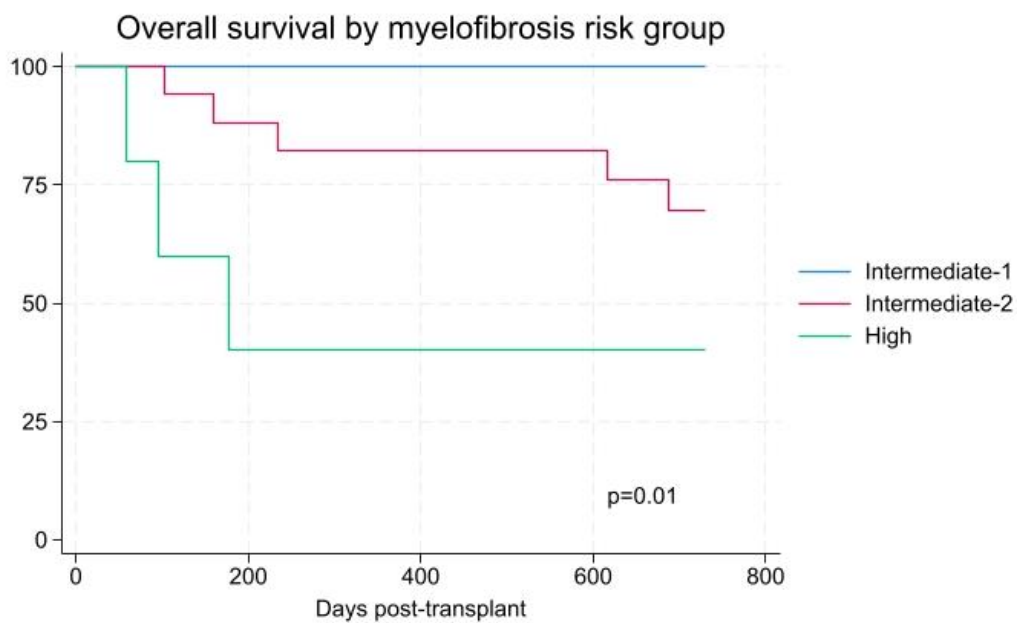


Figure 127: Overall survival by myelofibrosis DIPSS-Plus risk group; intermediate-1, intermediate-2 and high.

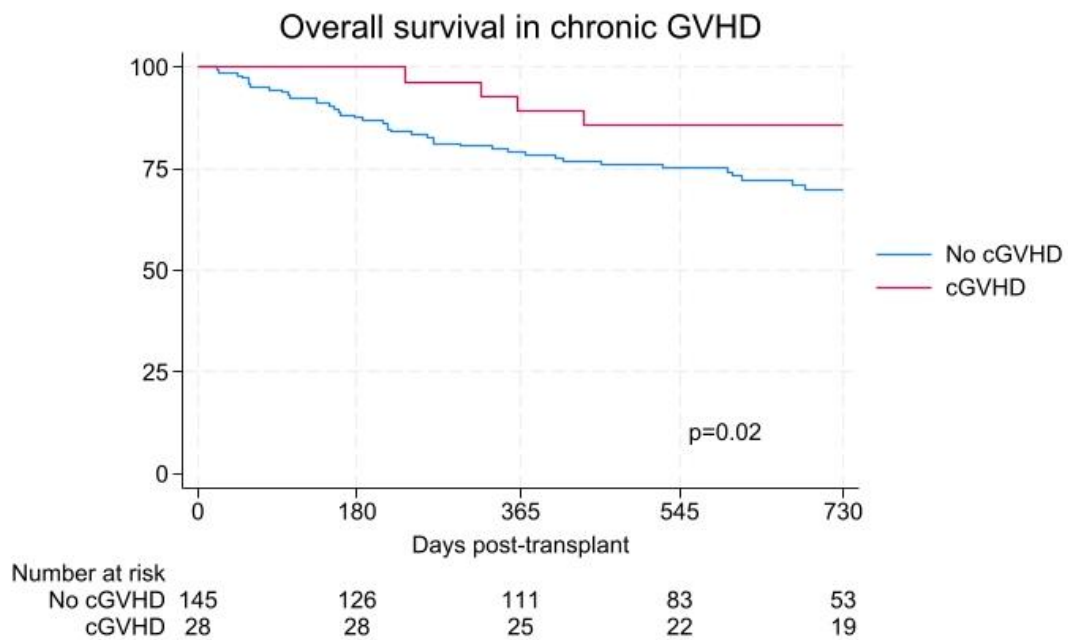


Figure 128: Overall survival in chronic GVHD vs. no chronic GVHD.

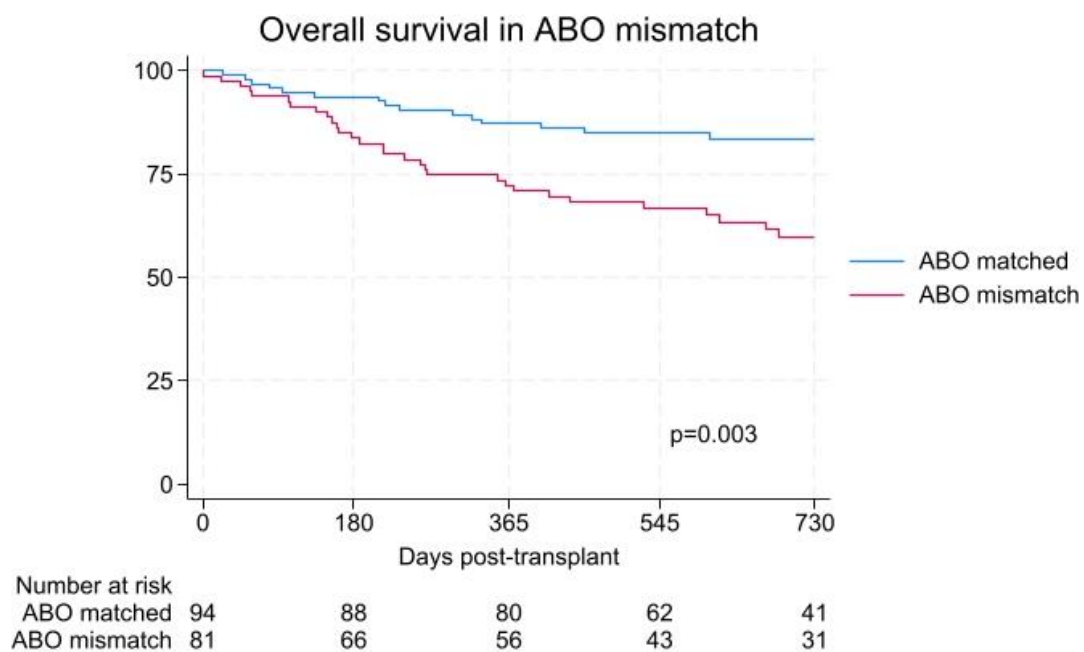


Figure 129: Overall survival in ABO mismatch vs. ABO matched.

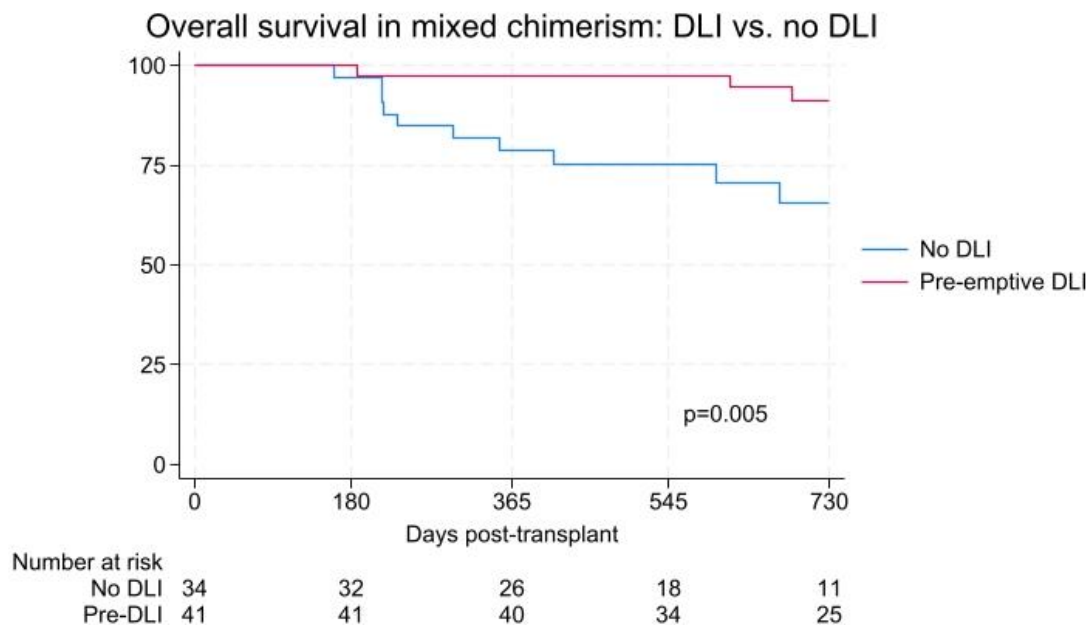


Figure 130: Overall survival in mixed chimerism beyond day 90, pre-emptive DLI vs. no DLI.

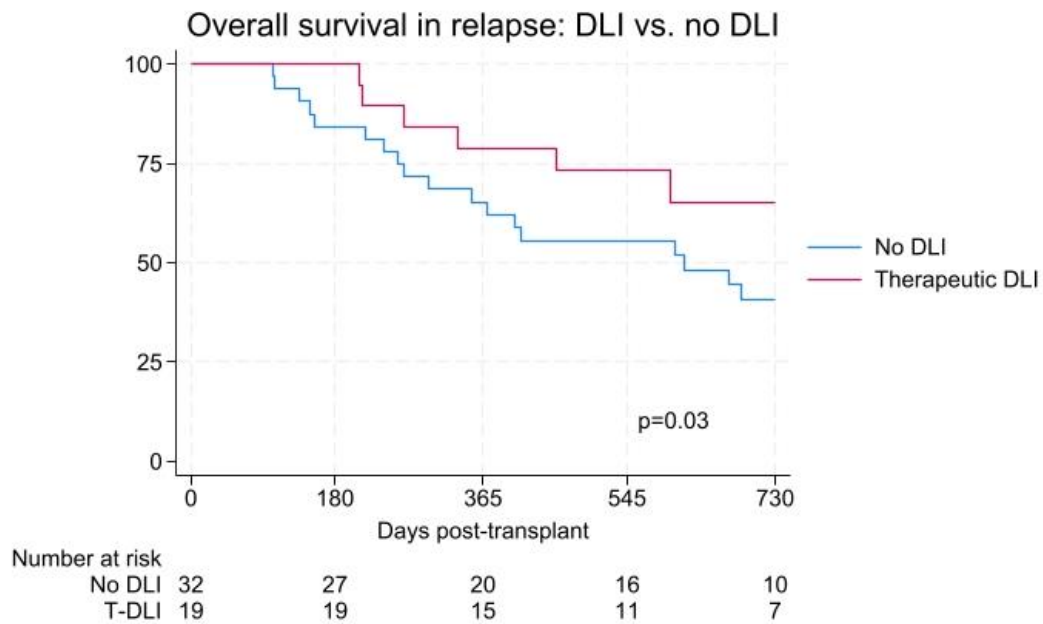


Figure 131: Overall survival in relapse; therapeutic DLI vs. no DLI.

Table 28: Pooled Overall survival

Variable	Univariable analysis			Multivariable analysis		
	Hazard Ratio	95% CI	P value	Hazard Ratio	95% CI	P value
Male recipient	0.79	0.44 – 1.43	0.43			
Recipient age	0.98	0.67 – 1.43	0.91			
Recipient age > 65	0.66	0.29 – 1.46	0.26			
Disease subtype	1.11	0.80 – 1.55	0.52			
KPS > 80	0.84	0.48 – 1.46	0.54			
aaHCT-CI 3+	1.29	0.75 – 2.26	0.35	1.32	0.74-2.36	0.33
CIBMTR DRI	1.74	1.04 – 2.90	0.03	1.52	0.84-2.75	0.16
High						
Myelofibrosis	4.36	1.34 – 14.17	0.01			
high-risk						
Unrelated donor	1.59	0.89 – 2.84	0.10			
Donor age	0.98	0.96 – 1.01	0.18			
Cryopreserved graft	1.02	0.40 – 2.58	0.96			
ABO mismatch	2.34	1.30 – 4.15	0.003	2.11	1.16-3.82	0.01
High graft CD8 ⁺ T cells	1.92	1.07-3.45	0.02	1.83	1.01-3.29	0.04

aGVHD grade II-IV	0.72	0.41 – 1.28	0.26			
aGVHD grade III-IV	1.77	0.83 – 3.79	0.16			
cGVHD	0.36	0.12 – 0.98	0.04	0.32	0.11-0.92	0.03
DLI (all indications)	0.38	0.19 – 0.74	0.002			
Pre-emptive DLI	0.22	0.07 – 0.69	0.005			
Salvage DLI	0.43	0.18 – 0.98	0.03			

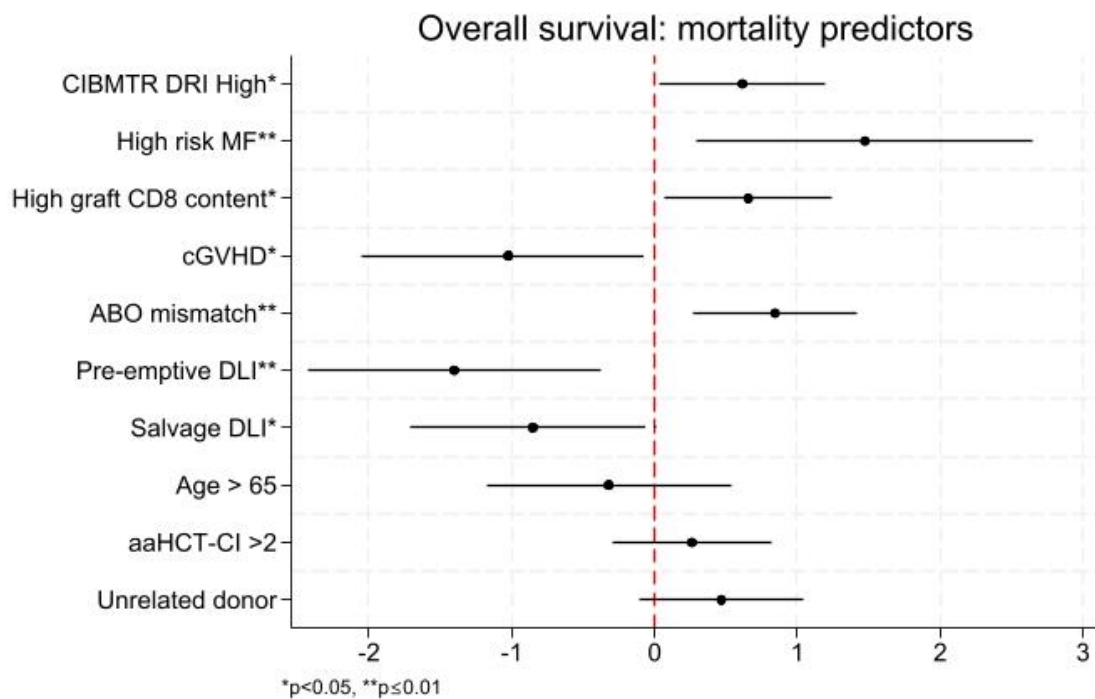


Figure 132: Coefficient plot of univariable survival analysis.

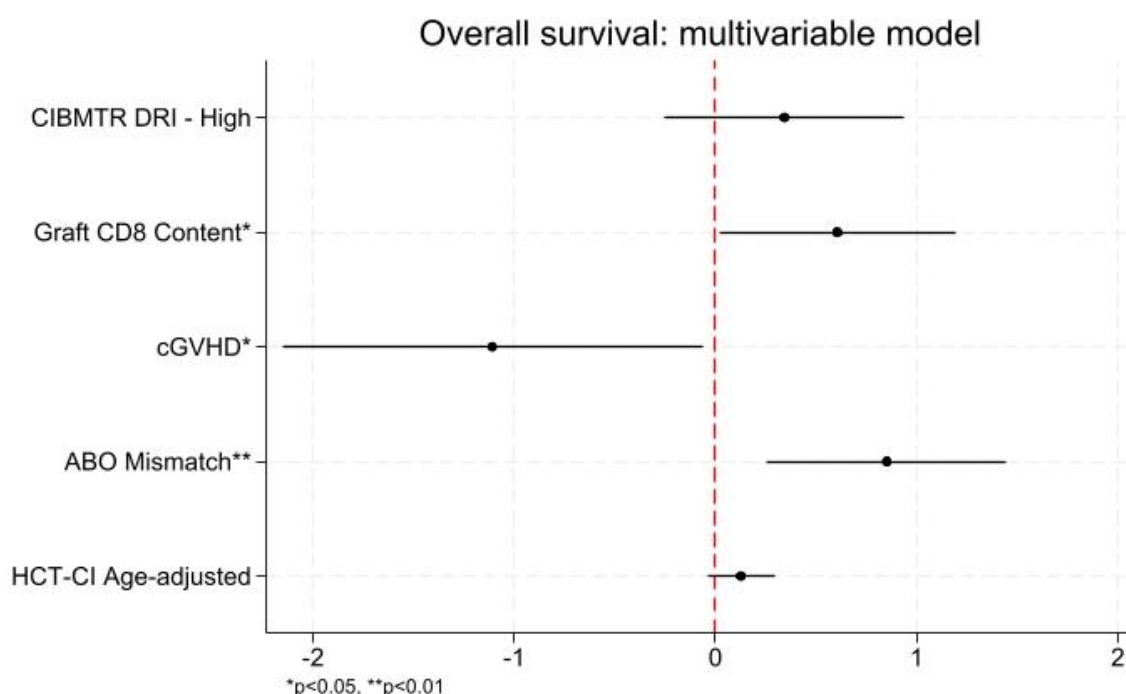


Figure 133: Coefficient plot of multivariable survival analysis.

Non-relapse mortality

NRM occurred in 19 patients at a median of 134 days (*Fig. 134*). The cumulative incidence of NRM at 1 and 2 years was 10.4% and 11% respectively. In AML the cumulative risk of NRM was 5.5%, 7.5%, 7.8% and 7.8% at 6 months, 1 year, 18 months and 2 years respectively. In MDS the cumulative risk at those same time-points was 7.9%, 10.9%, 11.5% and 11.5%. In MPN the cumulative risk of NRM was 11.5%, 15.8%, 16.8% and 16.8% respectively. The 2 year NRM risk in the myelofibrosis subgroup of MPNs was 17.5%. Univariable predictors of NRM included high-risk myelofibrosis (HR 8.61, 95% 1.91 – 38.69, $p=0.005$) (*Fig. 135*), grade III-IV acute GVHD (HR 5.29, 95% CI 2.15 – 13.04, $p=0.0003$) (*Fig. 136*), minor ABO mismatch (HR 3.33, 95% 1.62 – 8.84, $p=0.01$) (*Fig. 137*) and invasive fungal disease (HR 8.00, 95% CI 3.11 – 20.60, $p < 0.001$) (*Fig. 138*) (Table 29).

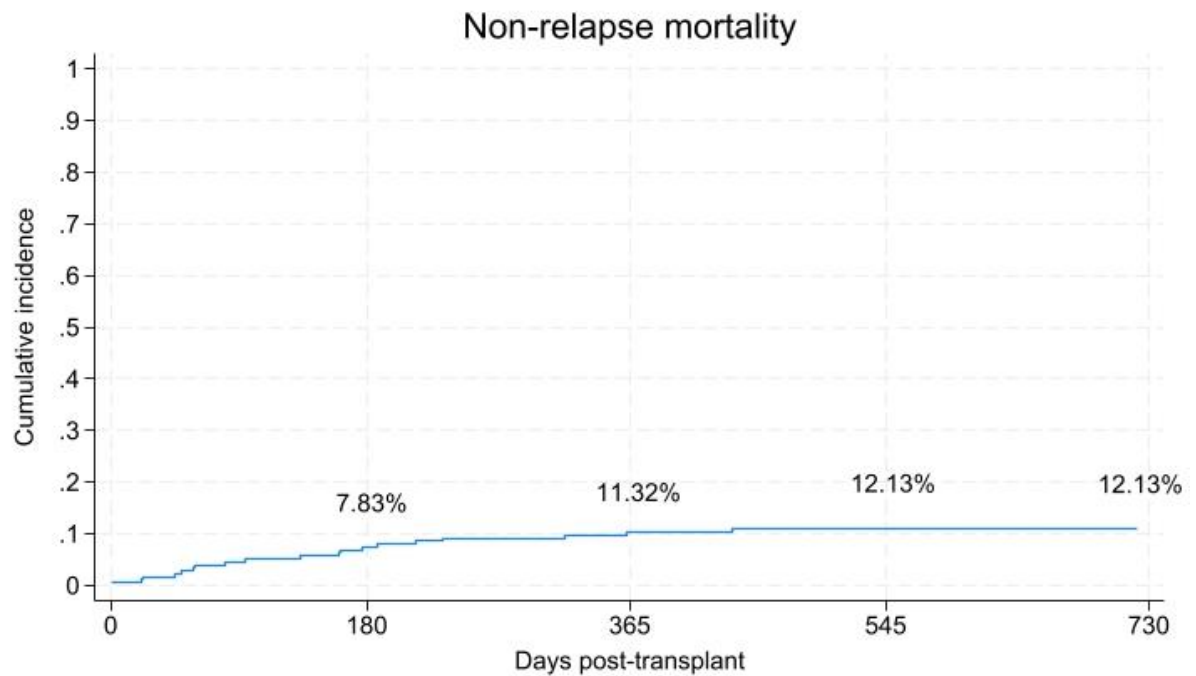


Figure 134: Non-relapse mortality cumulative incidence.

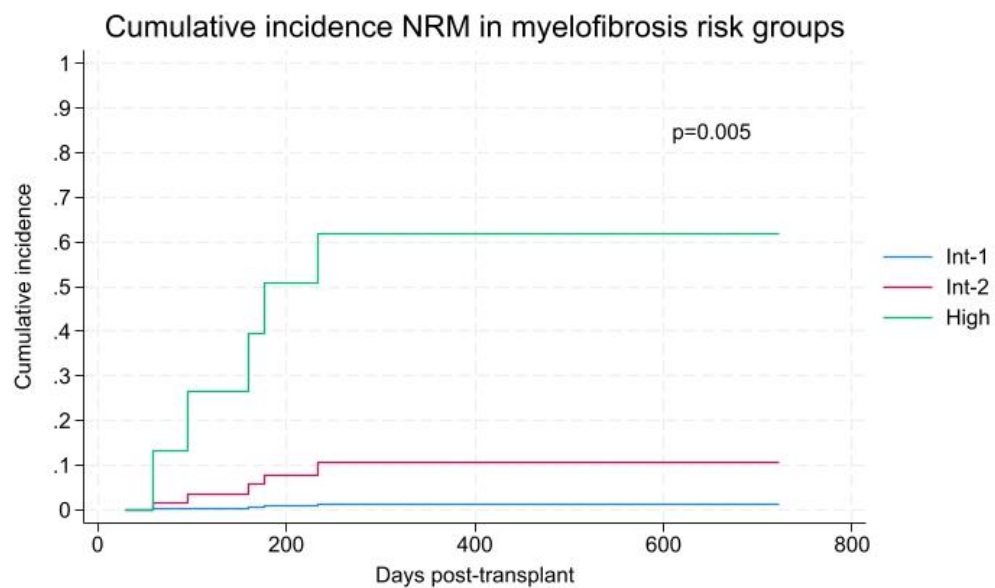


Figure 135: Non-relapse mortality cumulative incidence in myelofibrosis stratified by DIPSS-Plus risk group; intermediate-1, intermediate-2 and high.

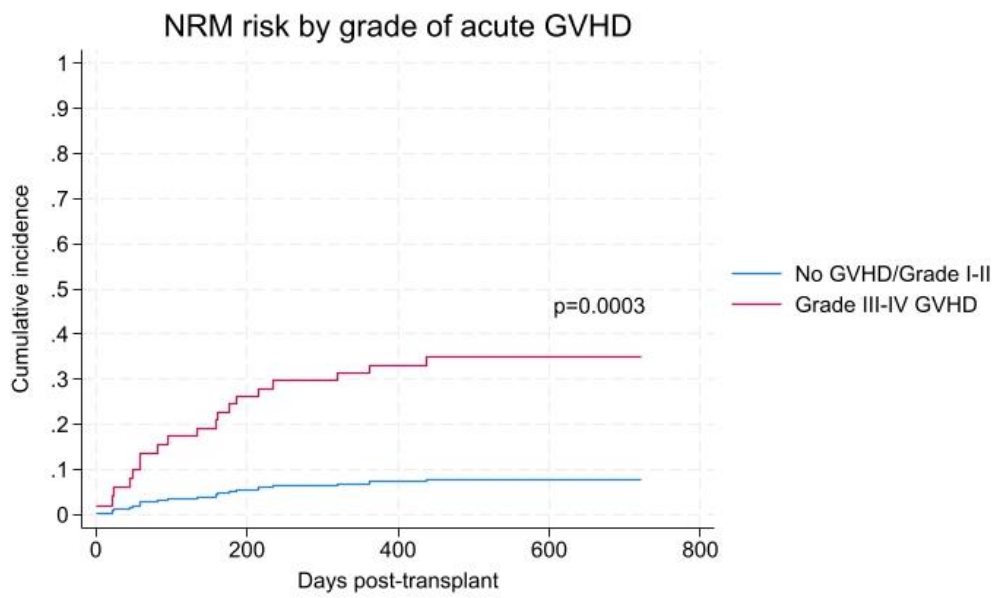


Figure 136: Non-relapse mortality in acute GVHD; no GVHD or grade I-II vs. grade III-IV.

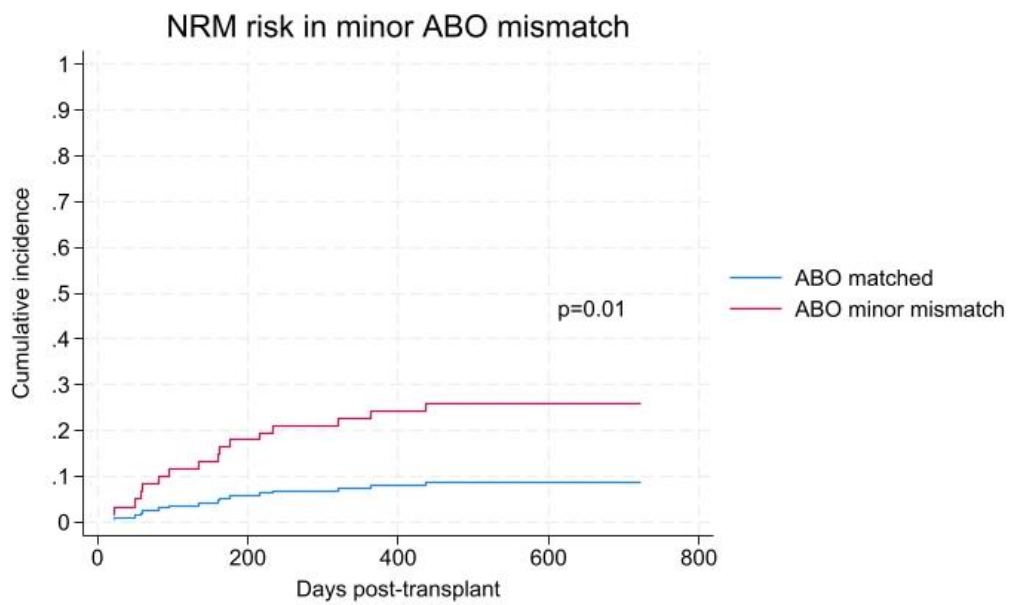


Figure 137: Non-relapse mortality in minor ABO mismatch vs. ABO matched.

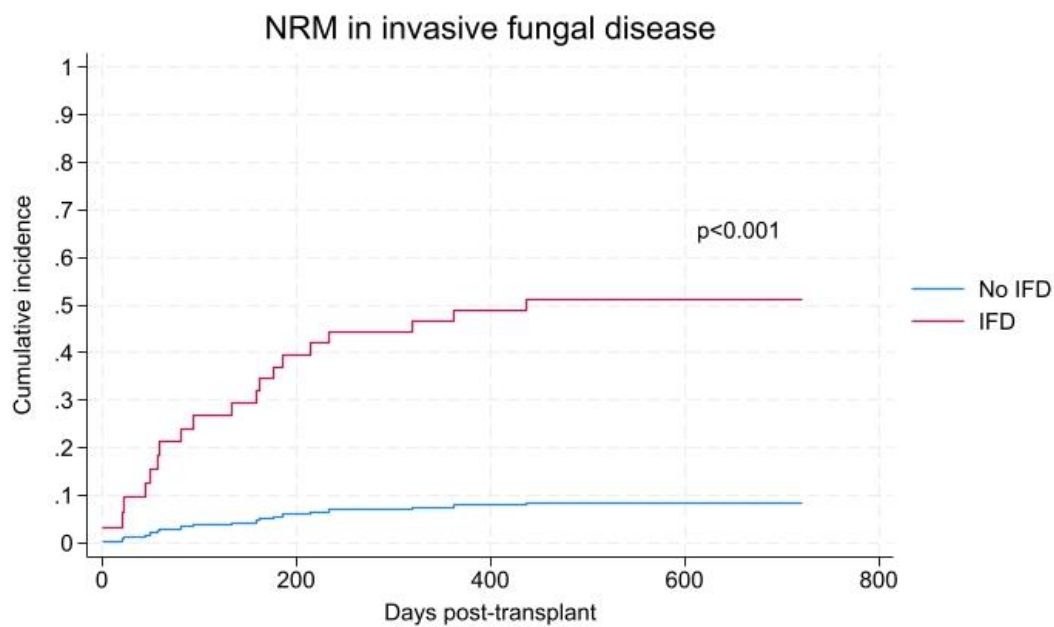


Figure 138: Non-relapse mortality in invasive fungal disease.

Table 29: Pooled Non-relapse mortality			
Variable	Hazard ratio	95% CI	P value
Male age	0.89	0.35 – 2.27	0.81
Recipient age	0.84	0.53 – 1.34	0.47
Disease subtype	1.50	0.95 – 2.37	0.07
High risk myelofibrosis	8.61	1.91 – 38.69	0.005
KPS > 80	1.11	0.45 – 2.72	0.81
aaHCT-CI 3+	1.77	0.71 – 4.40	0.21
Unrelated donor	1.82	0.69 – 4.72	0.22
Donor age	0.97	0.94 – 1.01	0.13
Graft cryopreservation	0.40	0.56 – 2.88	0.36
Minor ABO mismatch	3.33	1.26 – 8.84	0.01
aGVHD grade II-IV	1.01	0.41 – 2.47	0.98
aGVHD grade III-IV	5.29	2.15 – 13.04	0.0003
cGVHD	1.40	0.49 – 4.04	0.53
Invasive fungal disease	8.00	3.11 – 20.60	<0.001
IgG < normal at D180	6.04	1.06 – 34.43	0.04

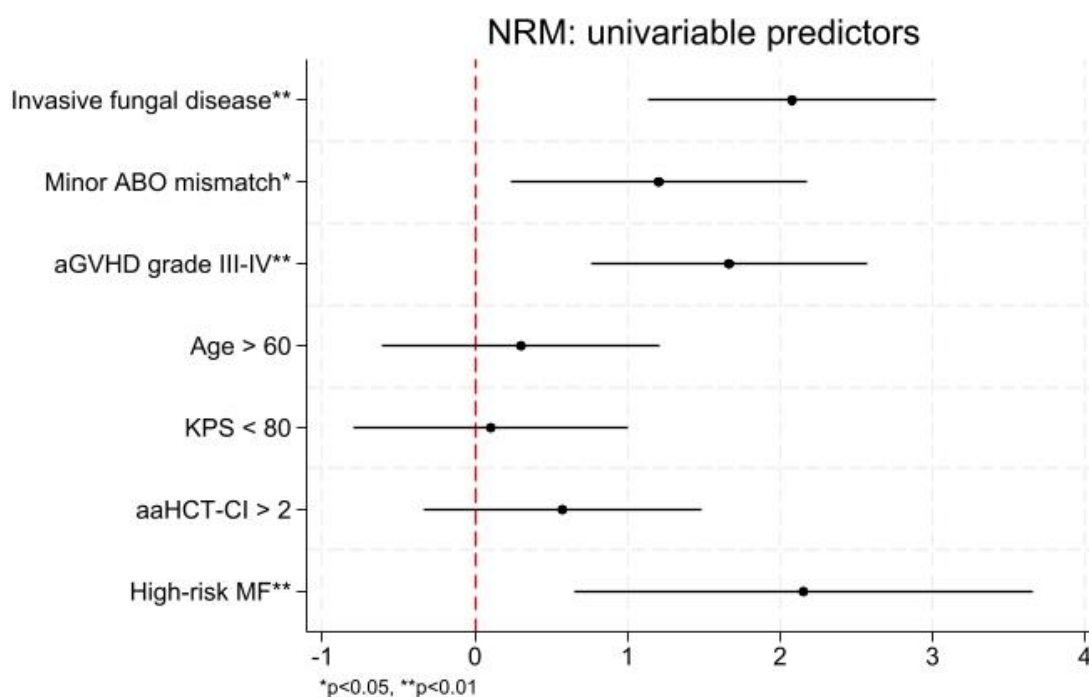


Figure 139: Coefficient plot of univariable analysis in NRM.

Relapse

Relapse occurred in 53 patients at a median of 204 days, range 30 – 2186 (Fig. 140). 24 relapses occurred within the AML group, 16 in MDS and 13 in MPN. The cumulative incidence of relapse was 19.5% and 27% at 1 and 2 years respectively. The cumulative incidence of relapse in AML at 6 months, 1 year, 18 months and 2 years respectively was 10.5%, 18%, 23% and 25% respectively. In MDS at the same time-points the risk was 11.5%, 19.5%, 25% and 27.5%. In MPN the risk was 13.5%, 22.5%, 28% and 30.5% respectively. Univariable predictors of relapse included CIBMTR DRI high-risk disease (HR 1.86, 95% CI 1.18 – 2.94, $p=0.007$) (Fig. 141), high-risk AML (HR 1.82, 95% CI 1.05 – 3.19, $p=0.03$) (Fig. 142), high-risk MDS (HR 2.07, 95% CI 1.04 – 4.15, $p=0.03$) (Fig. 143), high graft T cell content (HR 2.16, 95% CI 1.23 – 3.82, $p=0.002$) (Fig. 144-147), chronic GVHD (HR 0.40, 95% CI 0.17 – 0.94, $p=0.03$) (Fig. 148), cryopreserved stem cell graft (HR 2.38, 95% CI 1.11 – 5.10, $p=0.02$) (Fig. 149), ABO mismatch (HR 1.79, 95% CI 1.04 – 3.08, $p=0.03$) (Fig. 150) and pre-emptive DLI use in mixed chimerism beyond 90 days (HR 0.33, 95% CI 0.15 – 0.80, $p=0.01$) (Fig. 151) (Table 30, Fig. 152 and 153). Relapse was not affected by pre-ATLG lymphocyte count at any cut-off (unquantifiable vs. quantifiable ALC: HR 1.47, 95% CI 0.78 – 2.77, $p=0.22$).

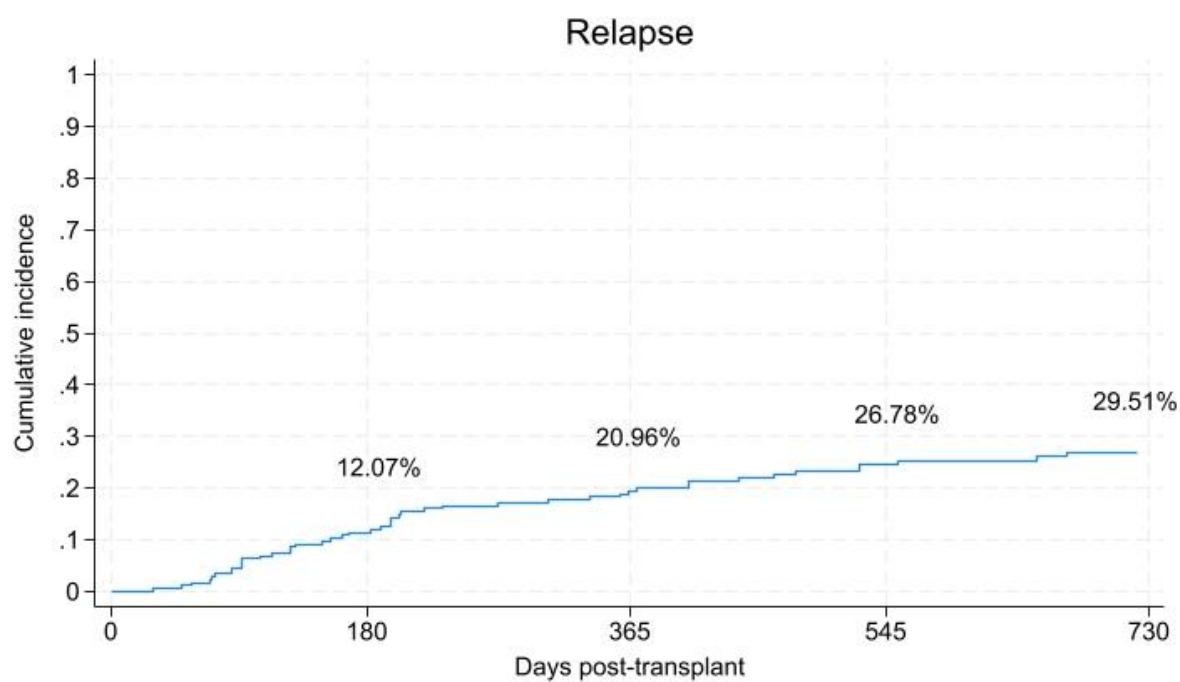


Figure 140: Relapse cumulative incidence.

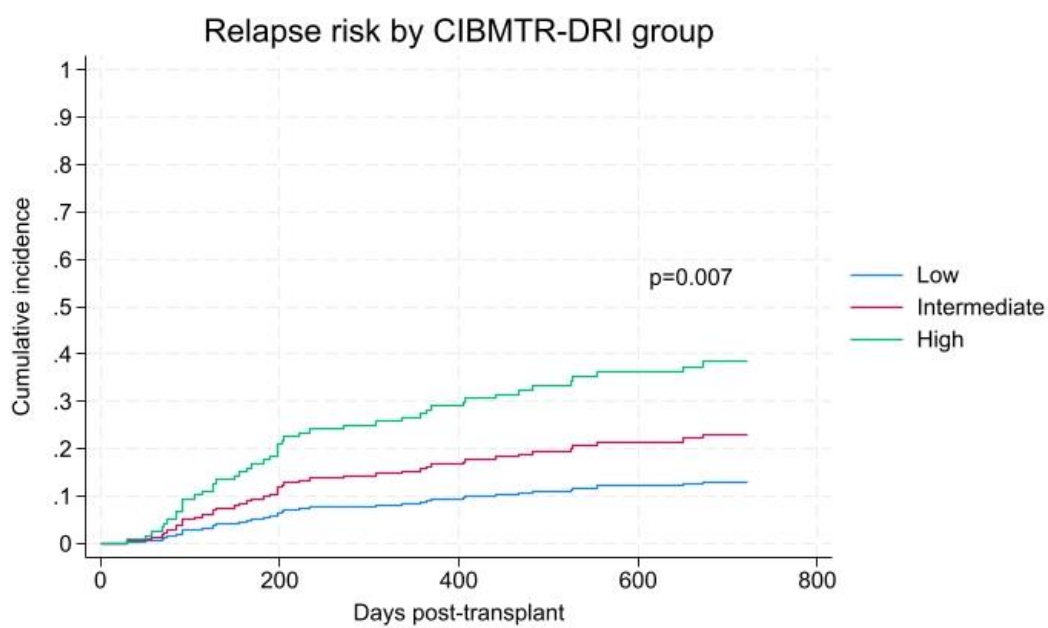


Figure 141: Relapse cumulative incidence stratified by CIBMTR-DRI risk group; low, intermediate and high.

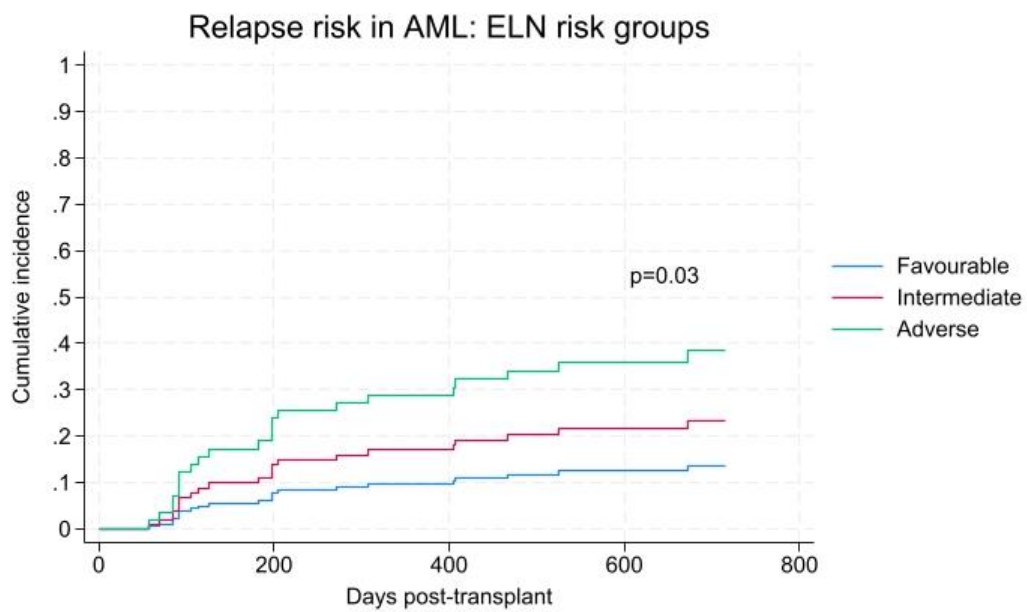


Figure 142: Relapse cumulative incidence in AML stratified by ELN 2017 prognostic group.

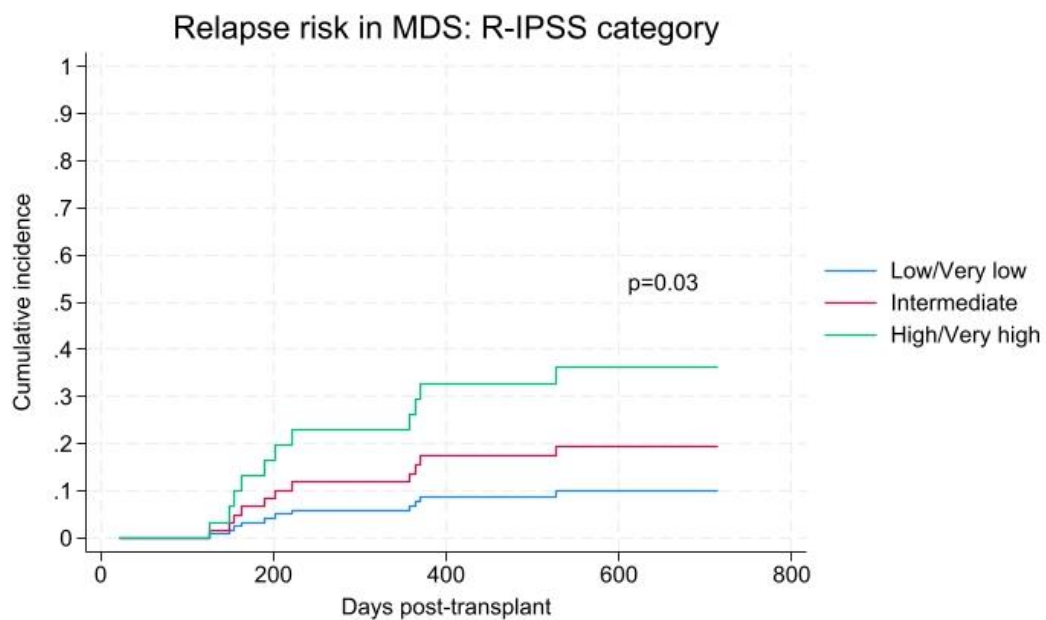


Figure 143: Relapse incidence in MDS stratified by R-IPSS risk group.

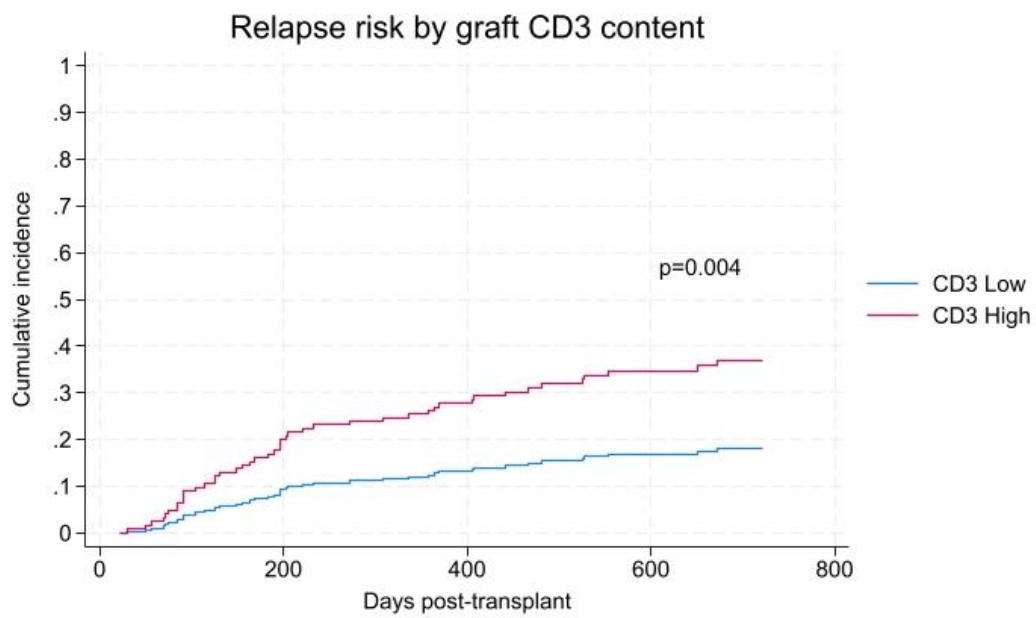


Figure 144: Relapse risk by graft CD3⁺ T cell content.

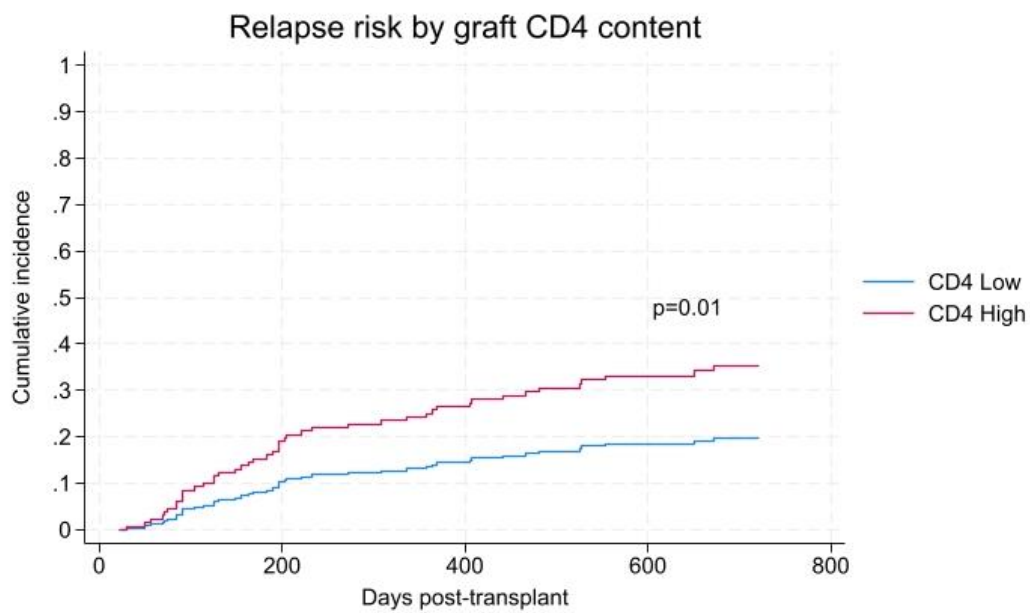


Figure 145: Relapse risk by graft CD4⁺ T cell content.

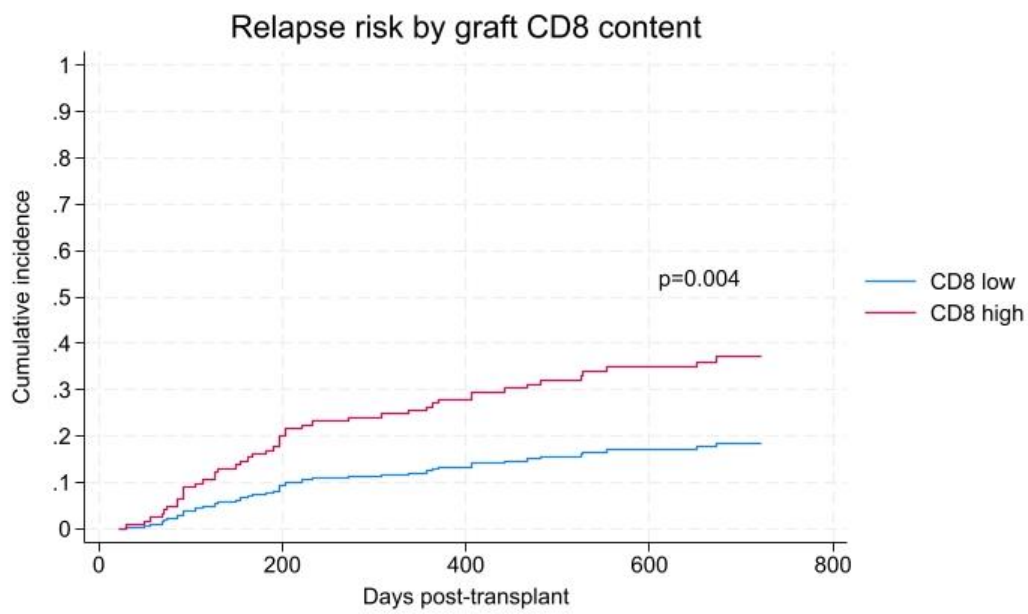


Figure 146: Relapse risk by graft CD8⁺ T cell content.

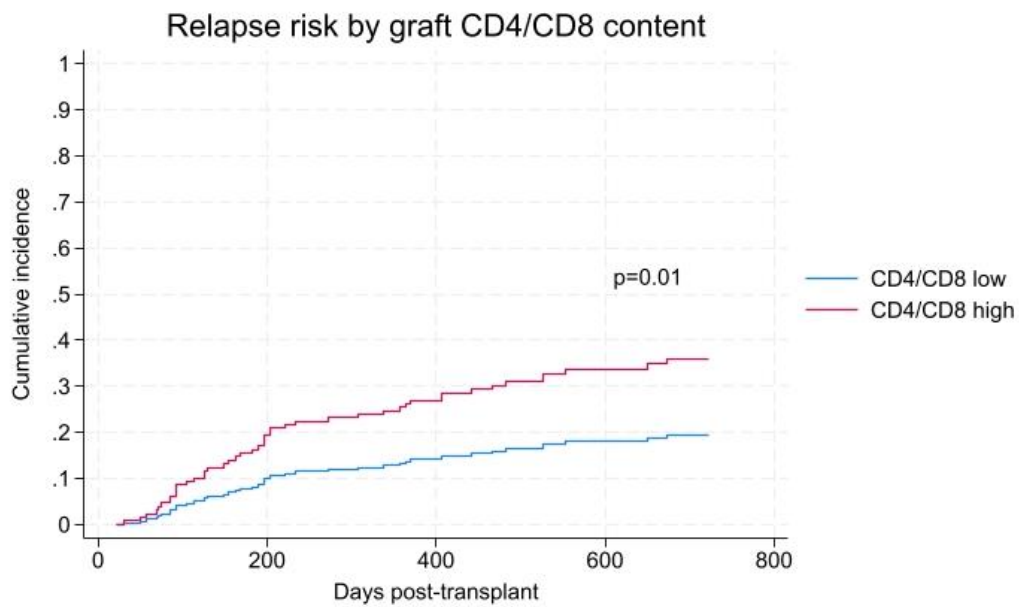


Figure 147: Relapse risk by graft CD4⁺/CD8⁺ T cell content.

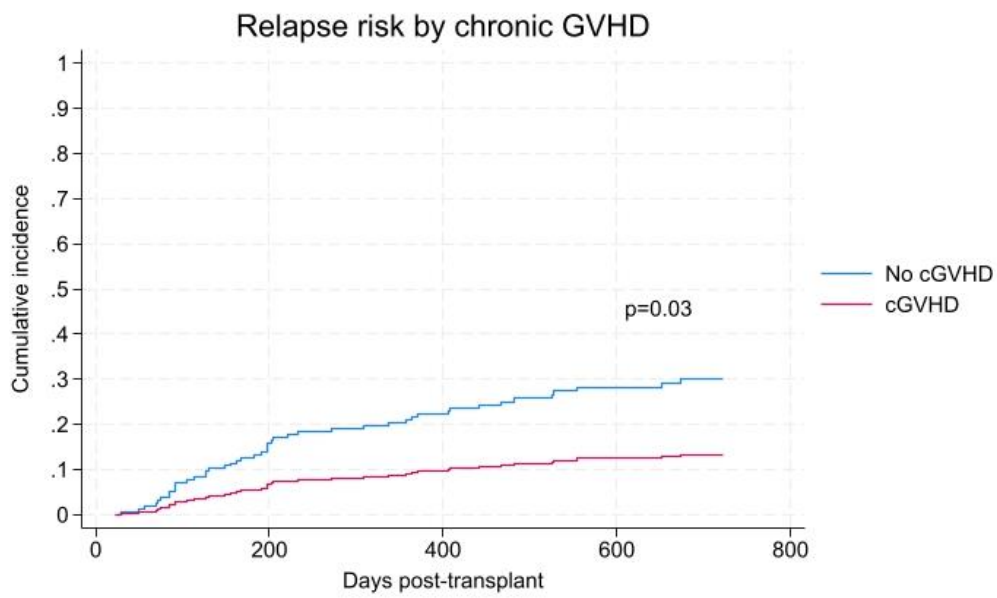


Figure 148: Relapse risk by chronic GVHD; chronic GVHD vs. no chronic GVHD.

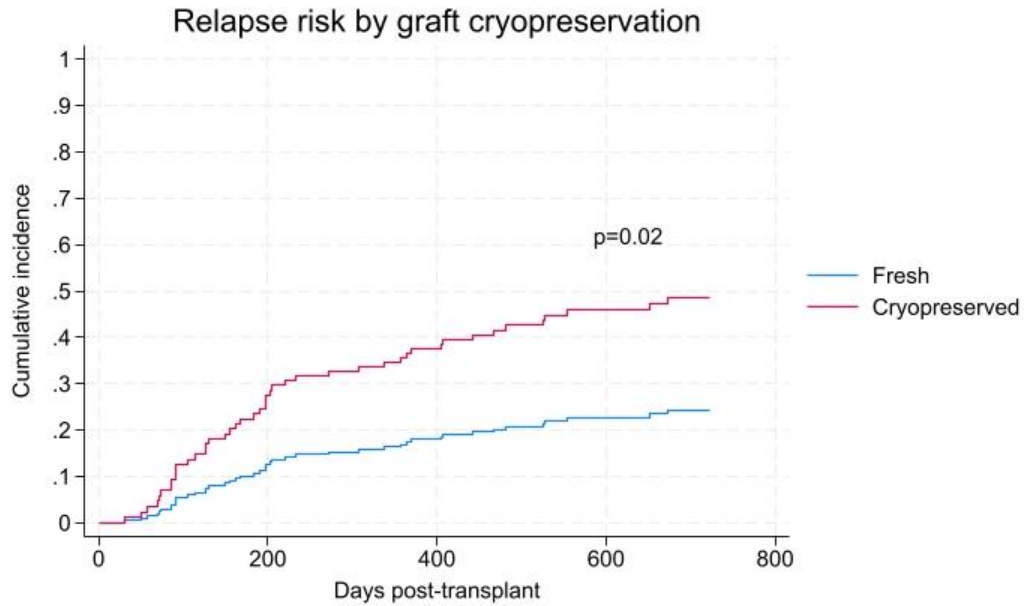


Figure 149: Relapse risk by graft preservation; fresh vs. cryopreserved.

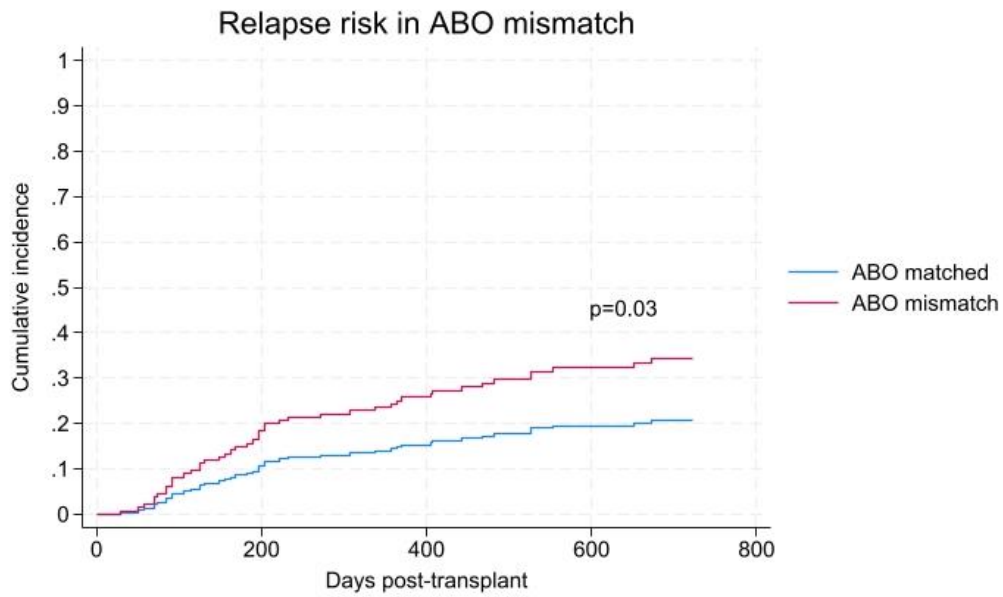


Figure 150: Relapse risk by ABO mismatch status.

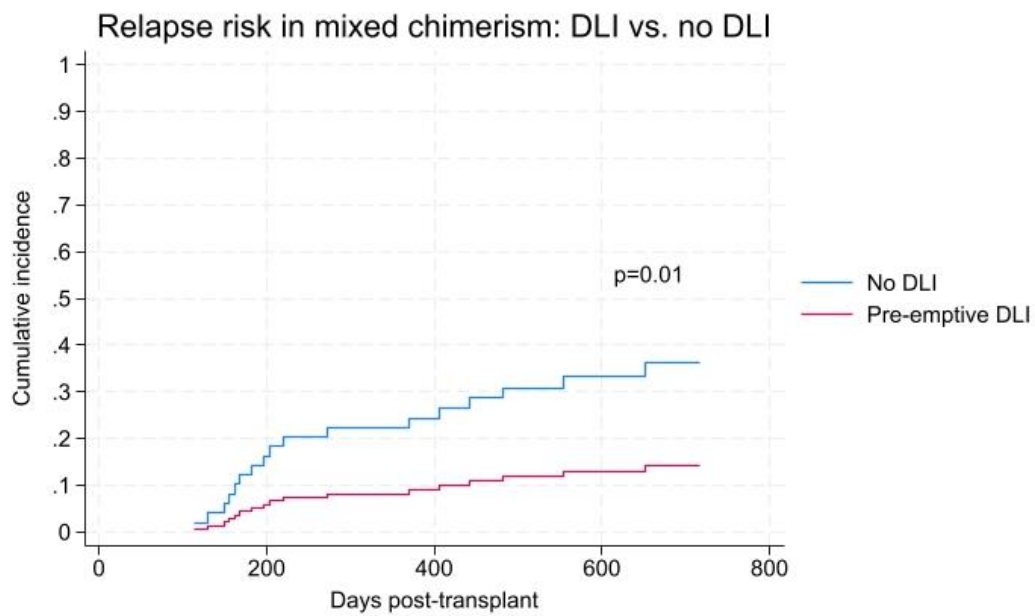


Figure 151: Relapse risk in mixed chimerism beyond day 90; DLI vs. no DLI.

Table 30: Pooled relapse

Variable	Univariable analysis			Multivariable analysis		
	Hazard ratio	95% CI	P value	Hazard ratio	95% CI	P value
Male recipient	0.71	0.39 – 1.26	0.23			
Recipient age	1.03	0.69 – 1.52	0.86			
Disease subtype	1.12	0.80 – 1.56	0.49			
CIBMTR-DRI High	1.86	1.18 – 2.94	0.007	1.71	0.95-3.08	0.07
AML Adverse risk	1.82	1.05 – 3.19	0.03			
MDS High risk	2.07	1.04 – 4.15	0.03			
Unrelated donor	1.10	0.64 – 1.88	0.72			
Donor age	0.99	0.98 – 1.02	0.88			
Graft cryopreservation	2.38	1.11 – 5.10	0.02	3.03	1.42-6.47	0.004
ABO mismatch	1.79	1.04 – 3.08	0.03	1.71	1.01-2.92	0.04
High graft CD3 ⁺	2.3	1.29 – 4.11	0.004	2.19	1.17-4.08	0.01
High graft CD4 ⁺	1.97	1.18 – 3.46	0.01			
High graft CD8 ⁺	2.29	1.29 – 4.06	0.004			
High graft CD4 ⁺ /CD8 ⁺	2.07	1.18 – 3.63	0.01			
ALC at ATG > 0.1	0.78	0.36 – 1.68	0.52			
ALC pre-conditioning	1.10	0.87 – 1.39	0.42			
aGVHD grade II-IV	0.67	0.39 – 1.16	0.15			
aGVHD grade III-IV	0.40	0.13 – 1.20	0.10			
cGVHD	0.40	0.17 – 0.94	0.03	0.32	0.12-0.81	0.01
Mixed chimerism D90	1.41	0.81 – 2.45	0.21			
Pre-emptive DLI	0.33	0.14 – 0.80	0.01			

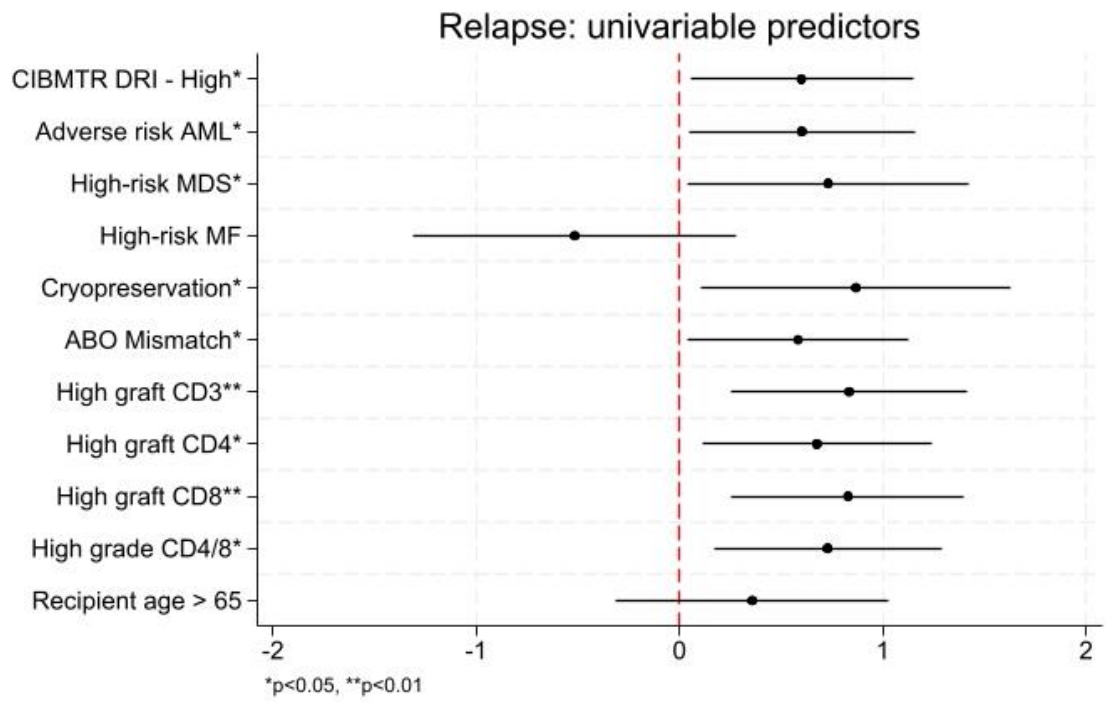


Figure 152: Coefficient plot, univariable analysis of relapse.

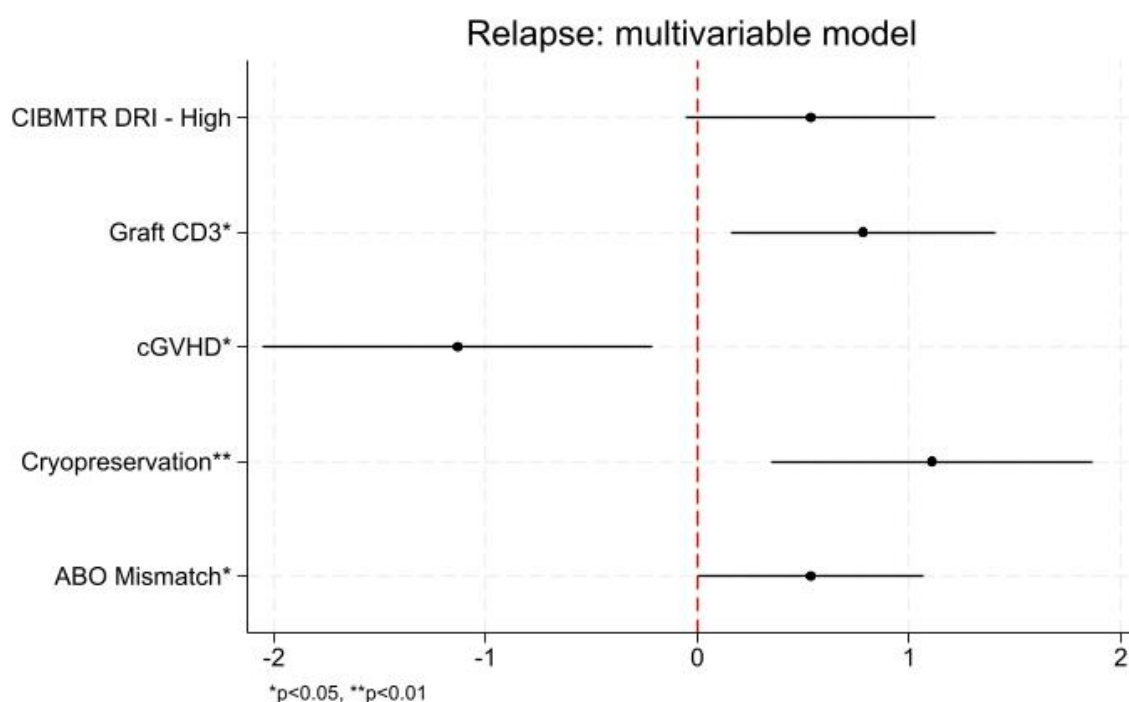


Figure 153: Coefficient plot: multivariable analysis of relapse.

Acute and chronic GVHD

Acute all-grade GVHD occurred in 105/175 patients (60.34%), with 81/105 experiencing grade II-IV disease and 19/105 experiencing grade III-IV disease (Fig. 154, 155). The median time to onset of acute GVHD was 71 days. 83/105 patients required systemic steroids and 19/83 required steroid-sparing treatment. Univariable associations with grade II-IV acute GVHD were tacrolimus use instead of ciclosporin (HR 1.59, 95% CI 1.02 – 2.50, p=0.04) and early calcineurin supratherapeutic levels (HR 4.38, 95% CI 1.34 – 14.23, p=0.01) (Fig. 156). Associations with grade III-IV disease were MDS or MPN rather than AML (HR 2.18, 95% CI 1.31 – 3.51, p=0.001) (Fig. 157), unrelated donors (HR 3.18, 95% CI 1.07 – 9.48, p=0.03) (Fig. 158), tacrolimus use (HR 2.54, 95% CI 1.03 – 6.28, p=0.04) (Fig. 159) and donor age <40 (HR 3.85, 95% CI 1.30 – 12.50, p=0.01) (Fig. 160). Chronic GVHD occurred in 28/175 patients (Fig. 161) and univariable predictors of incidence were prior acute grade II-IV or III-IV GVHD (HR 4.69, 95% CI 1.93 – 11.36, p=0.0006) (Fig. 162) and female donors with male recipients (HR 2.61, 95% CI 1.01 – 6.87, p=0.05) (Fig. 163) (Table 31, Fig. 164-166).

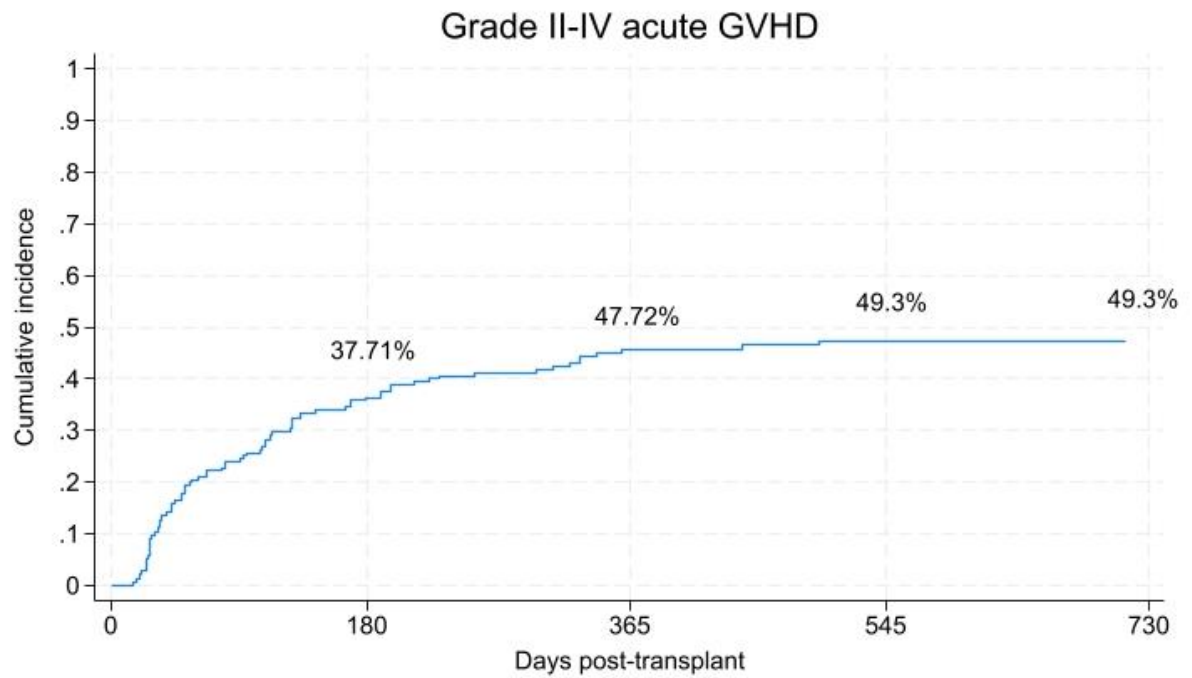


Figure 154: Cumulative incidence of grade II-IV acute GVHD.

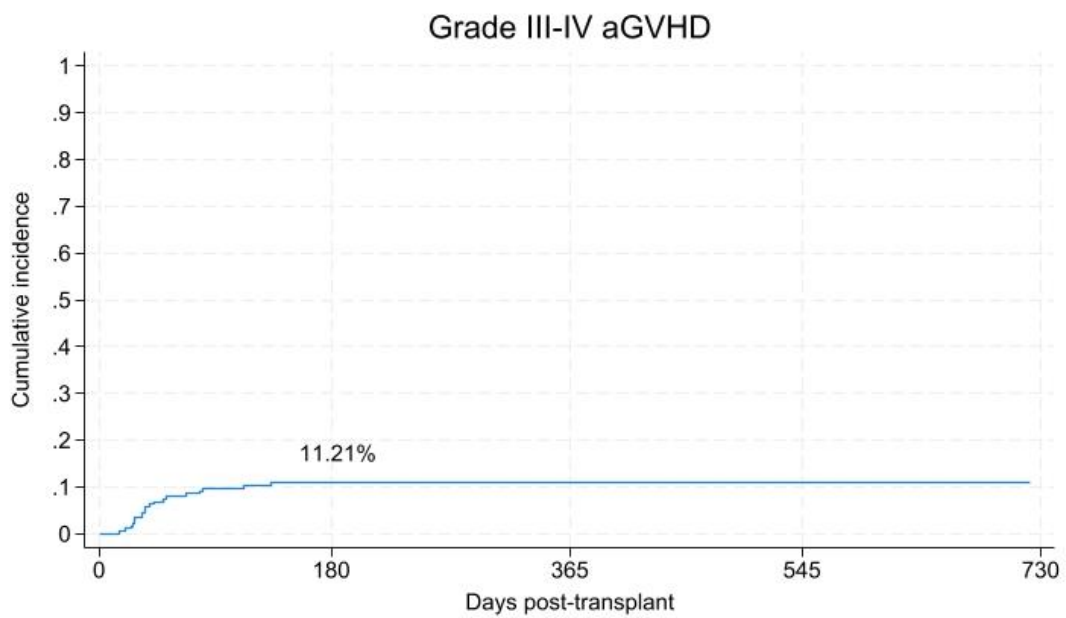


Figure 155: Cumulative incidence of grade III-IV acute GVHD.

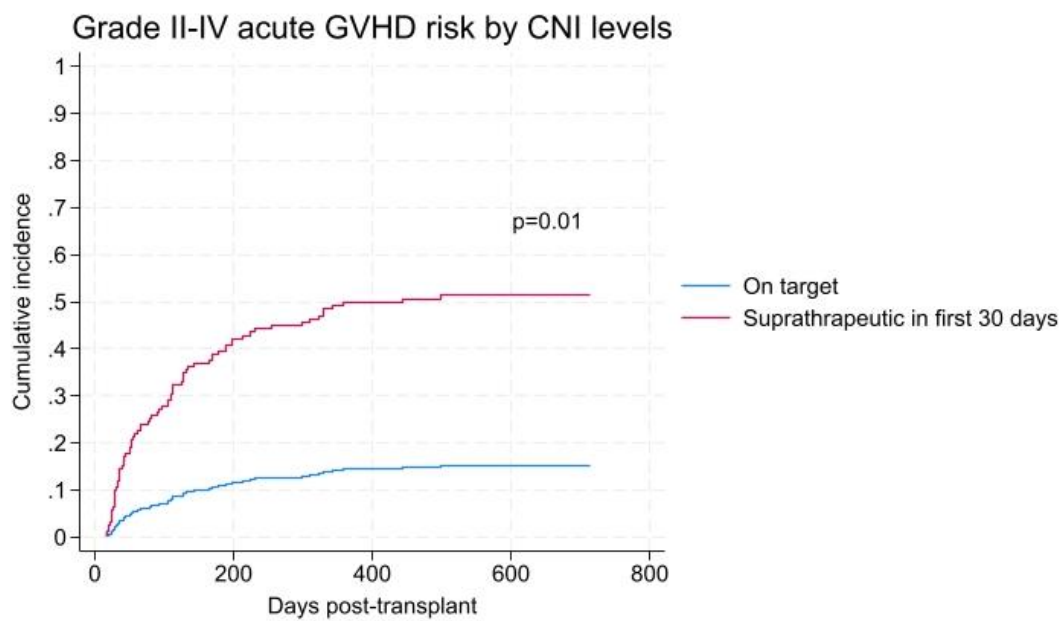


Figure 156: Cumulative incidence of acute GVHD by CNI levels in the first 30 days of transplant; on-target vs. suprathrapeutic on more than one occasion.

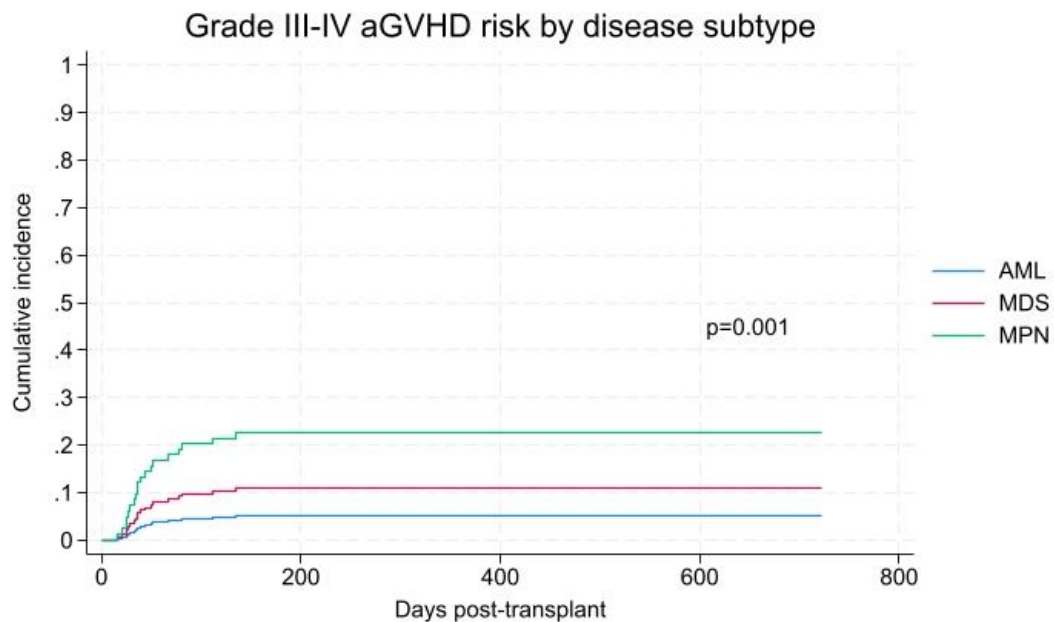


Figure 157: Cumulative incidence of acute grade III-IV GVHD by disease; AML, MDS and MPN.

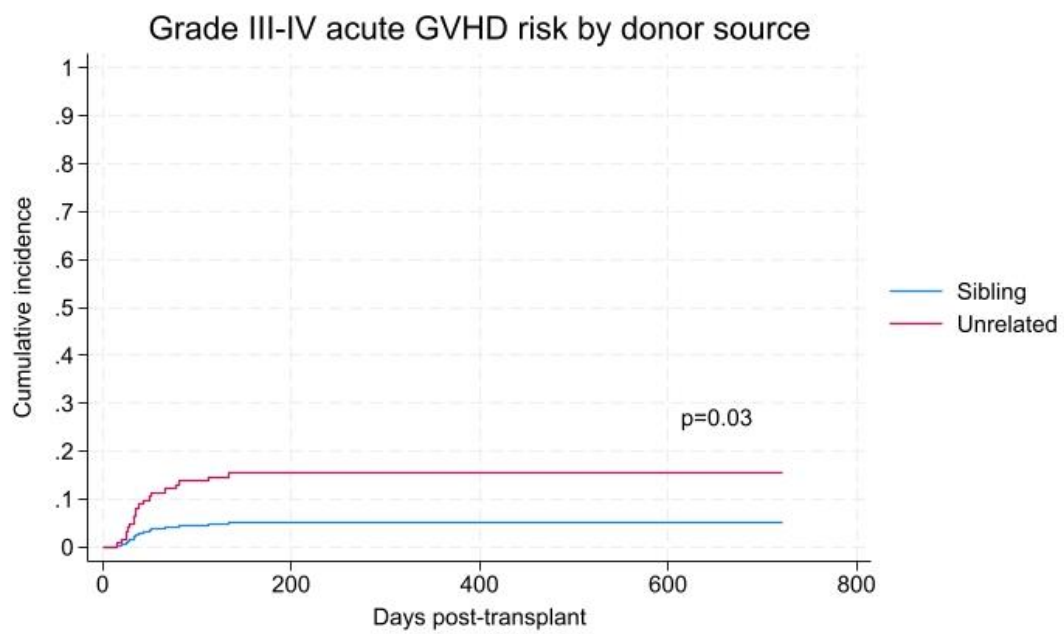


Figure 158: Cumulative incidence of grade III-IV acute GVHD by donor source; sibling vs. unrelated.

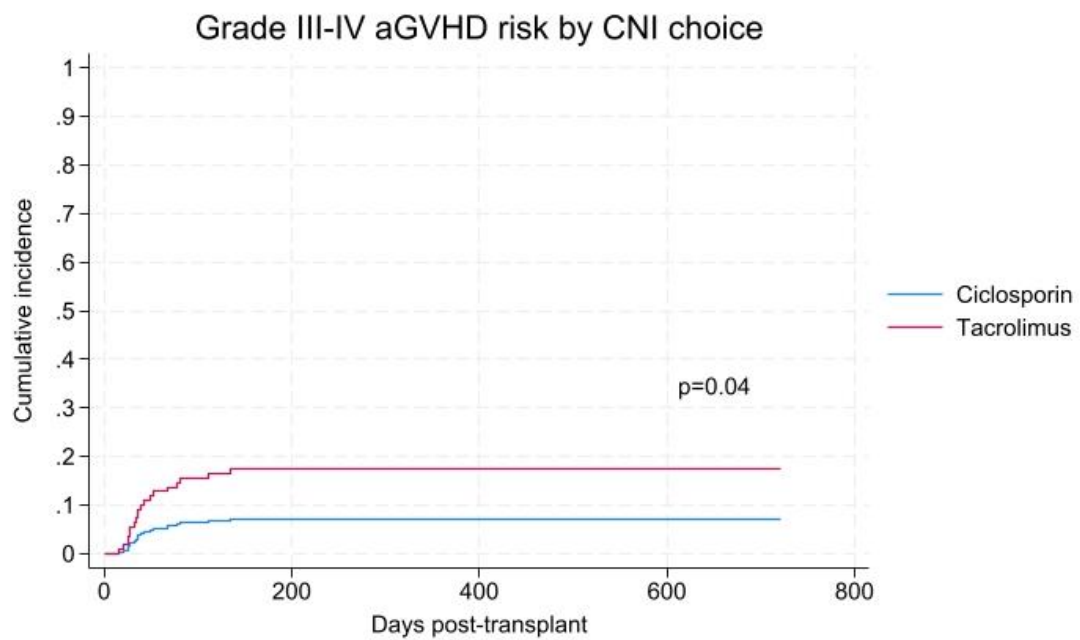


Figure 159: Cumulative incidence of grade III-IV acute GVHD by calcineurin inhibitor; ciclosporin vs. tacrolimus.

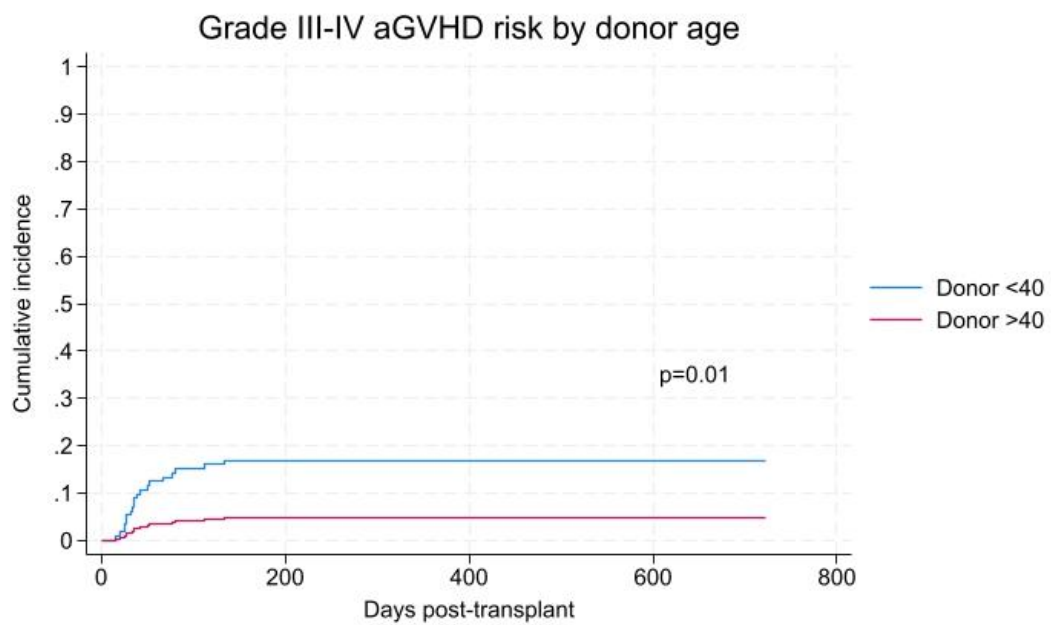


Figure 160: Cumulative incidence of grade III-IV acute GVHD by donor age; above and below 40.

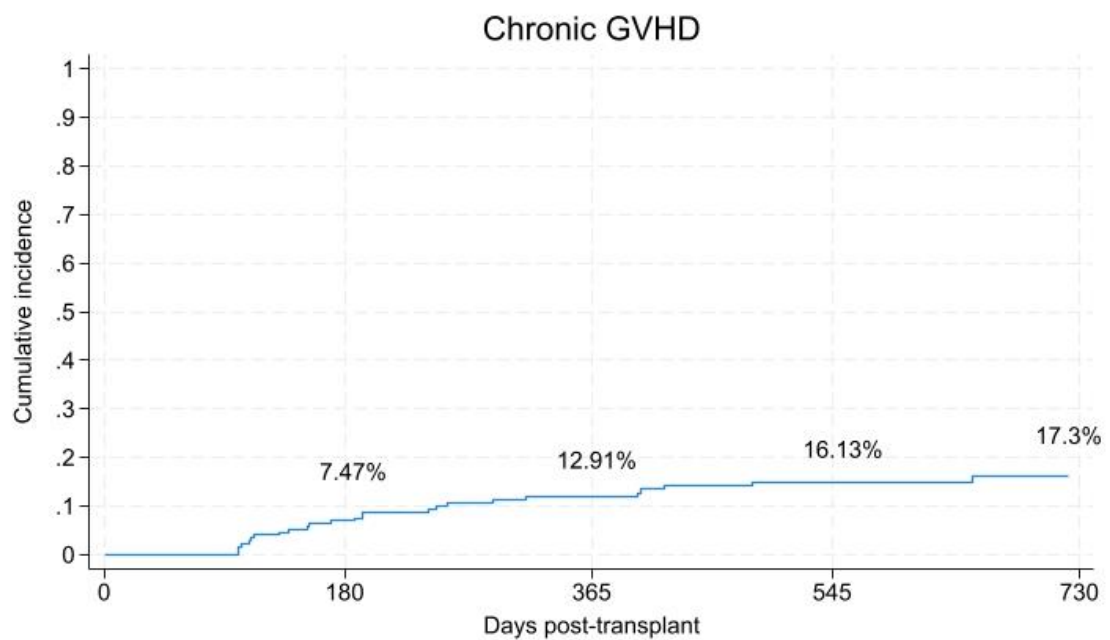


Figure 161: Chronic GVHD cumulative incidence.

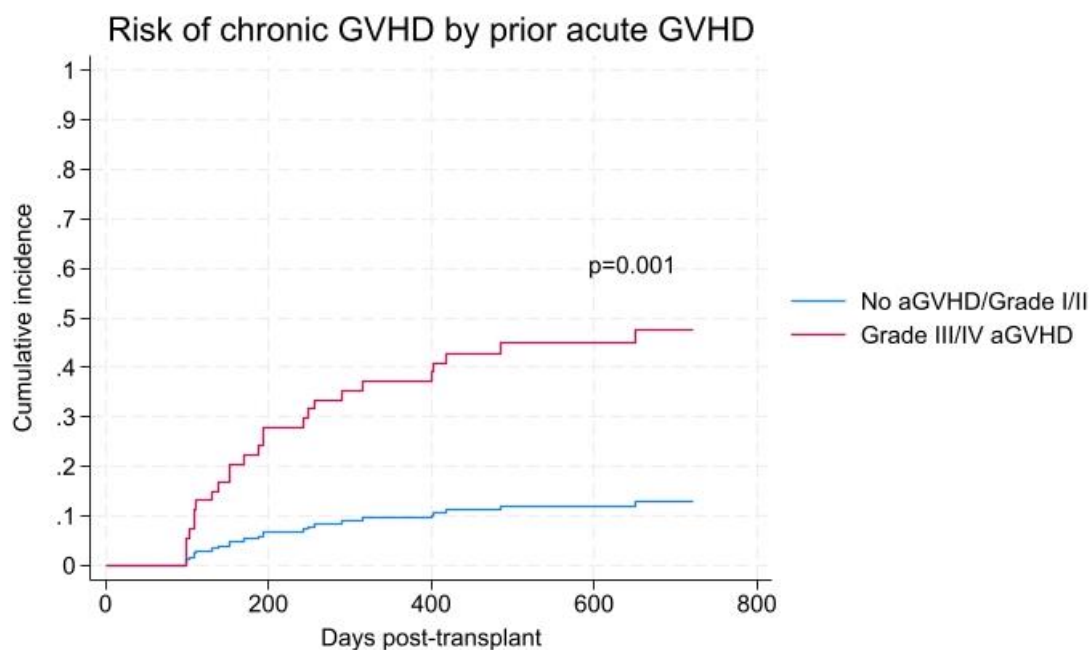


Figure 162: Cumulative incidence of chronic GVHD by prior acute GVHD occurrence; no acute GVHD or grade I-II vs. grade III-IV.

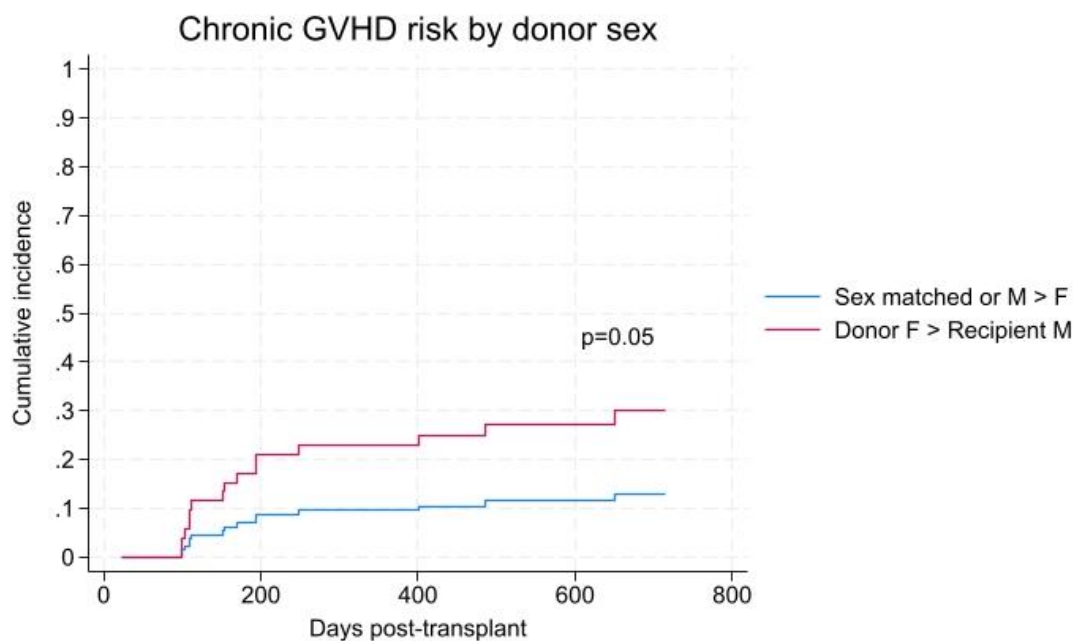


Figure 163: Cumulative incidence of chronic GVHD by donor/recipient sex mismatch; female donor to male recipient vs. sex matched or male donor to female recipient.

Table 31: Pooled GVHD

Variable	Hazard ratio	95% CI	P value
Acute GVHD II-IV			
Early CNl toxicity	4.38	1.34 – 14.23	0.01
Tacrolimus use	1.59	1.02 – 2.50	0.04
Acute GVHD III-IV			
Male recipient	0.71	0.27 – 1.86	0.48
Recipient age	1.01	0.91 – 1.12	0.91
MDS/MPN vs. AML	2.18	1.31 – 3.51	0.001
Unrelated donor	3.18	1.07 – 9.48	0.03
ABO mismatch	1.06	0.43 – 2.60	0.89
Donor age <40	3.84	1.30 – 12.50	0.01
Donor F > M	1.65	0.50 – 5.45	0.40
recipient			
ALC at ATG > 0.1	0.96	0.29 – 3.24	0.95
ALC pre-conditioning	1.55	0.97 – 2.47	0.06
Tacrolimus use	2.54	1.03 – 6.28	0.04
Chronic GVHD			
Male recipient	0.91	0.41 – 1.98	0.91
Recipient age	0.95	0.89 – 1.03	0.20
Recipient age > 65	0.66	0.20 – 2.17	0.49
Disease subtype	1.18	0.75 – 1.84	0.46
Unrelated donor	0.66	0.31 – 1.41	0.28
HLA mismatch	0.61	0.78 – 4.72	0.63
ABO mismatch	0.73	0.33 – 1.56	0.41
Donor age < 25	0.84	0.33 – 2.11	0.71
Donor age < 40	1.32	0.61 – 2.88	0.48
Donor F > M	2.61	1.01 – 6.87	0.05
recipient			
Cryopreserved graft	1.03	0.32 – 3.34	0.94
ALC at ATG > 0.1	1.24	0.47 – 3.26	0.66
ALC pre-conditioning	0.87	0.41 – 1.85	0.71
Tacrolimus use	1.31	0.61 – 2.83	0.49
aGVHD grade II-IV	4.69	1.93 – 11.36	0.0006
aGVHD grade III-IV	4.62	1.86 – 11.48	0.001

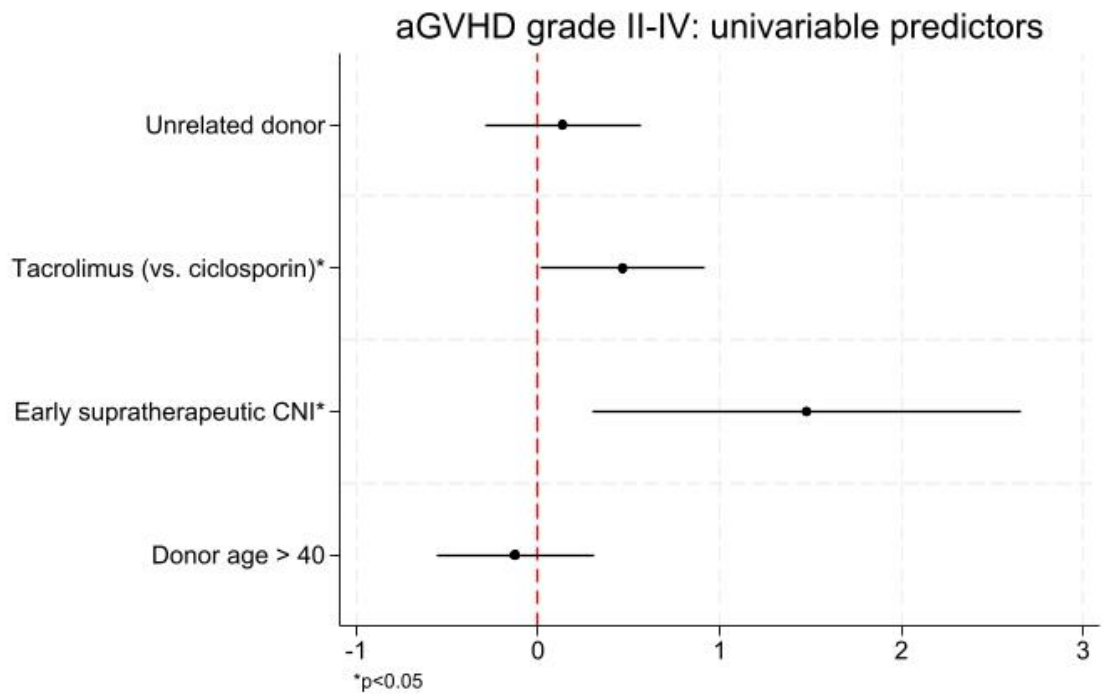


Figure 164: Coefficient plot: univariable analysis in acute GVHD grade II-IV.

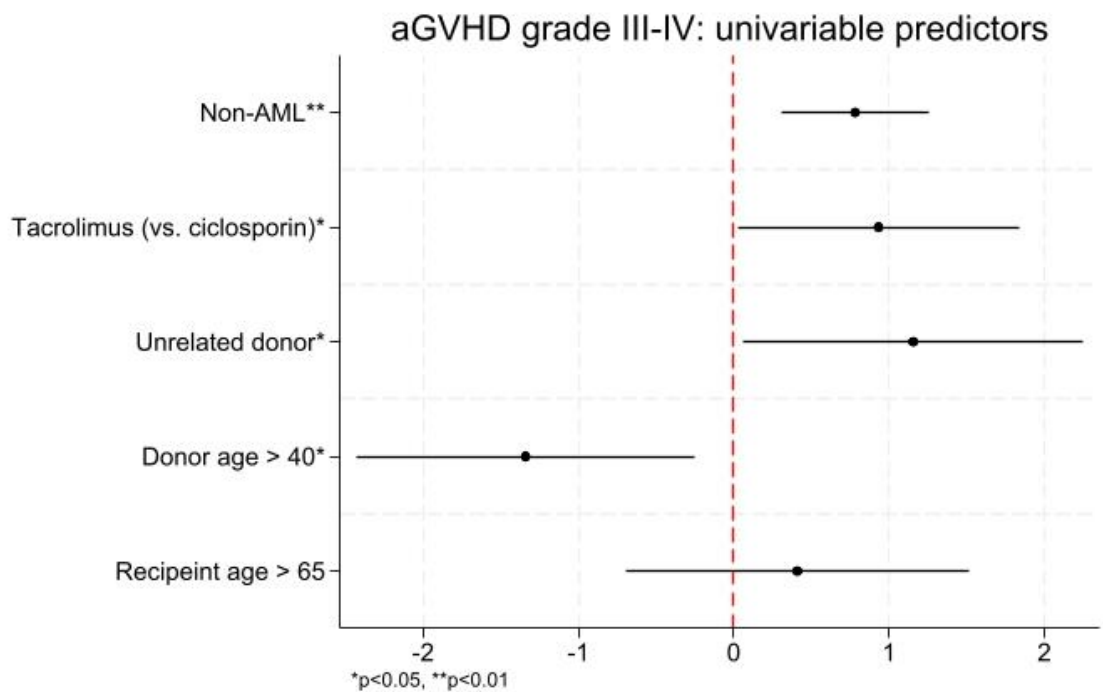


Figure 165: Coefficient plot: univariable analysis in acute GVHD grade III-IV.

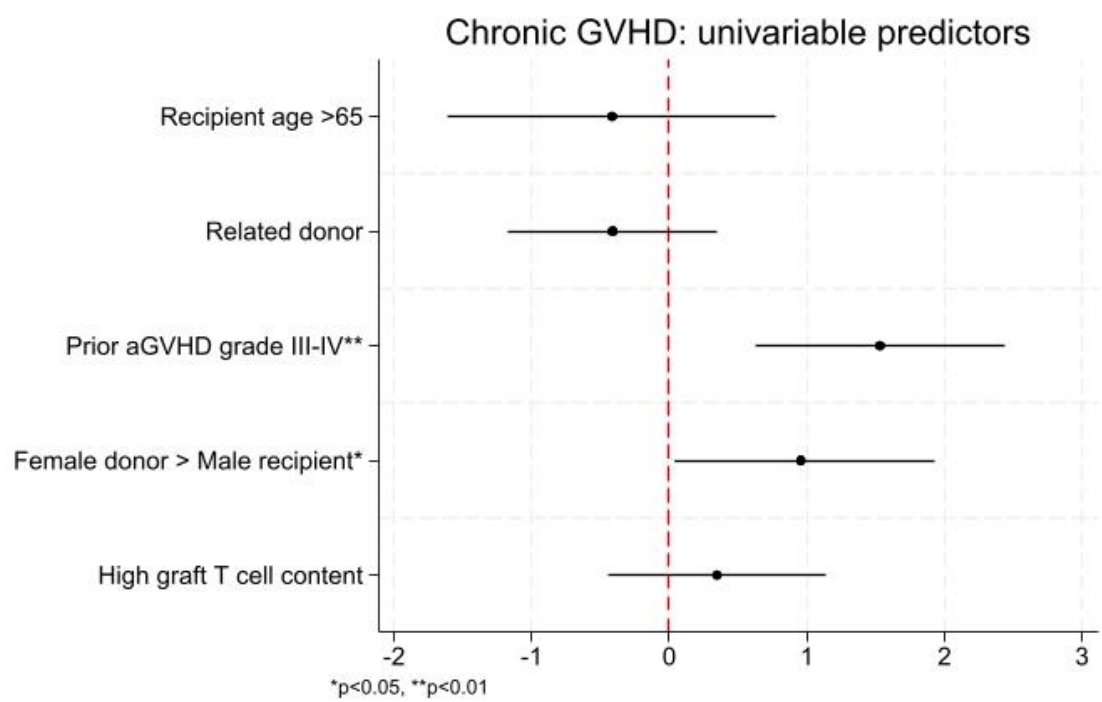


Figure 166: Coefficient plot: univariable analysis in chronic GVHD.

Chimerism and donor lymphocyte infusion

Mixed donor chimerism recorded in 84/163 cases at day 90 or beyond. 64 patients received 113 DLI doses at a median of 186 days post-transplant. 42/84 patients with a mixed chimerism received a pre-emptive DLI. 33/42 patients who received a pre-emptive DLI subsequently attained a full donor chimerism. 3 patients with full donor chimerism received a prophylactic DLI in remission. 19 received a therapeutic DLI as part of salvage for post-transplant relapse. OS in relapsed patients who did not receive a DLI was 742 days as compared to 1087 days in those who did.

Immune reconstitution

T cell reconstitution as measured by CD4 recovery was modest at the 3-, 6- and 12-month time-points, with a median of 68.5, 112.5 and 158 x 10⁶/L respectively (Table 32). A trend toward reduced CD4 recovery at 6 months was seen in those who experienced prior grade III-IV acute GVHD ($p=0.06$) but at all other time-points recovery was not significantly affected by antecedent acute or chronic GVHD. No difference in recovery was seen based on disease type, recipient weight, donor source, donor age, ATLG dose level, stem cell graft CD3⁺ or CD4⁺ T cell content or choice of calcineurin inhibitor. In vivo recipient lymphocyte volume at the time of ATLG infusion had no impact on CD4 recovery at any time-point. Recipient age 70 or above was associated with diminished CD4 recovery at 6 and 12 months ($p=0.05$) but in the larger groups of patients below this age, there was no significant association with age and recovery rate. B cell/plasma cell reconstitution as measured by IgG was more robust, with median levels at 3, 6 and 12 months falling within the normal range and not affected by donor age, donor source or ATLG dose. However, those who did not have normal IgG levels by 6 months post-transplant experienced inferior survival ($p=0.05$).

Reactivation of cytomegalovirus (CMV) occurred in 48 patients, with a case fatality rate of 2%. There was a 45.2% relative risk reduction of CMV reactivation in the years of routine letermovir prophylaxis (2020 onwards). Epstein-Barr virus (EBV) reactivation occurred in 73 patients, with an 8.2% risk of radiological or biopsy-proven PTLD in those who experienced reactivation. All cases of PTLD were successfully treated with rituximab monotherapy. Adenovirus (ADV) reactivation occurred in 18 patients with a case fatality rate of 11.1%. There was one case of *Toxoplasma gondii* reactivation, which directly caused an NRM event. *Pneumocystis jirovecii* occurred in 4 and contributed to an NRM event in 1. Early phase Sars-CoV-2 infection occurred in 21 patients, although incomplete data on later endemic disease was not universally available. Influenza B was the primary cause of death in 1 patient. Invasive fungal disease occurred in 9 patients with a case fatality rate of 33%, all occurring in the setting of neutropenia, steroid-use or other immunosuppression.

Table 32: Immune reconstitution						
	ALC (x10 ⁹ /L)		CD4 (x10 ⁶ /L)		IgG (g/L)	
All patients						
Day 100	0.8 (0 – 3.7)		68.5 (3 – 541)		8.08 (0.54 – 16)	
Day 180	0.9 (0.1 – 8.7)		112.5 (6 – 559)		8.08 (1.52 – 27.43)	
Day 365	1.1 (0.2 – 7.1)		158 (26 – 690)		8.66 (1.12 – 38.39)	
Age > 65		<i>P</i>		<i>P</i>		<i>P</i>
Day 100, age ≥ 65	0.9 (0.2–3.7)	0.04	86 (3-489)	0.08	7.39 (1.6 – 9.87)	0.21
Day 100, age < 65	0.75 (0–3.5)		60 (5-541)		8.36 (0.54-16)	
Day 180, age ≥ 65	1.25 (0.3–4.5)	0.05	105 (24-457)	0.85	7.89 (1.52-16.8)	0.82
Day 180, age < 65	0.9 (0.1–8.7)		112.5 (6-559)		8.39 (2.25-27.4)	
Day 365, age ≥ 65	1.1 (0.2–5.6)	0.73	127 (26-555)	0.19	10.95 (3.1-18.7)	0.09
Day 365, age < 65	1.1 (0.2–7.1)		167 (27-690)		8.13 (1.12-38.3)	
ATLG Dose		<i>P</i>		<i>P</i>		<i>P</i>
Day 100, ATLG 30mg/kg	0.7 (0.2-3.5)	0.11	61.5 (7-541)	0.91	8.73 (4.01-15.34)	0.02
Day 100, ATLG 60mg/kg	0.8 (0-3.7)		81.5 (3-489)		7.38 (0.54-16)	
Day 180, ATLG 30mg/kg	0.9 (0.2-4.5)	0.07	95 (18-457)	0.18	7.9 (2.17-14.23)	0.12
Day 180, ATLG 60mg/kg	1 (0.1-8.7)		130 (6-559)		8.8 (1.52-27.43)	
Day 365, ATLG 30mg/kg	0.9 (0.3-3.7)	0.0003	135 (34-690)	0.06	8.24 (3.03-16.22)	0.23
Day 365, ATLG 60mg/kg	1.4 (0.2-7.1)		193 (26-555)		8.81 (1.12-38.39)	
ALC first day of ATLG		<i>P</i>		<i>P</i>		<i>P</i>
Day 100, ALC ≤ 0.1	0.8 (0-3.7)	0.59	132 (3-541)	0.25	7.85 (0.54-15.34)	0.25
Day 100, ALC > 0.1	0.7 (0.2-3.3)		60 (39-104)		8.72 (6.15-16)	
Day 180, ALC ≤ 0.1	0.9 (0.1-4.5)	0.94	113.5 (6-559)	0.30	8.43 (1.52-27.43)	0.41
Day 180, ALC > 0.1	1 (0.1-8.7)		102.5 (19-295)		7.12 (2.25-27.3)	
Day 365, ALC ≤ 0.1	1.1 (0.2-7.1)	0.78	160.5 (26-690)	0.74	8.49 (1.12-38.39)	0.55
Day 365, ALC > 0.1	1.15 (0.5-4.8)		127 (40-493)		8.81 (5.71-22.41)	

EASIX

The EASIX score was calculated pre-conditioning, at day 0, GVHD onset and 1-year post-transplant. The mean score at pre-transplant was 3.02, 15.66 at day 0, 3.47 at onset in all-grade acute GVHD and 3.9 in grade II-IV disease. At 1-year the mean score was 2.12 (Table 32).

The pre-conditioning EASIX score was predictive of overall survival at a cut-off of 3 and above (HR 2.04, 95% CI 1.17 – 3.56, $p=0.01$) (Fig. 167), The score was also predictive of NRM at a cut-off of 3 and above (HR 2.56, 95% CI 1.05 – 6.28, $p=0.03$) (Fig. 168). Furthermore, the score was predictive of grade III-IV acute GVHD above a threshold of 3 (HR 2.52, 95% CI 1.03 – 6.17, $p=0.04$) (Fig. 169). The day 0 EASIX score was not predictive of OS, NRM or relapse. At a cut-off of 15 it was predictive of subsequent acute grade III-IV GVHD (HR 2.72, 95% CI 1.10 – 6.75, $p=0.03$) (Fig. 170). The EASIX score at GVHD onset was predictive for survival at a cut-off of 3 (HR 2.74, 95% CI 1.23 – 6.12, $p=0.01$) (Fig. 171). The EASIX score at 1-year post-transplant was predictive for survival at a cut-off of 1 (HR 14.57, 95% CI 1.89 – 112.32, $p=0.0003$) (Fig. 172) and relapse at a cut-off of 1 (HR 3.59, 95% CI 1.29 – 10.04, $p=0.01$) (Fig. 172) (Table 32 and Fig. 174).

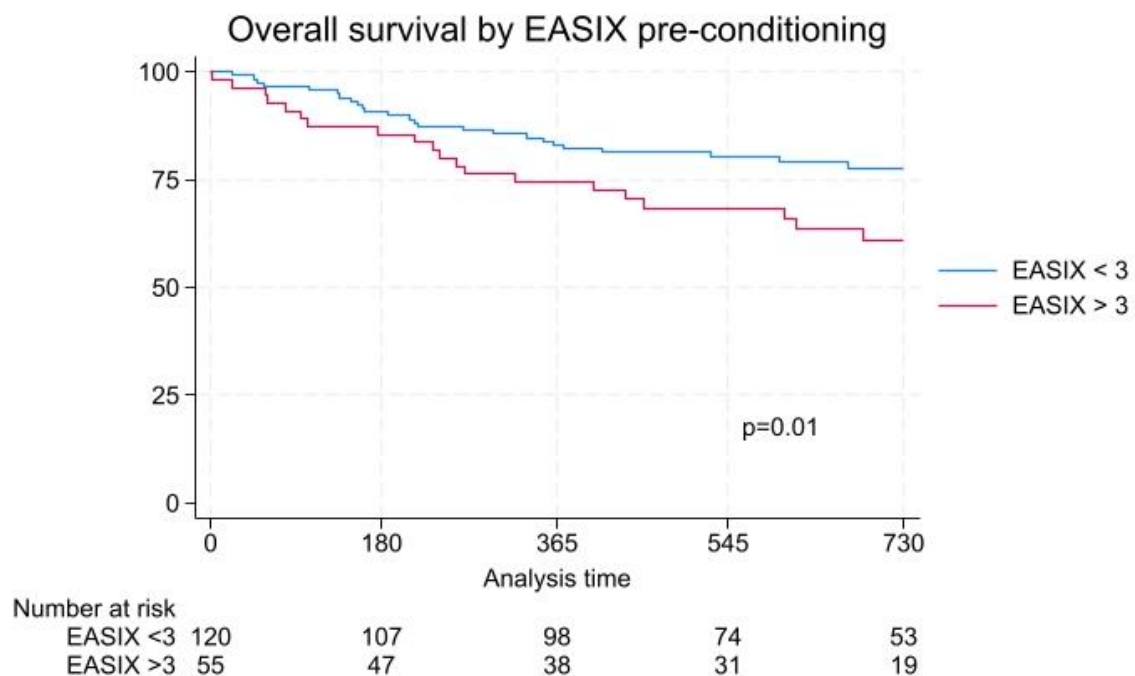


Figure 167: Overall survival by EASIX at pre-conditioning time-point; above and below 3.

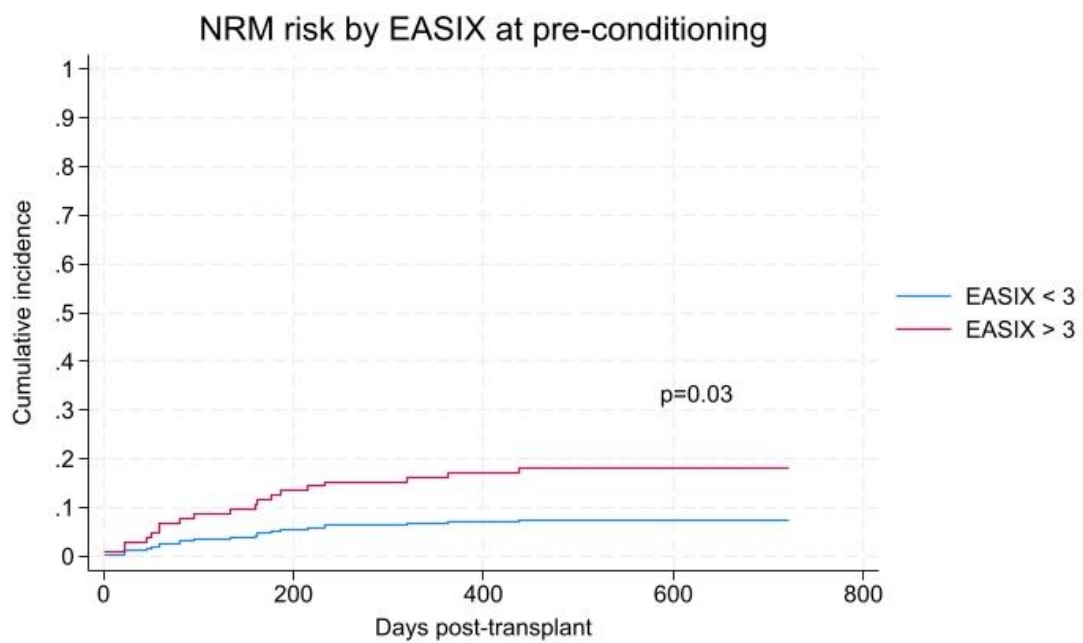


Figure 168: Cumulative incidence of NRM by EASIX at pre-conditioning time-point; above and below 1.

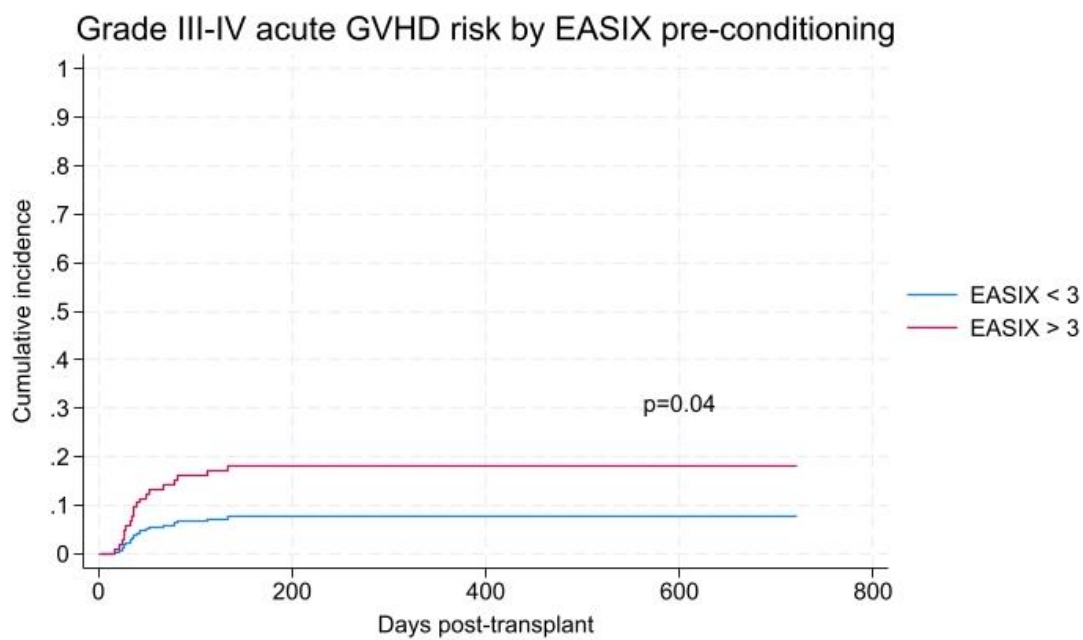


Figure 169: Cumulative incidence of grade III-IV acute GVHD by EASIX at pre-conditioning time-point; above and below 3.

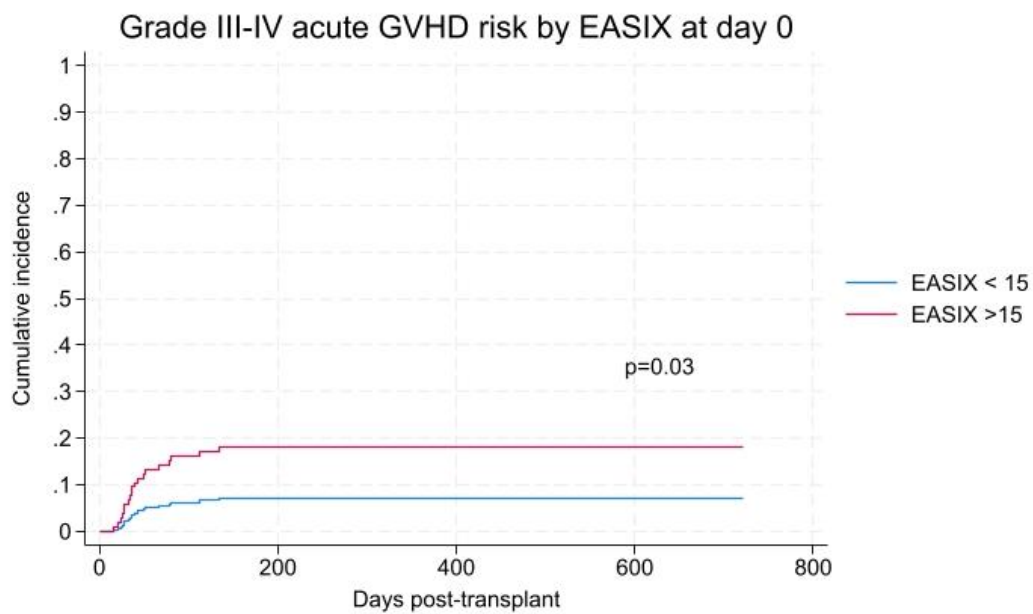


Figure 170: Cumulative incidence of grade III-IV acute GVHD by EASIX score at day 0 time-point; above and below 15.

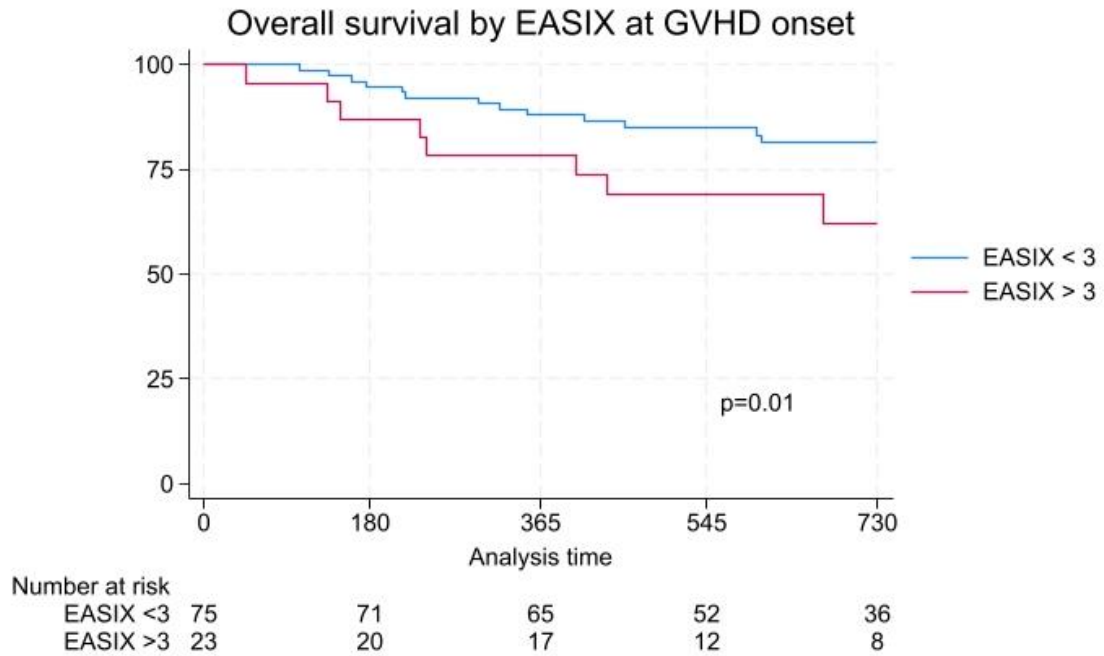


Figure 171: Overall survival by EASIX score at GVHD onset; above and below 3.

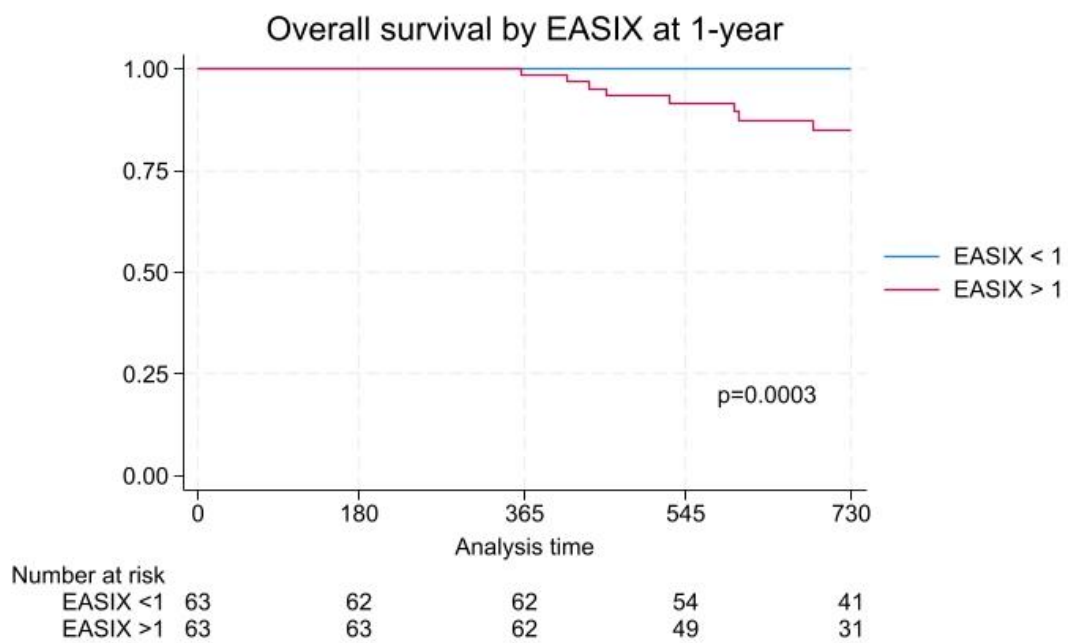


Figure 172: Overall survival by EASIX score at 1-year; above and below 1.

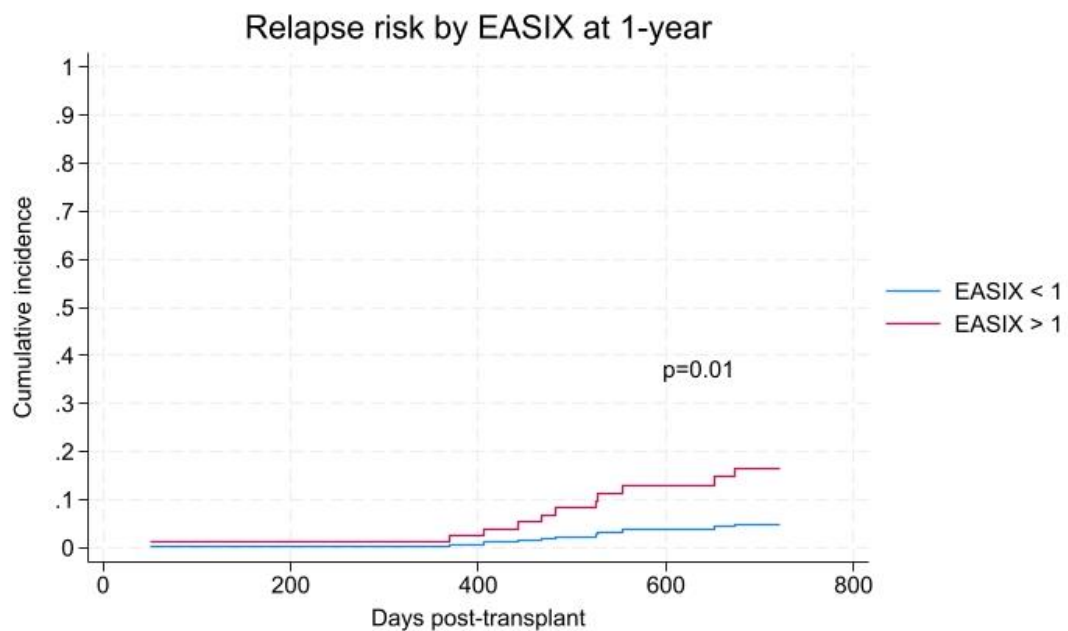


Figure 173: Cumulative incidence of relapse by EASIX at 1-year; above and below 1.

Table 33: Pooled EASIX

Time-point	Mean	SD	Median	Range
Pre-conditioning	3.02	3.03	1.83	0.30 – 17.54
Day 0	15.66	18.39	11.51	1.07 – 180.09
At GVHD	3.47	5.96	2.03	0.40 – 51.43
At 1-year	2.12	2.64	1.27	0.29 – 19.34

Variable	Cut-off	Hazard ratio	95% CI	P value
Overall survival				
Pre-conditioning	3	2.04	1.17 – 3.56	0.01
Day 0	15	1.20	0.68 – 2.13	0.52
At GVHD	3	2.74	1.23 – 6.12	0.01
At 1-year	1	14.57	1.89 – 112.32	0.0003
NRM				
Pre-conditioning	3	2.26	1.05 – 6.28	0.03
Day 0	15	1.80	0.73 – 4.42	0.19
At GVHD	3	3.47	0.88 – 13.70	0.07
Acute GVHD				
Pre-conditioning	3	2.52	1.03 – 6.17	0.04
Day 0	15	2.72	1.10 – 6.76	0.03
Relapse				
Pre-conditioning	3	1.39	0.80 – 2.42	0.24
Day 0	15	0.79	0.44 – 1.41	0.41
At GVHD	3	1.68	0.71 – 3.93	0.23
At 1-year	1	3.59	1.29 – 10.04	0.01

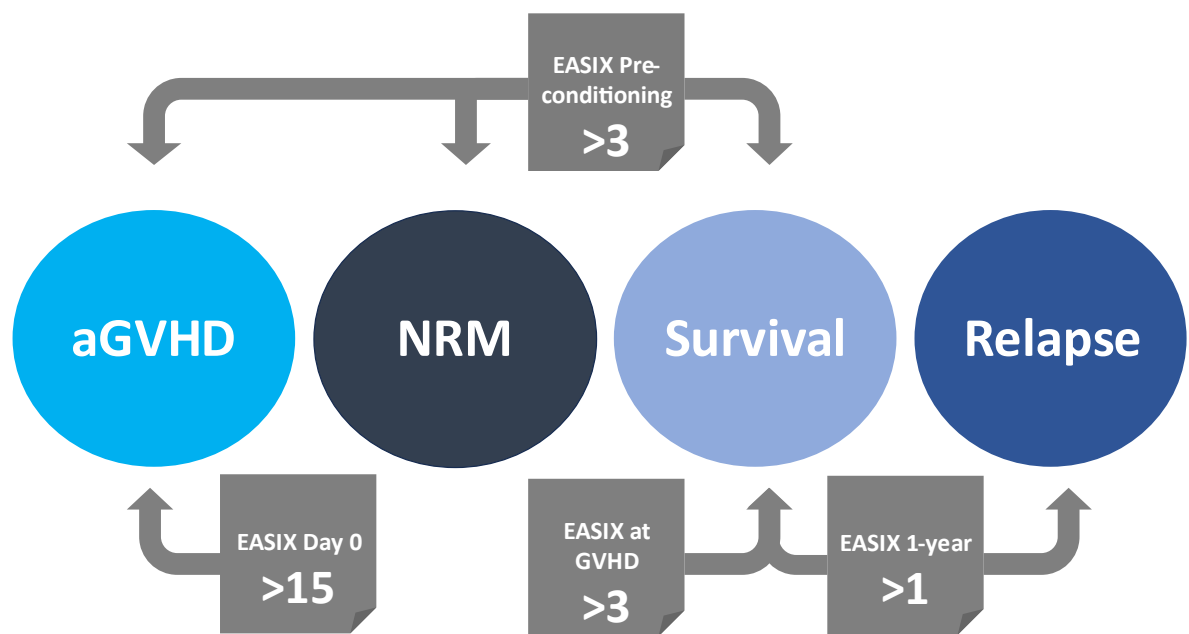


Figure 174: EASIX predictive model; time-points, cut-offs and predictive outcomes.

Results - cytokine analysis

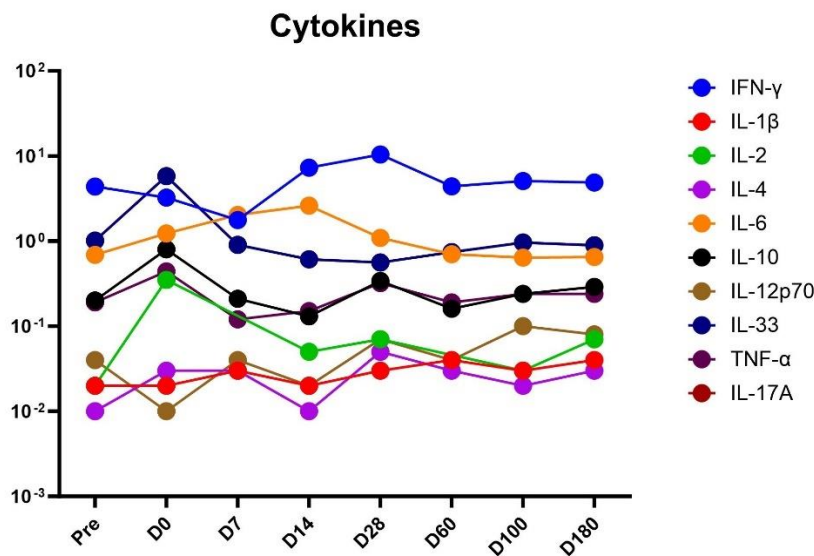


Figure 175: Overall median expression in all 32 patients of cytokines at sequential time-points from pre-transplant to day +180 (logarithmic representation).

The median expression intensity and trends of each individual cytokine over time are discussed in this section. As shown in Fig. 175, cytokine expression was variable both by analyte and by the timing of the sample. The following subsections review the temporal variances within each cytokine and thereafter their potential correlation with clinical outcomes including GVHD, relapse and death. Additional representative graphs for each cytokine are available in Appendix 4. 0

Interferon-gamma

IFN γ levels were quantifiable in all but two samples. The median pre-transplant IFN γ level was 4.38 pg/ml, which then dropped at day 0 to 3.24 pg/ml, further again at day 7 to 1.76 pg/ml before rising to 7.3 pg/ml at day 14. The peak IFN γ level was seen at day 28, with a median of 10.39 pg/ml. From day 60 onwards the levels showed a plateau with a median of 4.4, 5.08 and 4.86 at days 60, 100 and 180 respectively (Fig. 176, 177). The variance in IFN γ levels between the serial time-points was significantly different ($p=0.0003$). The distinct significant differences were between pre-transplant and day 28, day 0 and 14 and 28, day 7 and 14 and 28, day 28 and 60, 100 and 180.

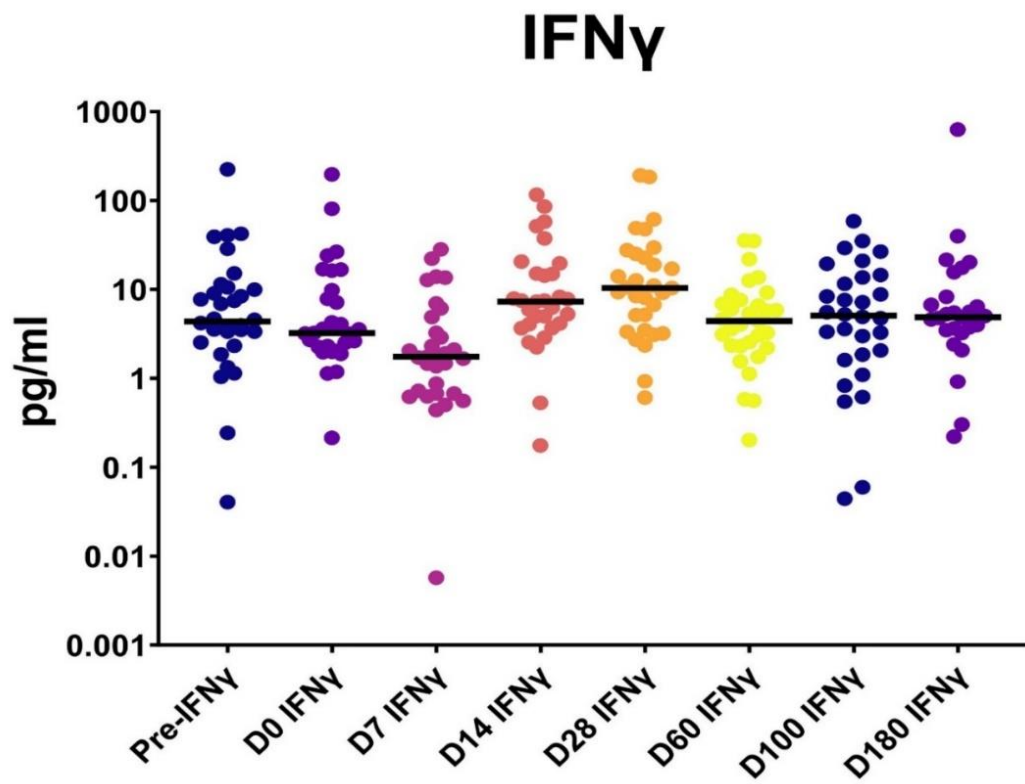


Figure 176: Interferon-gamma levels at serial time-points, logarithmic representation.

IFN γ

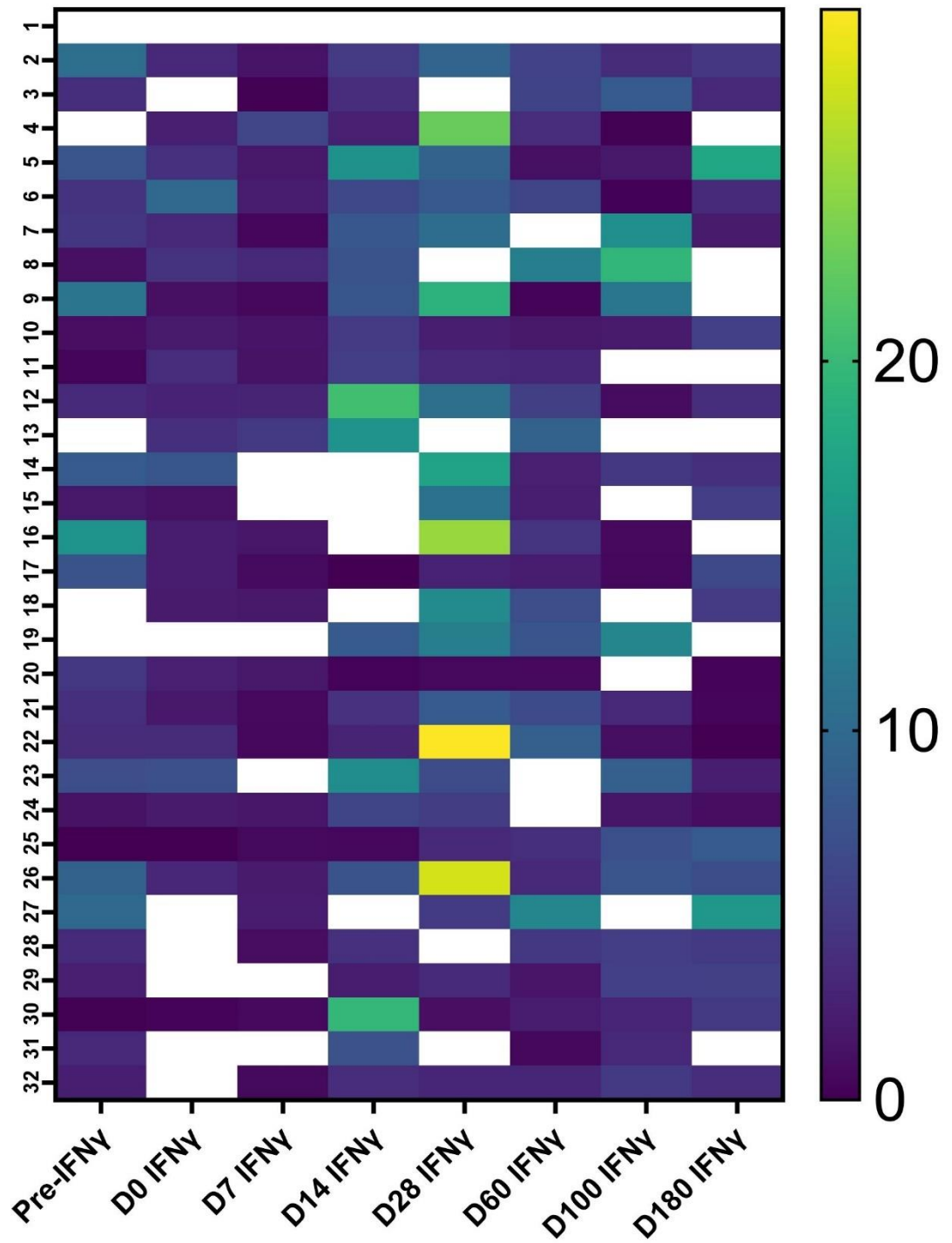


Figure 177: Interferon-gamma expression over serial time-points, significant outliers and no available sample at time-point represented by white box.

Interleukin-1 β

IL-1 β levels were quantifiable in 186/244 samples (Fig. 178, 179). Undetectable levels were present in a similar number of patients at each sequential time-however not consistently in the same patients. The median level was 0.02 pg/ml at the pre-transplant time-point and did not significantly change throughout, rising to a peak median of 0.04 pg/ml at day 180 post-transplant. Variance in median levels by time-point was not statistically significant ($p=0.98$).

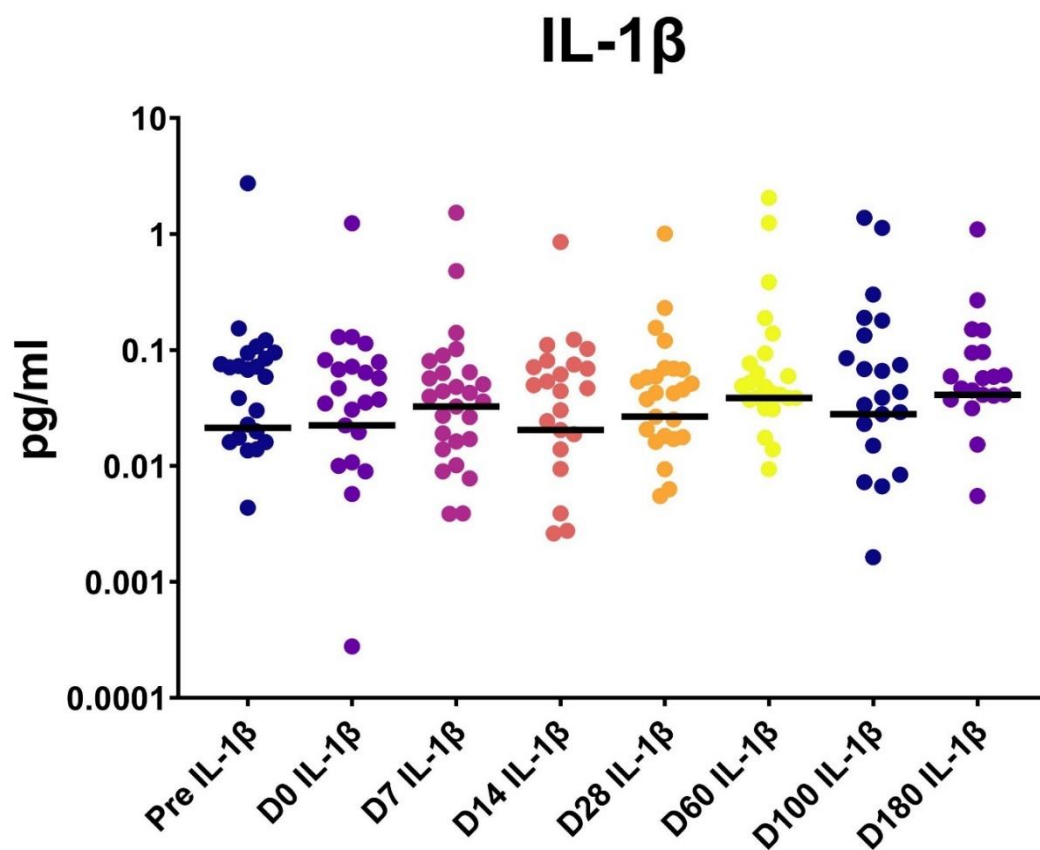


Figure 178: Interleukin-1-beta levels at serials time-points, logarithmic representation.

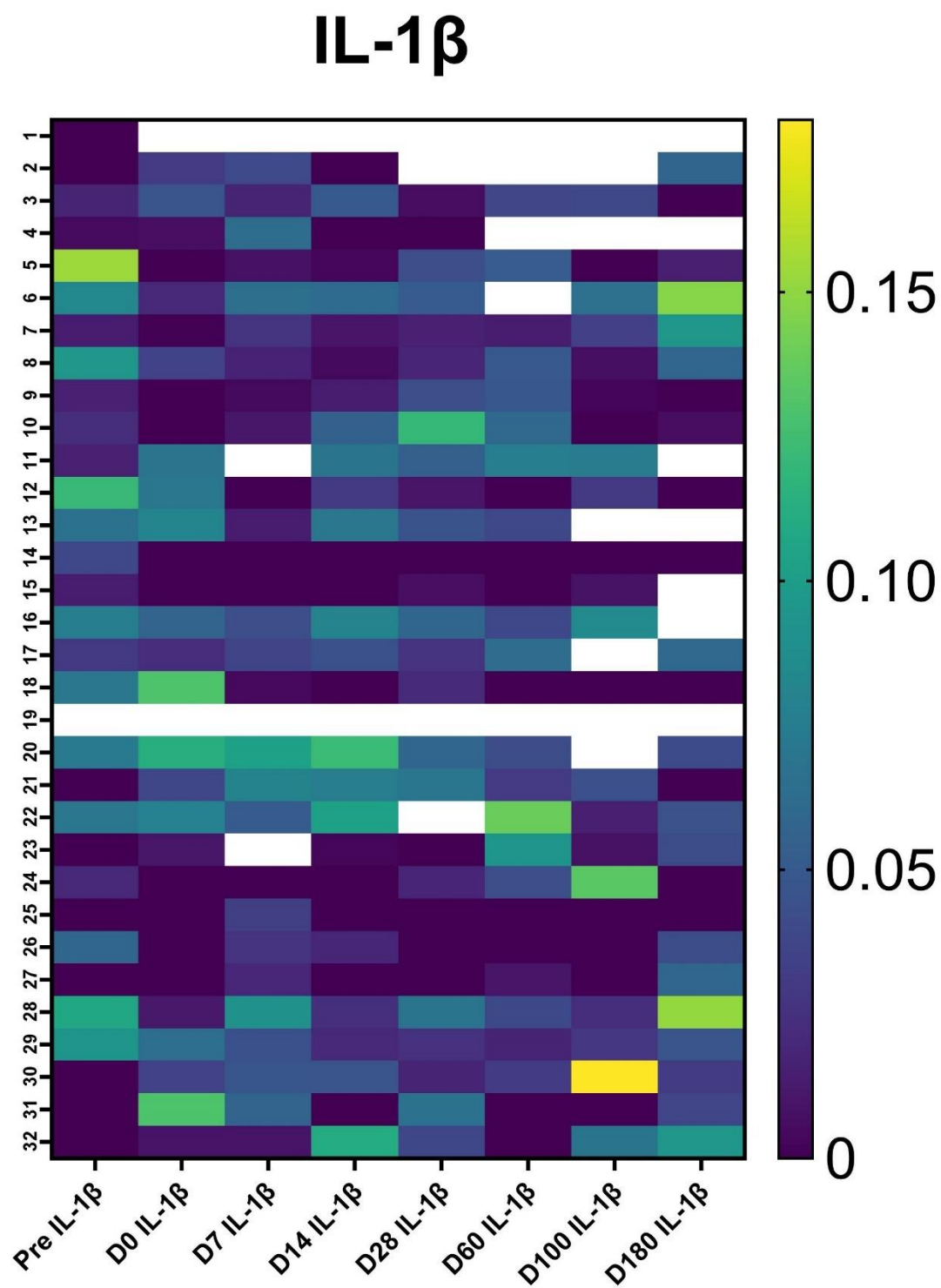


Figure 179: Interleukin-1-beta expression over serial time-points, significant outliers and no sample at given time-point represented by white box.

Interleukin-2

IL-2 was quantifiable in 142/244 samples (Fig. 180, 181). The median IL-2 level at the pre-transplant time-point was 0.02 pg/ml, which peaked at day 0 at a level of 0.35 pg/ml and thereafter remained broadly stable over subsequent time-points. There was significance in the variation between time-points ($p < 0.001$). The significant differences were between day 0 and 7, 14, 28, 60, 100 and 180.

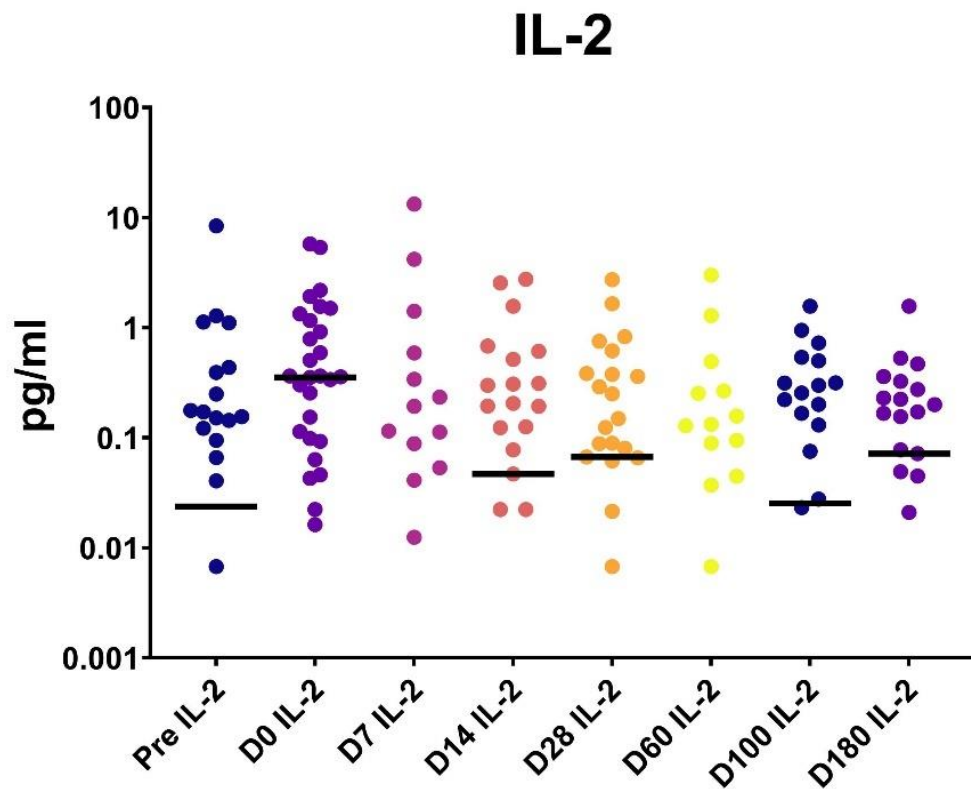


Figure 180: Interleukin-2 levels at serial time-points, logarithmic representation.

IL-2

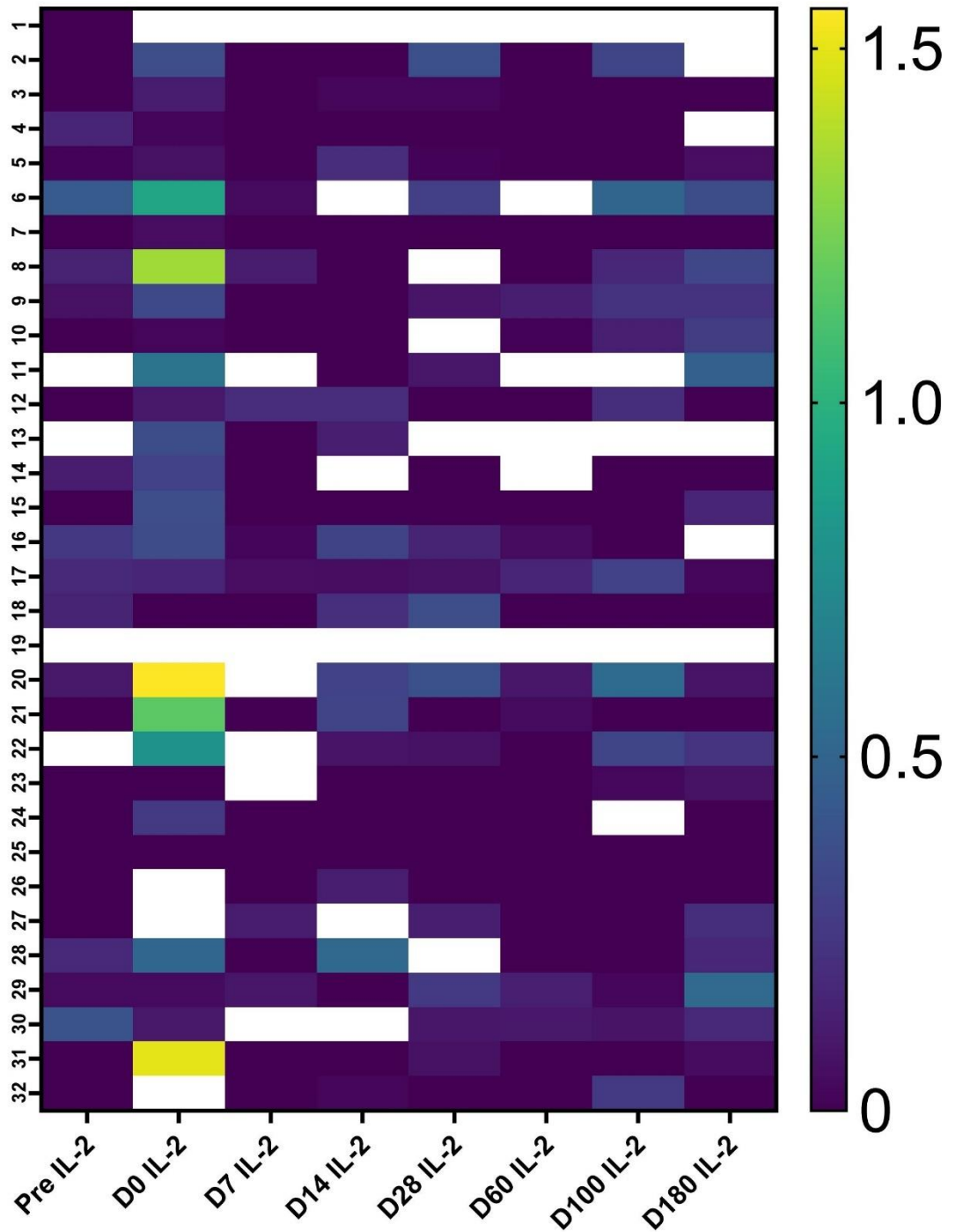


Figure 181: Interleukin-2 expression over serial time-points, significant outliers and no sample at given time-point represented by white box.

Interleukin-4

IL-4 levels were quantifiable in 168/244 samples (*Fig. 182, 183*). The median level at the pre-transplant time-point was the lowest in the series at 0.01 pg/ml, with a peak seen at day 28 of 0.05 pg/ml. There was no statistically significant variance in median levels between time-points ($p=0.28$) although a difference between the distinct time-points of pre-transplant and day 0 and 28, and day 0 and day 100 was noted.

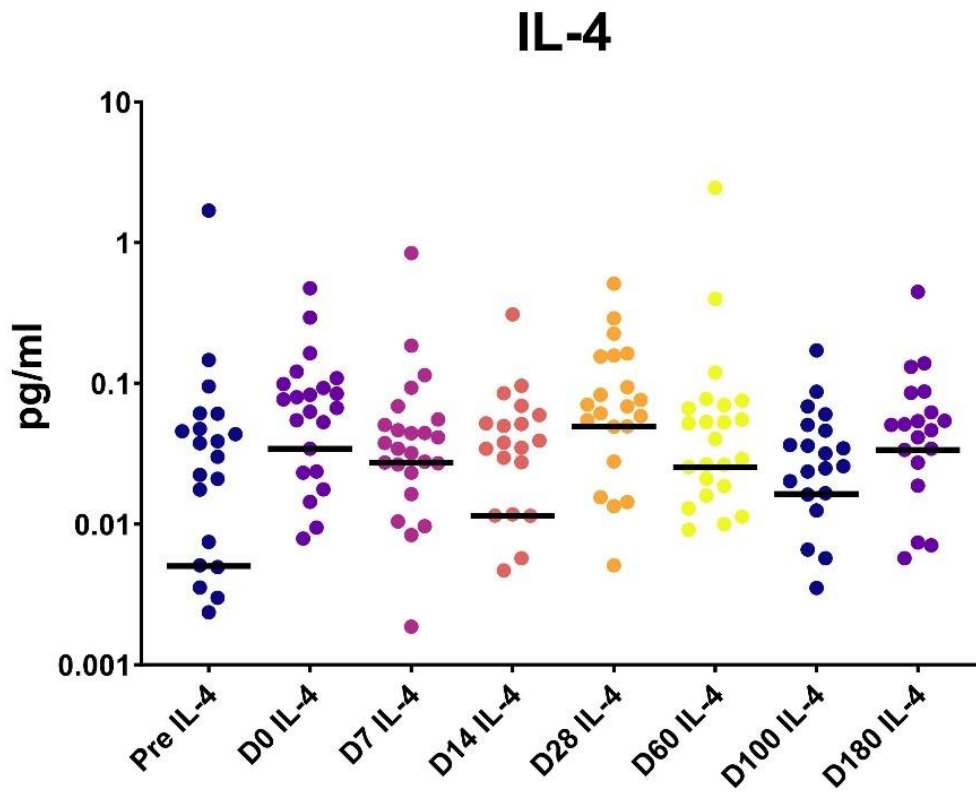


Figure 182: Interleukin-4 levels at serial time-points, logarithmic representation.

IL-4

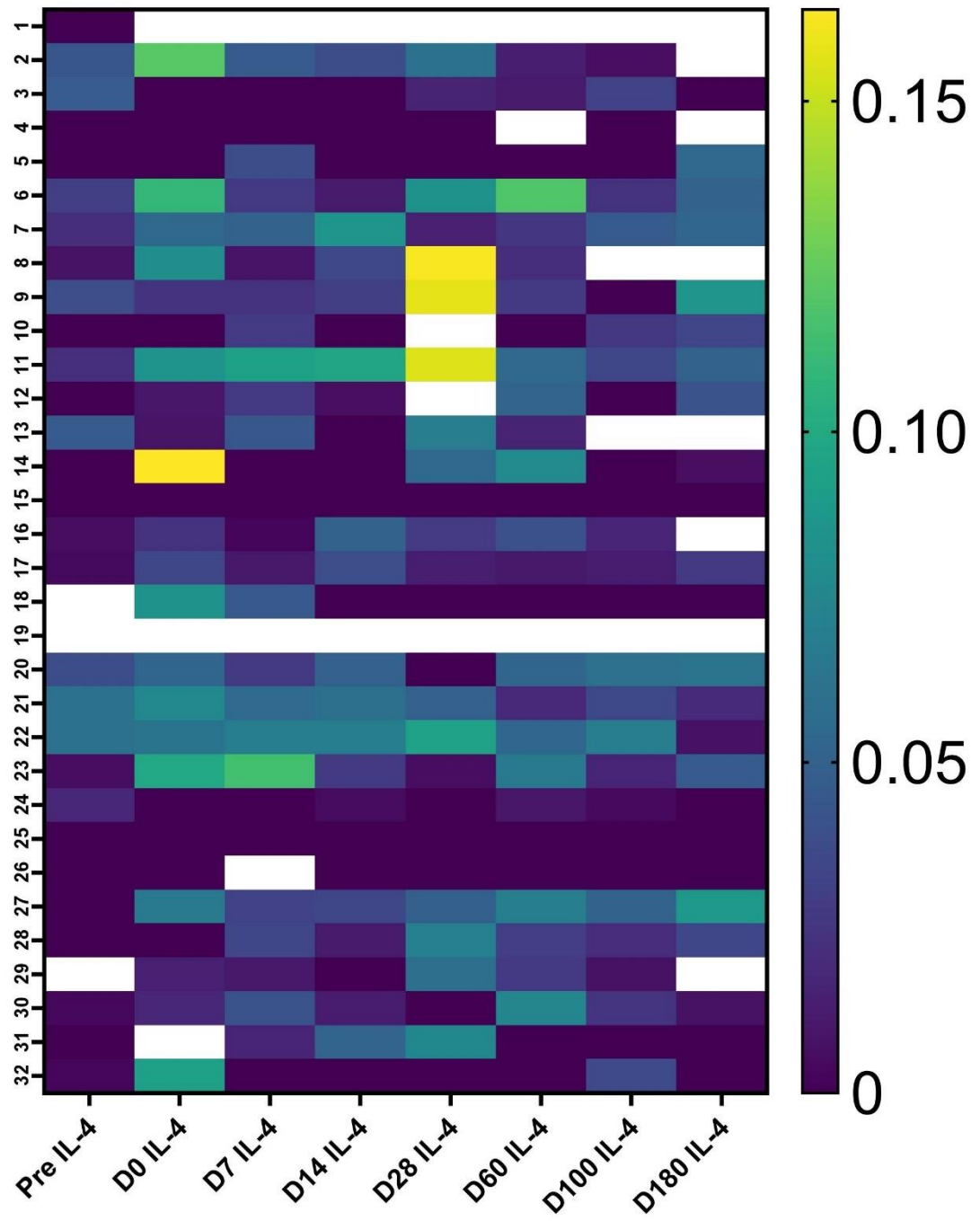


Figure 183: Interleukin-4 expression over serial time-points, significant outliers and no sample at given time-point represented by white box.

Interleukin-6

IL-6 levels were quantifiable in 234/244 samples (Fig. 184, 185). The peak median IL-6 level was at day 14 at 2.61 pg/ml, with increased levels seen between day 0 and day 28. Levels returned to the pre-transplant median of 0.69 pg/ml by day 60 post-transplant and remained stable thereafter. Markedly raised levels were noted in a small number of patients at the day 7, 14 and 28 time-points. There was a significant variance in levels between time-points ($p < 0.001$). The significant differences occurred between pre-transplant and day 7 and 14, day 0 and 7 and 14, day 7 and 28, 60, 100 and 180, and day 14 and 28, 60, 100 and 180.

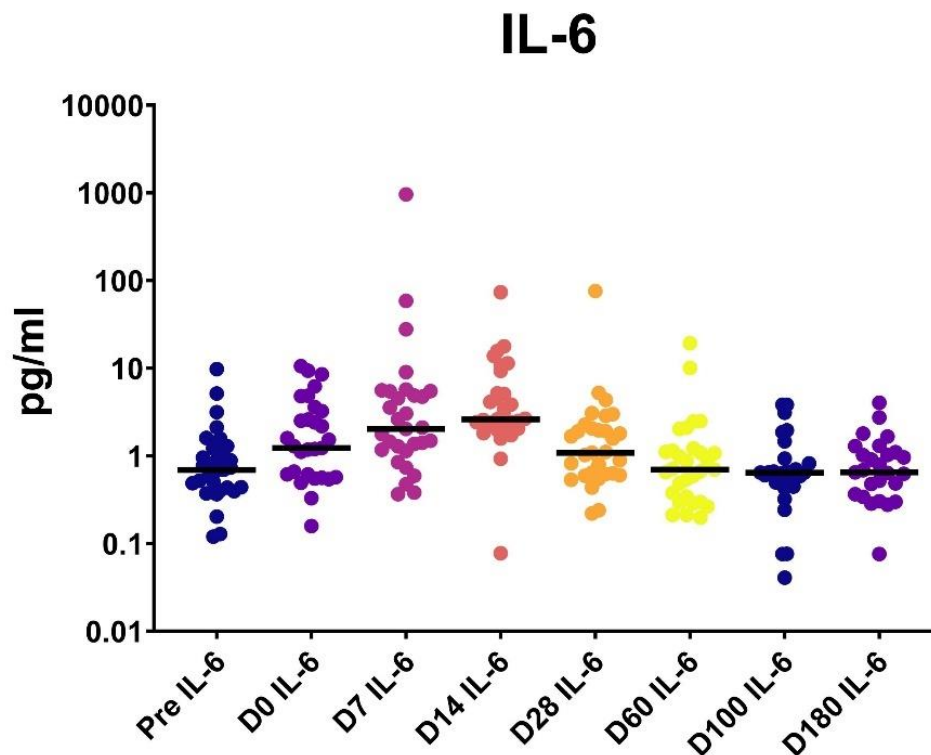


Figure 184: Interleukin-6 levels at serial time-points, logarithmic representation.

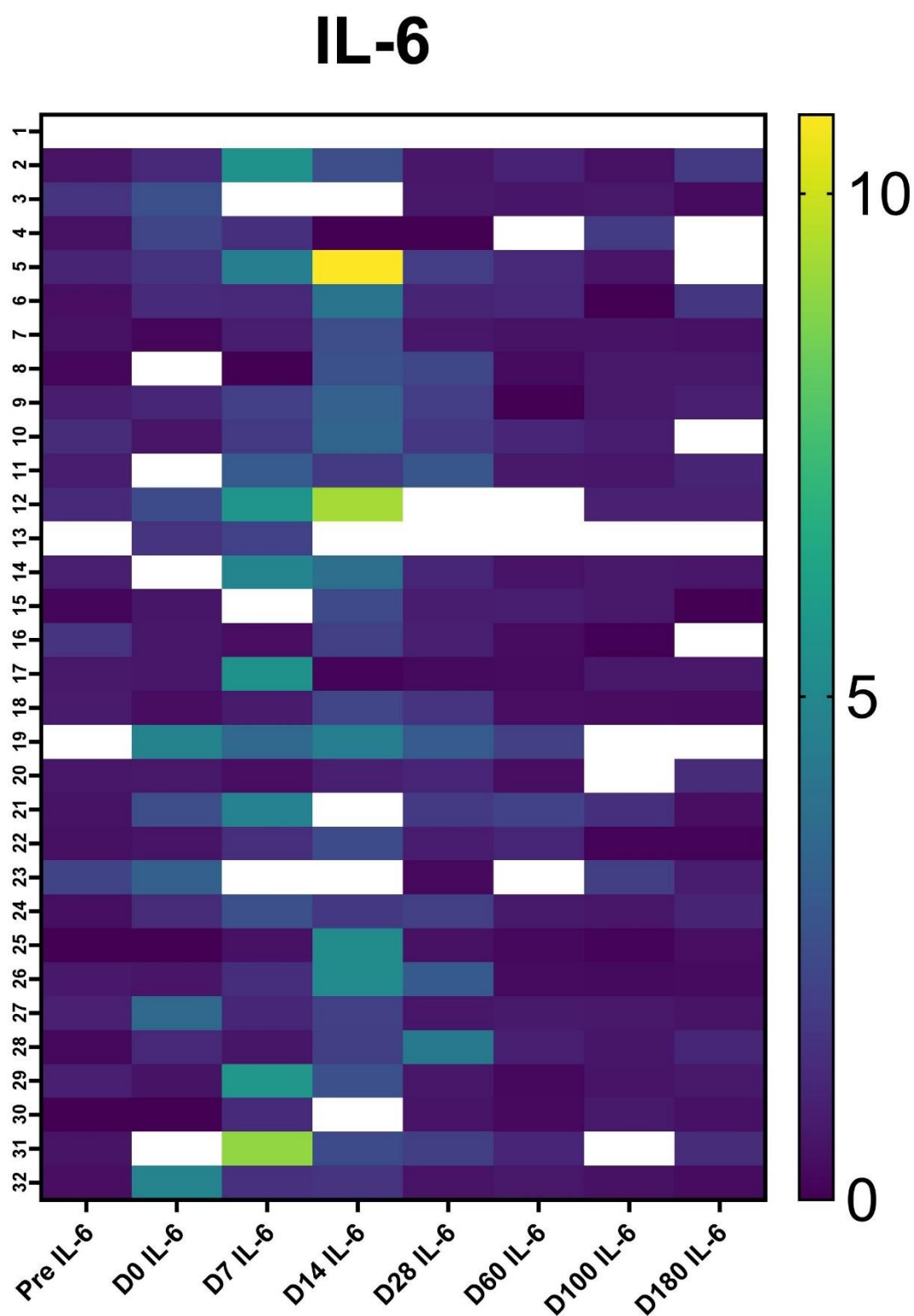


Figure 185: Interleukin-6 expression over serial time-points, significant outliers and no sample at given time-point represented by white box.

Interleukin-10

IL-10 was quantifiable in 238/244 samples (*Fig. 186, 187*). The peak levels were seen at day 0 at 0.80 pg/ml, and a nadir shortly after of 0.13 pg/ml on day 14 post-transplant. A markedly raised IL-10 level was noted in one patient at the day 7 time-point. Variation between time-points was significant ($p=0.0008$). The significant differences occurred between pre-transplant and day 0 and 28, day 0 and 7, 14, 60, 100 and 180, day 8 and 28, day 14 and 28 and day 28 and 60.

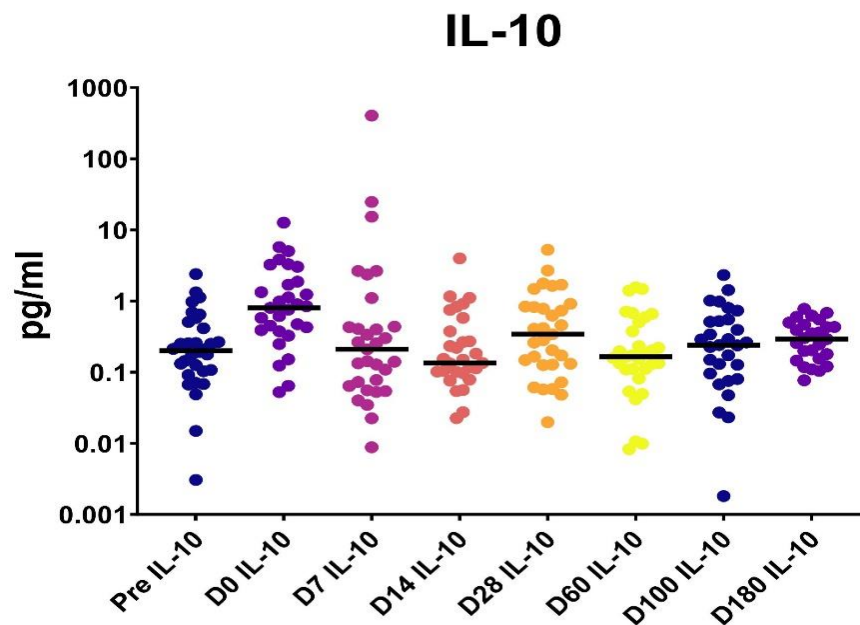


Figure 186: Interleukin-10 levels at serial time-points, logarithmic representation.

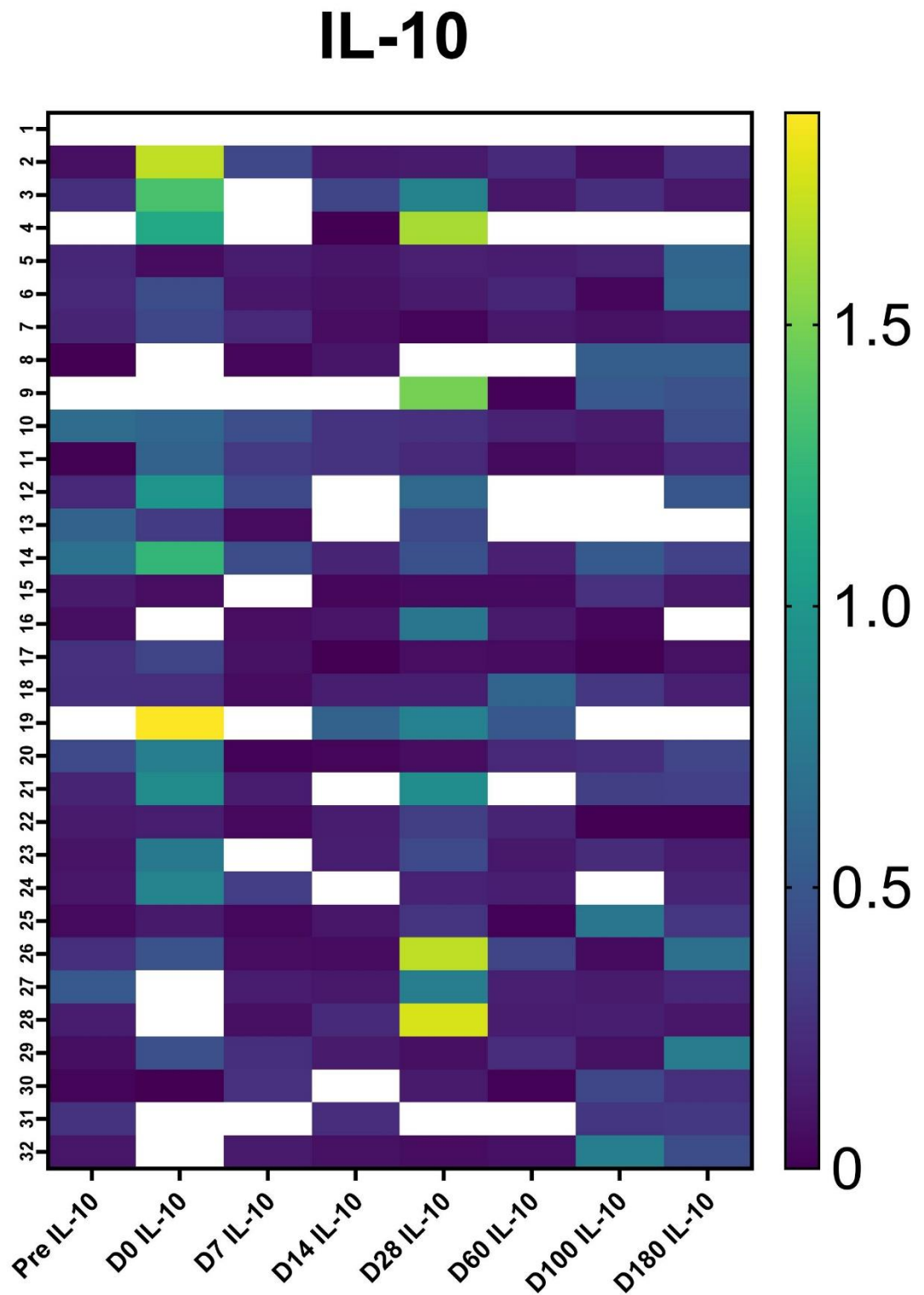


Figure 187: Interleukin-2 expression over serial time-points, significant outliers and no sample at given time-point represented by white box.

Interleukin-12p70

IL-12p70 was quantifiable in 161/244 samples (Fig. 188, 189). The peak median IL-12p70 occurred at day 100 post-transplant at 0.1 pg/ml, with the nadir of 0.1 pg/ml seen early at day 0. The overall variance in expression between time-points was not significantly different ($p=0.16$) although a difference between pre-transplant and day 100 and 180, and day 7 and 100 and 180 was noted.

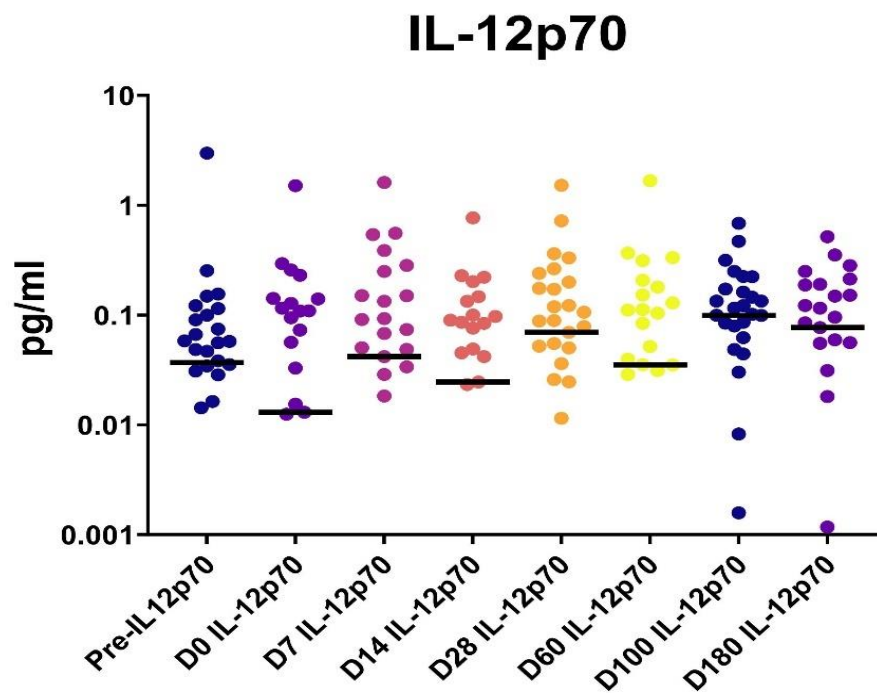


Figure 188: Interleukin-12p70 levels at serial time-points, logarithmic representation.

IL-12p70

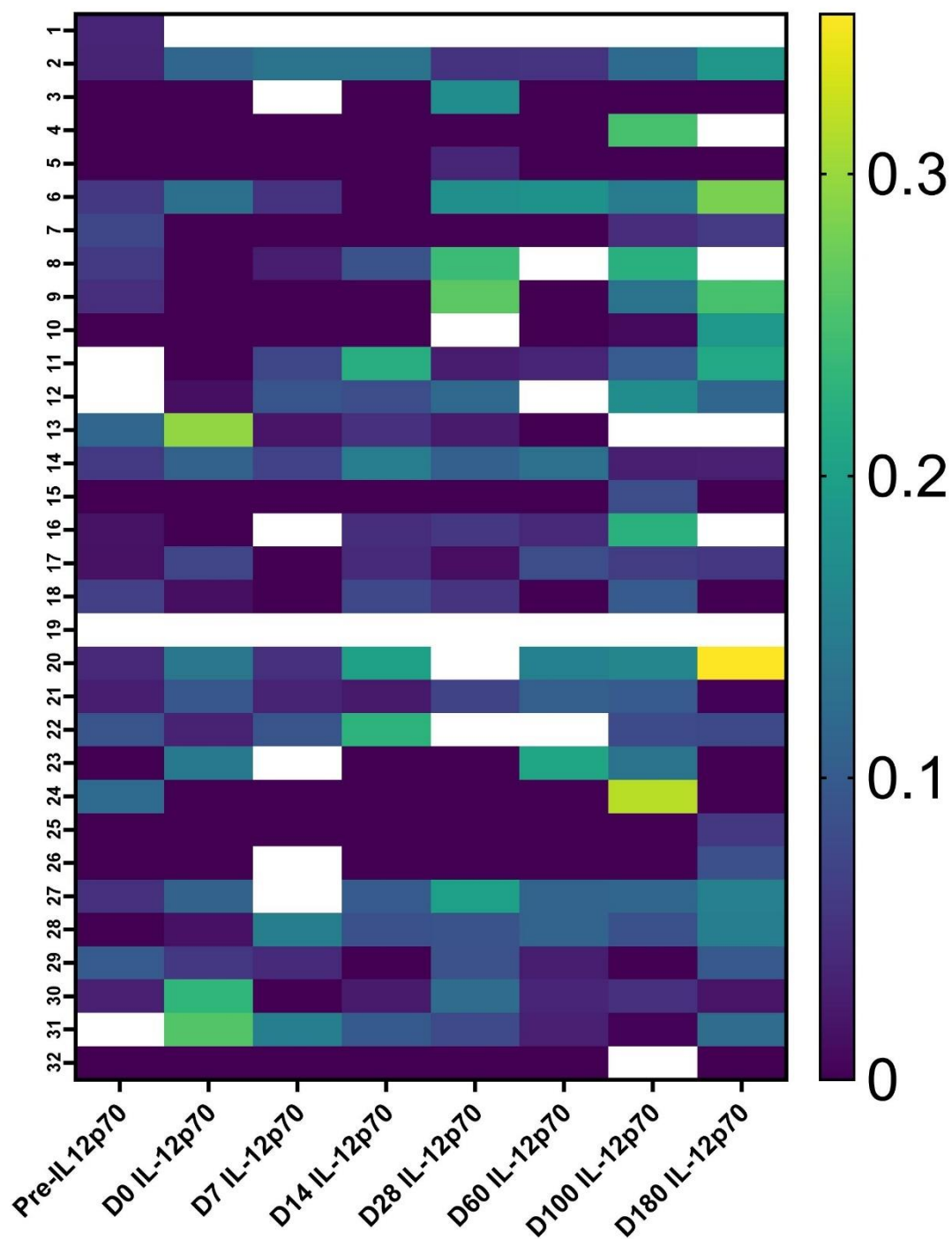


Figure 189: Interleukin-12p70 expression over serial time-points, significant outliers and no sample at given time-point represented by white box.

Interleukin-17A

IL-17A was quantified in 204/244 samples (Fig. 190, 191). The peak median level was 5.8 pg/ml at day 0, with a nadir of 0.56 pg/ml at day 28 post-transplant. There was a significant variance in median levels between time-points ($p < 0.0001$). The significant differences occurred between pre-transplant and day 0 and day 0 and 7, 14, 28, 60, 100 and 180.

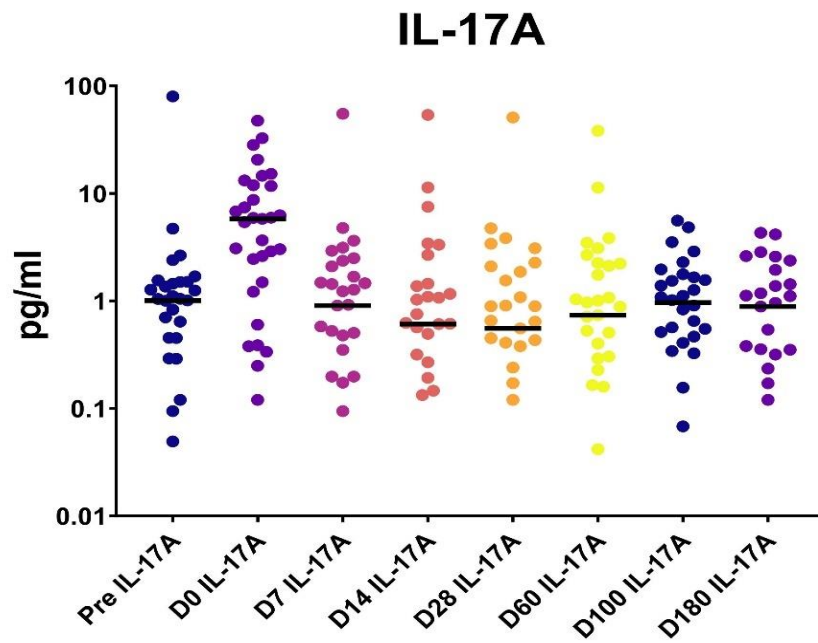


Figure 190: Interleukin-17A levels at serial time-points, logarithmic representation.

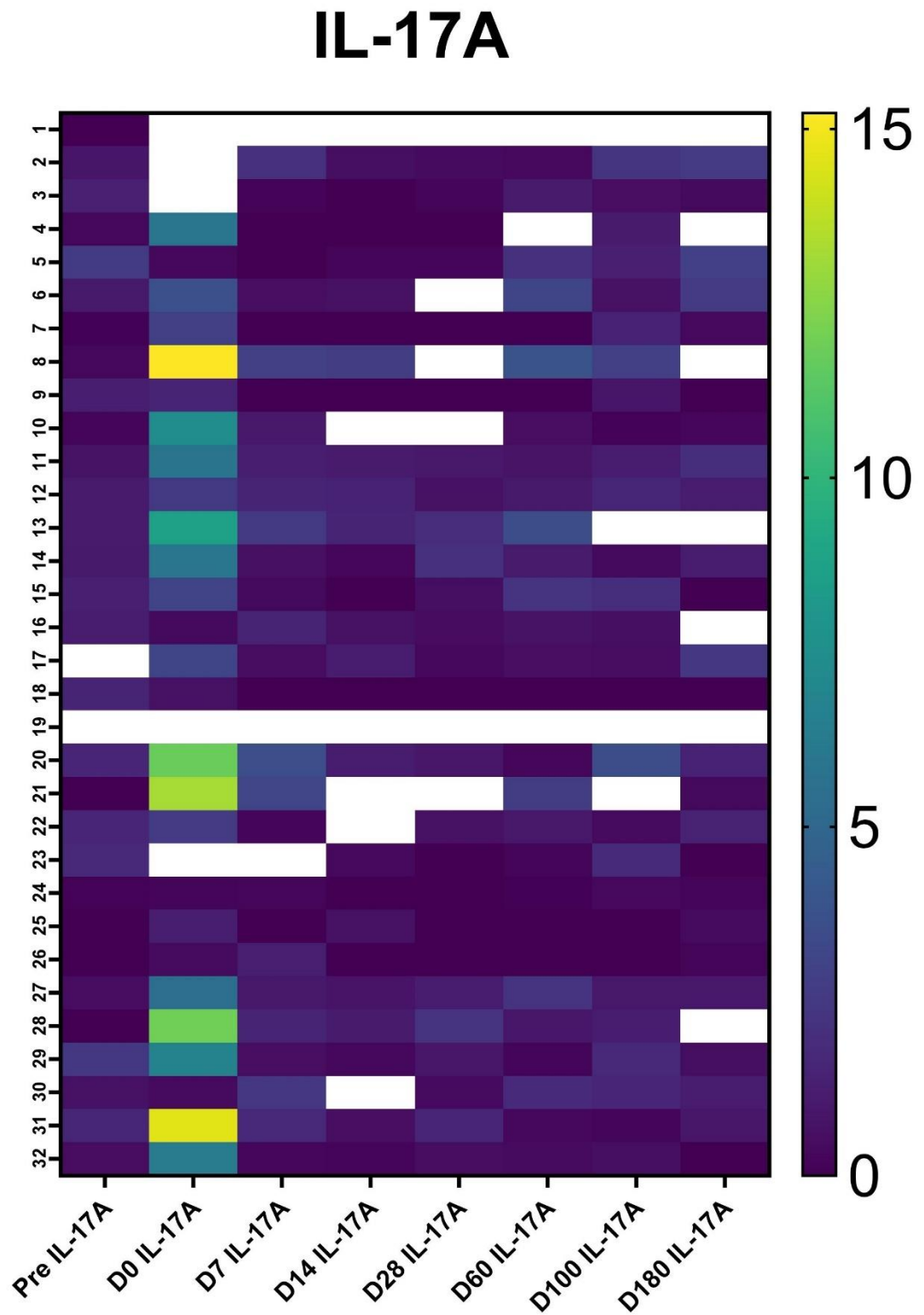


Figure 191: Interleukin-17A expression over serial time-points, significant outliers and no sample at given time-point represented by white box.

Interleukin-33

IL-33 was quantified in only 40/244 samples; therefore, the median was <0.01 pg/ml at all time-points, with a small number of patients having quantifiable levels at various time-points and only one patient with consistent levels over time (Fig. 192, 193). There was no significance variation in levels by time-point ($p=0.51$).

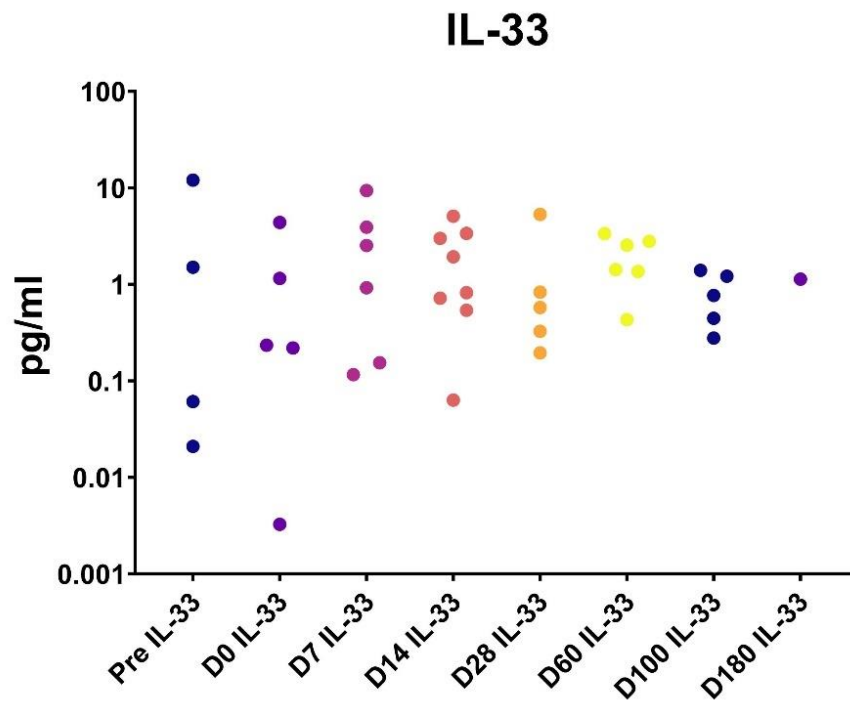


Figure 192: Interleukin-33 levels at serial time-points, logarithmic representation.

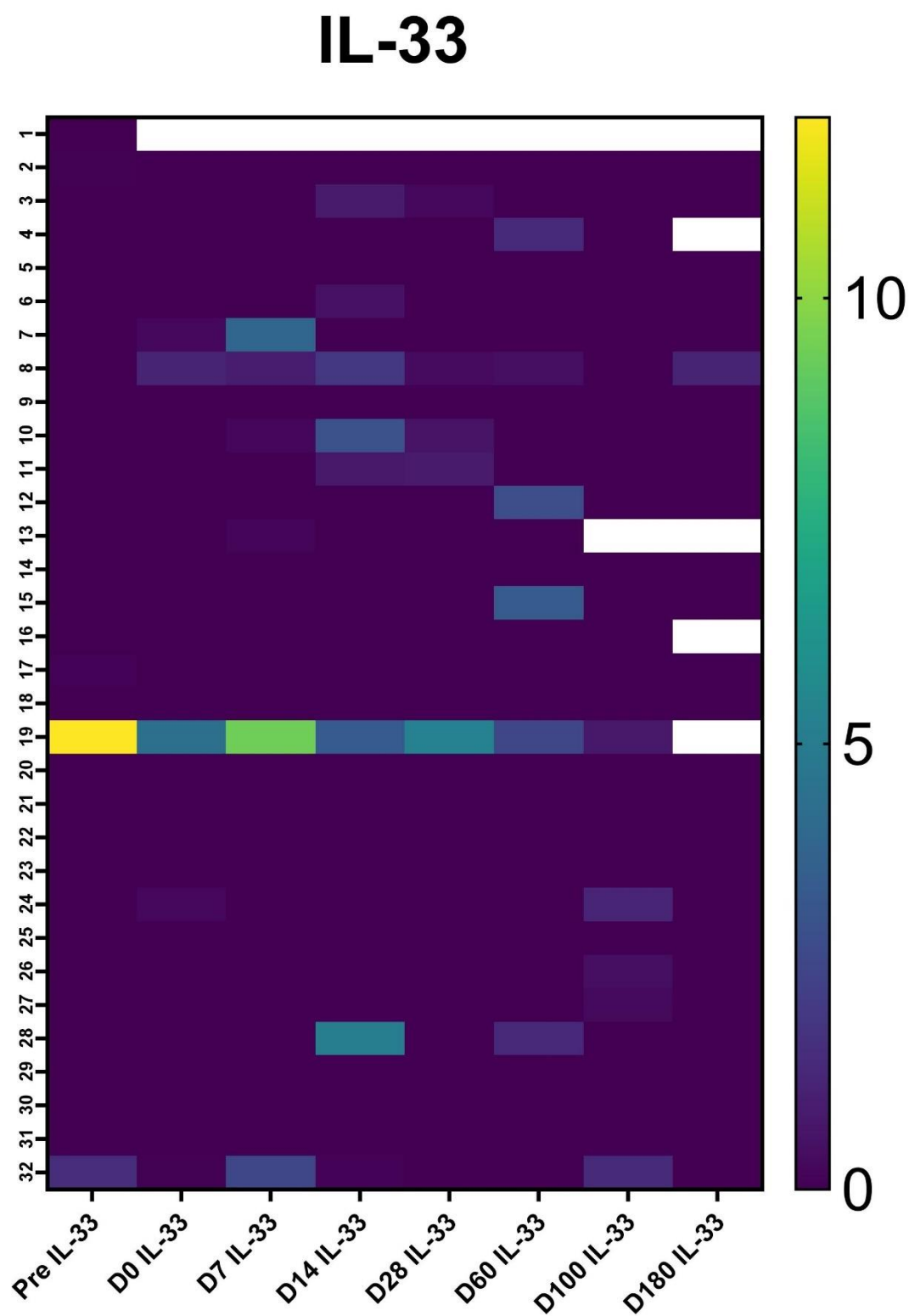


Figure 193: Interleukin-33 expression over serial time-points, significant outliers and no sample at given time-point represented by white box.

Tumour necrosis factor alpha

TNF α levels were quantifiable in 234/244 samples (*Fig. 194, 195*). The peak expression was at day 0, at a level of 0.44 pg/ml and the nadir was at day 7 at 0.12 pg/ml. One patient demonstrated a markedly raised level at the day 7 time point. The variance of median levels between time-points was small but significant ($p=0.04$). The significant differences between distinct time-points occurred between day 0 and 7, 14 and 60, day 7 and 28 and day 28 and 60.

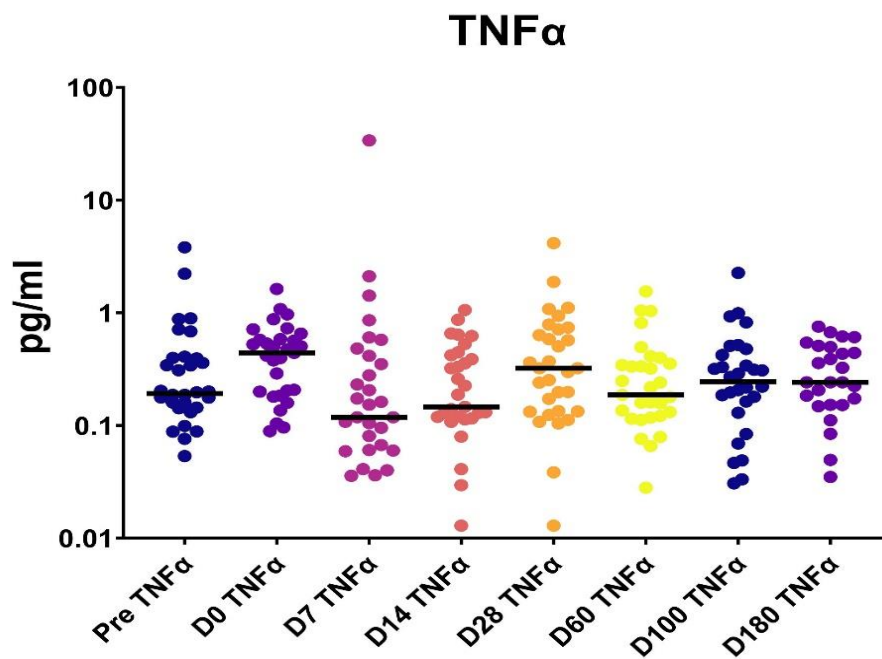


Figure 194: TNF α levels at serial time-points, logarithmic representation.

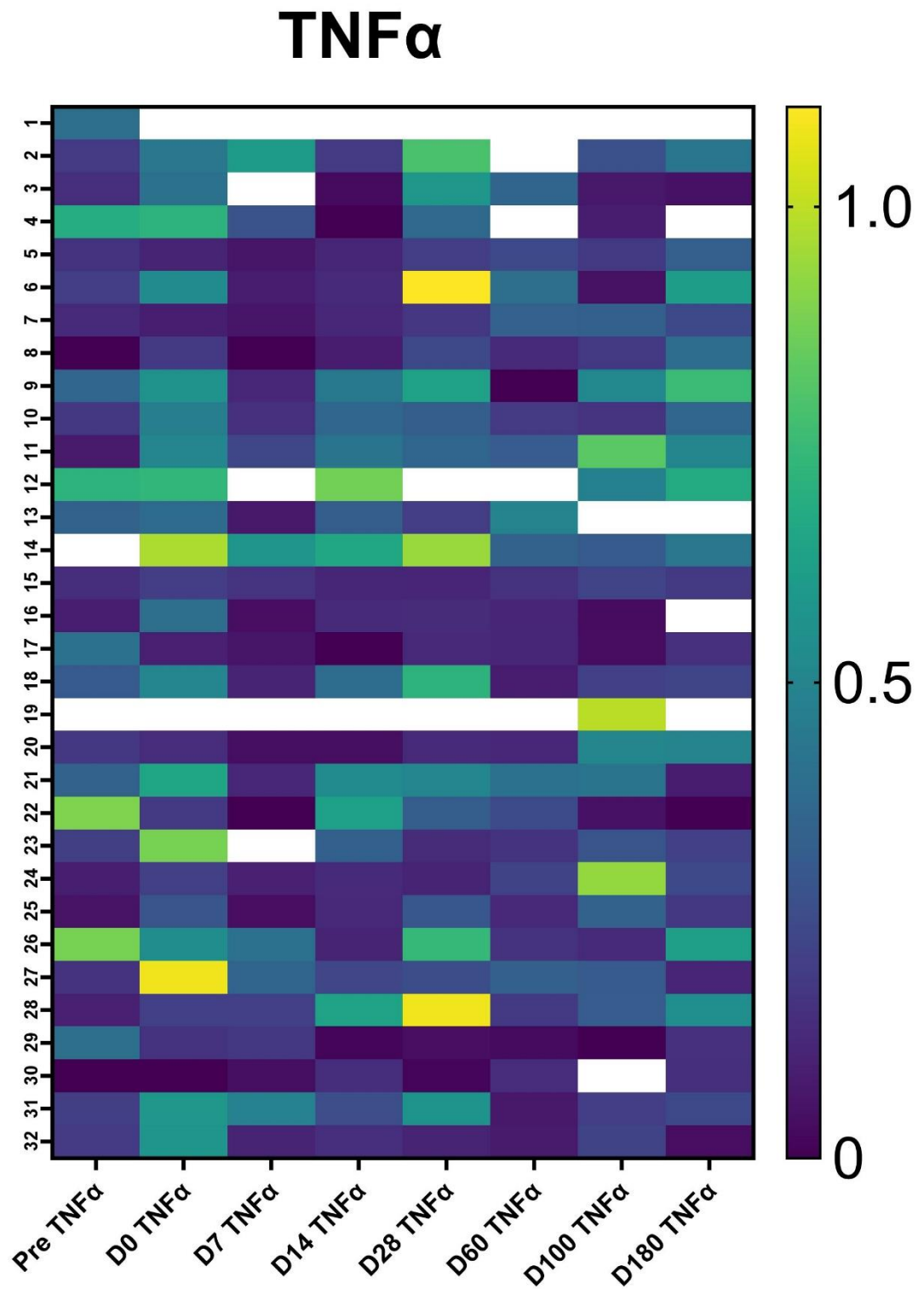


Figure 195: TNF α expression over serial time-points, significant outliers and no sample at given time-point represented by white box.

Cytokine interactions

At the pre-transplant time-point, the strongest correlation between individual cytokines was between IL1- β , IL-2, IL-4, IL-12p70, IL-17A and IL-33, and to a lesser extent in IL-6, IL-10 and TNF α , whereas no correlation was seen with IFN γ (Fig. 196). At day 0 the strongest association was between IL1- β and IL-12p70 and IL-33 (Fig. 197). A similar pattern as seen pre-transplant re-emerged at day 7, with a cluster of associations involving IL1- β , IL-4, IL-12p70, IL-17A and IL-33 but a weak association with IL-2 at this time-point (Fig. 198). A second correlation group was seen involving IL-2, IL-6, IL-10 and TNF α . At day 14 the strongest association was between IL-1 β and IL-17A, followed by IL-4 and IL-12p70. Weak negative correlations involving IL-4, IL-33 and IFN γ first occurred at this time-point (Fig. 199).

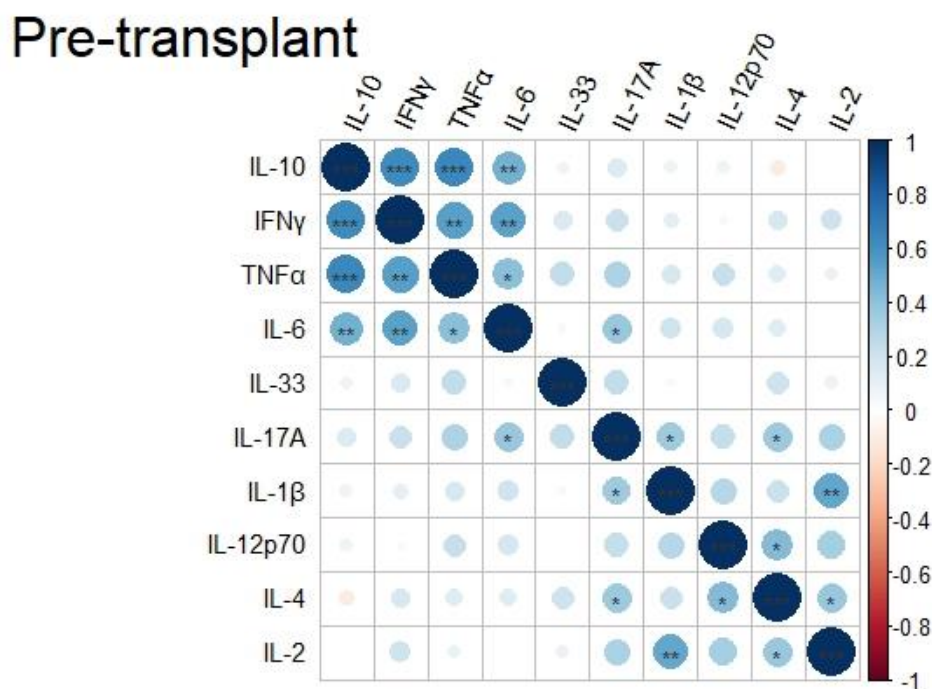


Figure 196: Correlation matrix of cytokines at the pre-transplant time-point. Red hues indicate negative correlation and blue indicate positive correlation.

Day 0

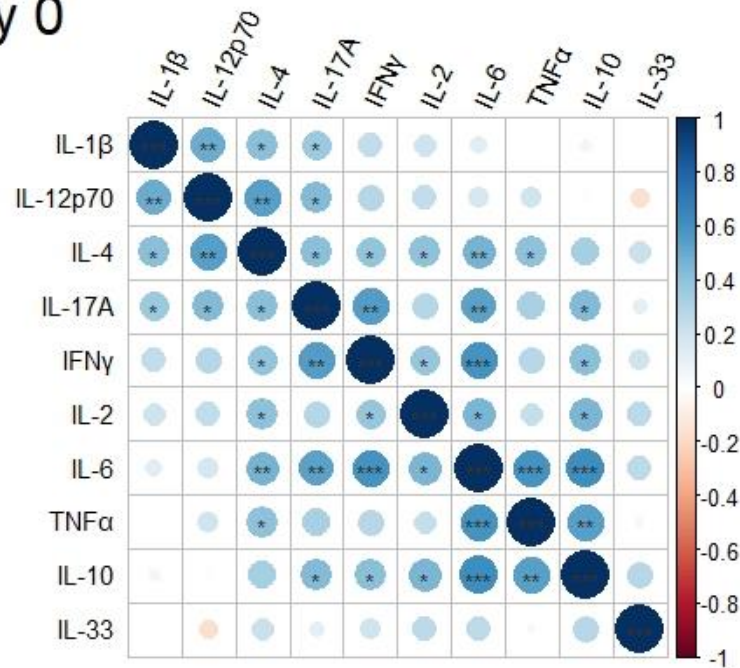


Figure 197: Correlation matrix of cytokines at the day 0 time-point. Red hues indicate negative correlation and blue indicate positive correlation.

Day 7

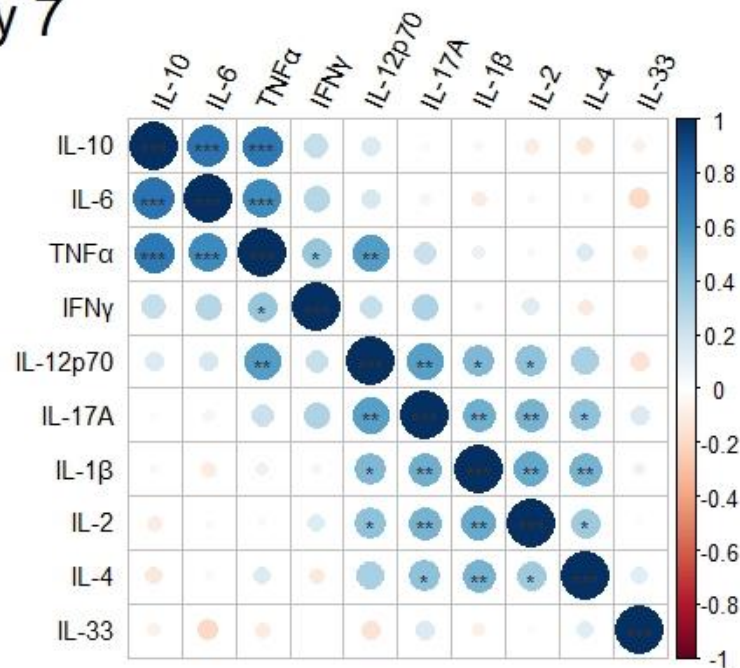


Figure 198: Correlation matrix of cytokines at the day 7 time-point. Red hues indicate negative correlation and blue indicate positive correlation.

Day 14

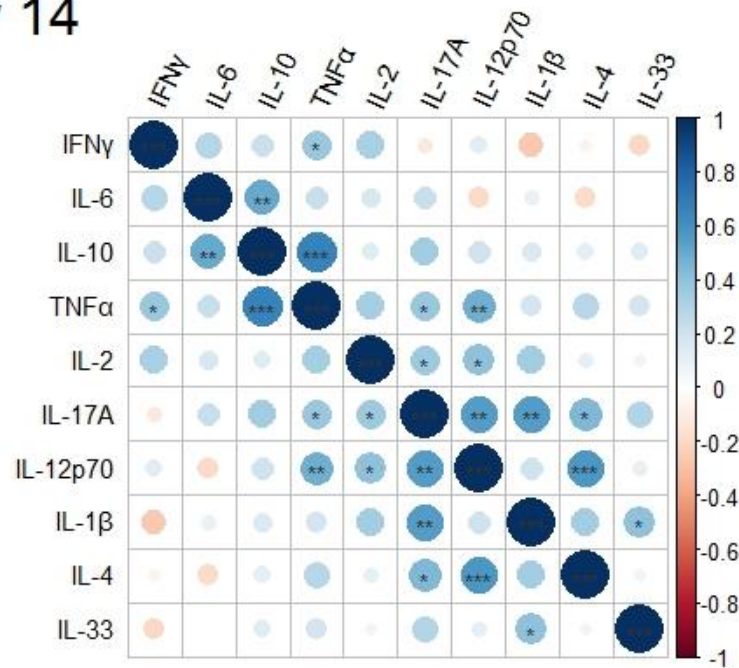


Figure 199: Correlation matrix of cytokines at the day 14 time-point. Red hues indicate negative correlation and blue indicate positive correlation.

By day 28 the pattern seen at pre-transplant and day 7 occurred again, with associations between IL1- β , IL-2, IL-4, IL-12p70, IL-17A and IL-33 (Fig. 200). At day 60 correlation occurred between IL-2, IL-12p70 and IL-17A and furthermore between IL-1 β and IL-4 (Fig. 201). Associations appeared weakest at the day 100 time-point, with the weakest again being IFN γ and all other cytokines (Fig. 202). At day 180 there was an association between IL-2 and IL-4 and further between IFN γ and IL-33, in addition to a weak negative correlation between IL-1 β and IL-6 and IL-1 β and IL-10 (Fig. 203). Global cytokine co-expression is demonstrated in Fig. 204.

Day 28

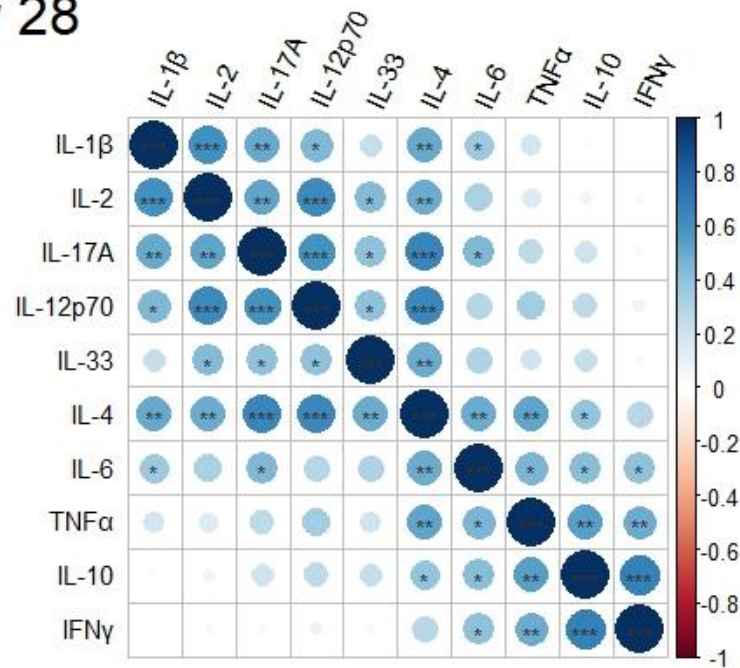


Figure 200: Correlation matrix of cytokines at the day 28 time-point. Red hues indicate negative correlation and blue indicate positive correlation.

Day 60

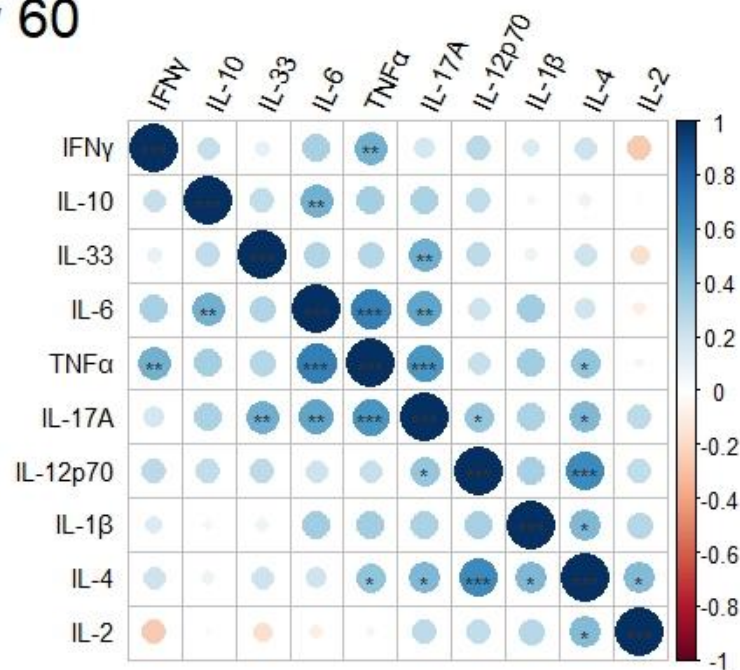


Figure 201: Correlation matrix of cytokines at the day 60 time-point. Red hues indicate negative correlation and blue indicate positive correlation.

Day 100

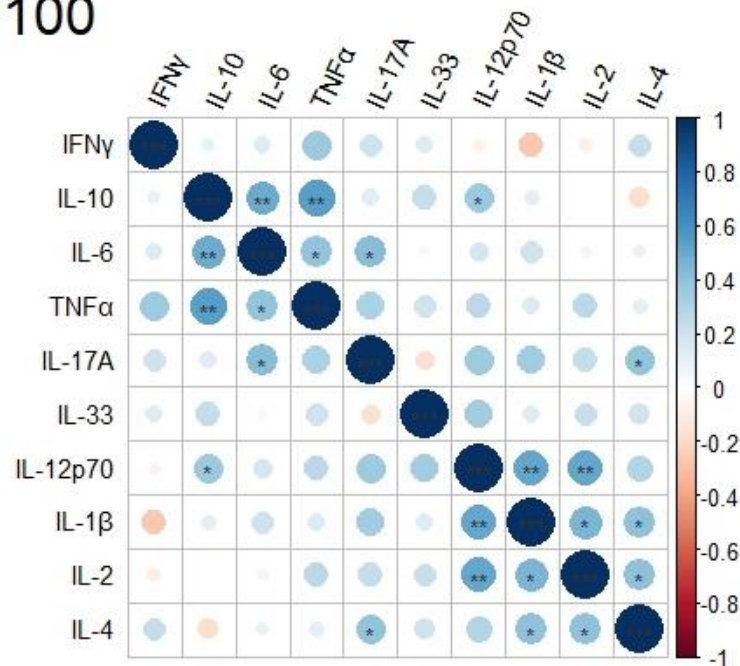


Figure 202: Correlation matrix of cytokines at the day 100 time-point. Red hues indicate negative correlation and blue indicate positive correlation.

Day 180

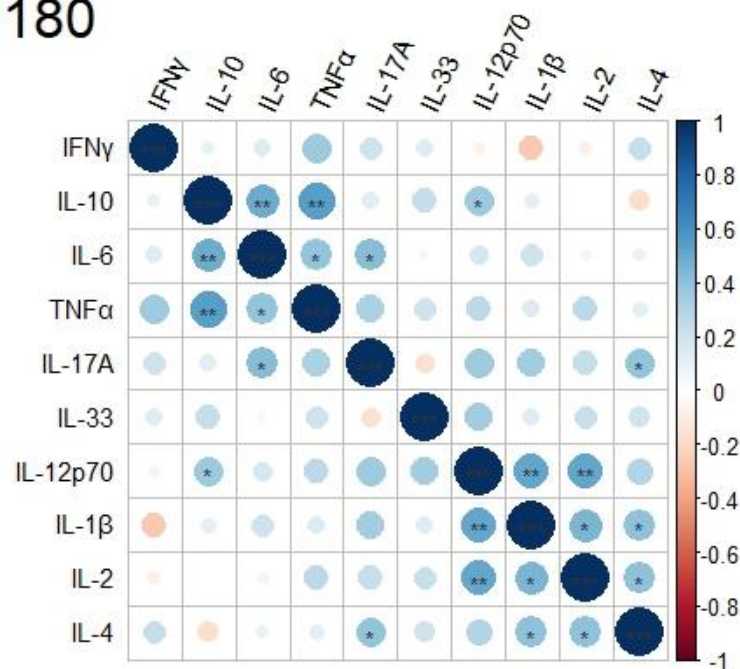


Figure 203: Correlation matrix of cytokines at the day 180 time-point. Red hues indicate negative correlation and blue indicate positive correlation.

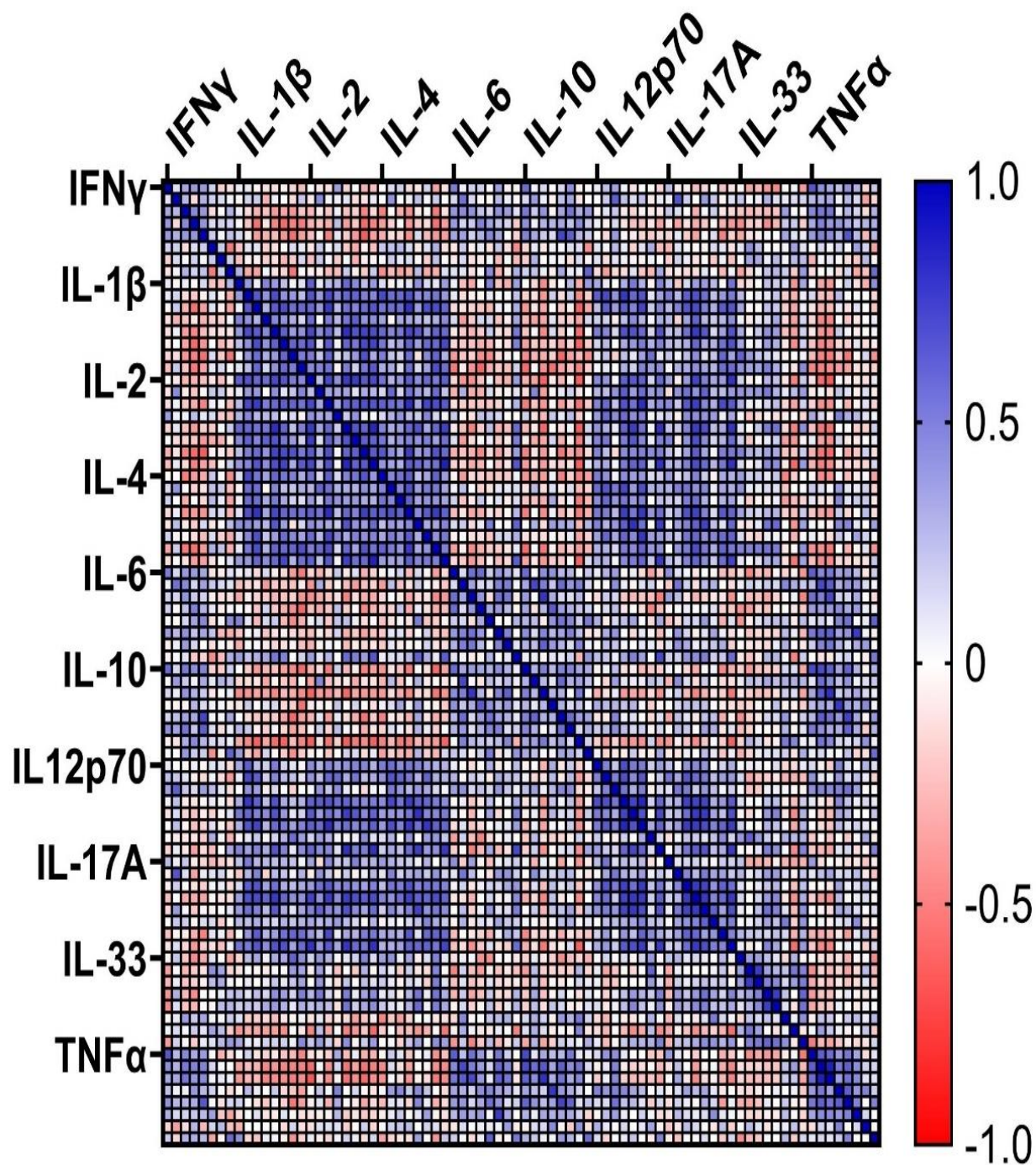


Figure 204: Correlation matrix of cytokine, amalgamated time-points. Red hues indicate negative correlation and blue indicate positive correlation. Each small square on the X or Y axis represents a sequential time-point from pre-transplant to day 180.

Correlative analysis

Cytokines per patient

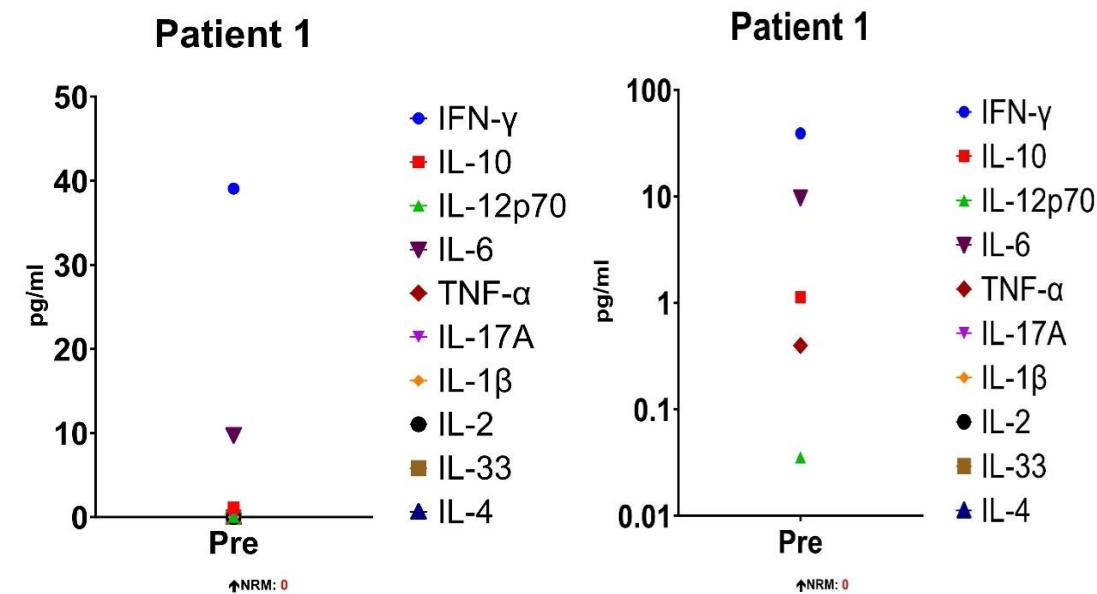


Figure 205: Patient 1 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 1 had adverse risk AML with a complex karyotype and experienced the shortest survival with an NRM event at day 0 due to bacterial sepsis on a background of prolonged aplasia pre-transplant. Only the pre-transplant cytokine levels were available, which identified a significant IFN γ level of 39.07, 34.39 pg/ml greater than the median for this time-point. Similarly a markedly raised IL-6 level was noted at 9.77 pg/ml, 9.08 greater than the median of 0.69 pg/ml. IL-10 was raised at 1.12, with a median for this time-point of 0.2 and TNF α and IL-12p70 were within one quartile of the median. The remaining cytokines including IL-1 β , IL-2, IL-4, IL-17A and IL-33 were undetectable.

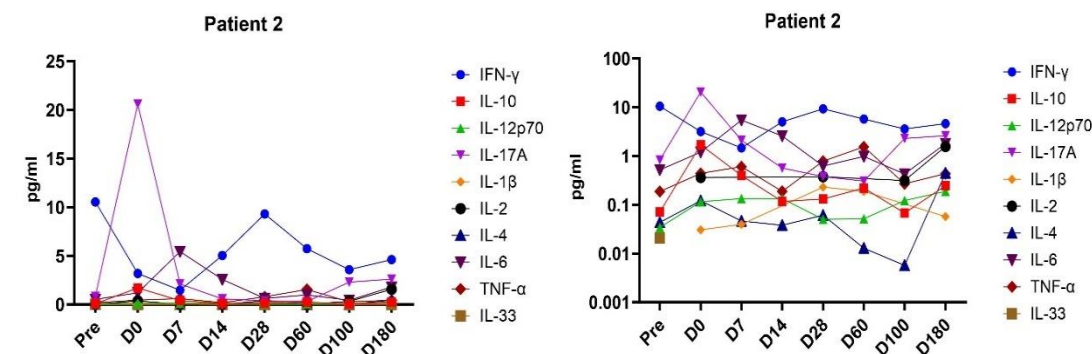


Figure 206: Patient 2 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 2 had intermediate risk AML with a normal karyotype and a *FLT-3 ITD* mutation. An IL-17A level one standard deviation from the mean was noted at day 0, however this patient did not develop any significant complications. Sorafenib maintenance was started at day 100 and an increase in IL-4 expression was noted by day 180. No complications had developed by a follow up of 701 days.

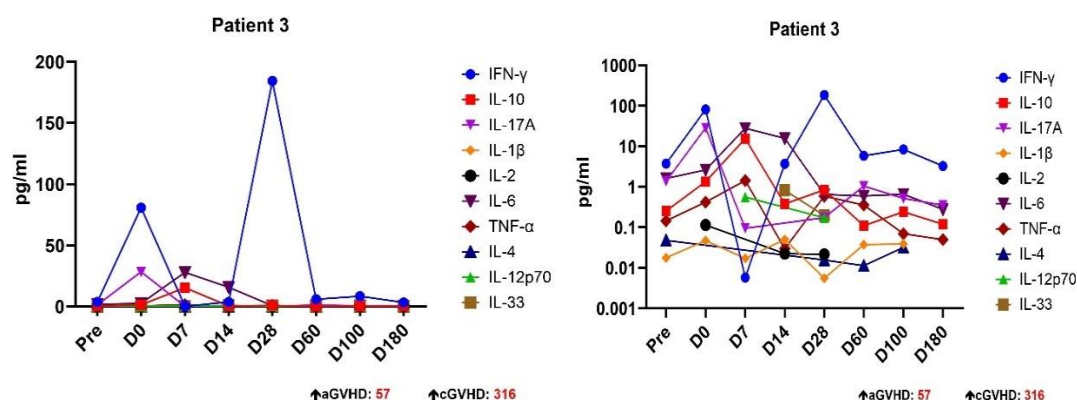


Figure 207: Patient 3 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 3 had intermediate risk AML with trisomy 21, isodicentric 21(p11.2) and a *DNMT3A* mutation. Grade II upper gastrointestinal acute GVHD occurred on day 57 and moderate and extensive chronic GVHD at day 316. A notable peak in IFN γ level of 184.15 pg/ml was seen at the time-point preceding acute gastrointestinal GVHD onset (day 28), when the median for this time-point was 10.39 pg/ml. A smaller but significantly raised IFN γ level of 80.71 pg/ml was seen at day 0, where the median was 3.24 pg/ml. No further complications occurred by a follow up of 548 days.

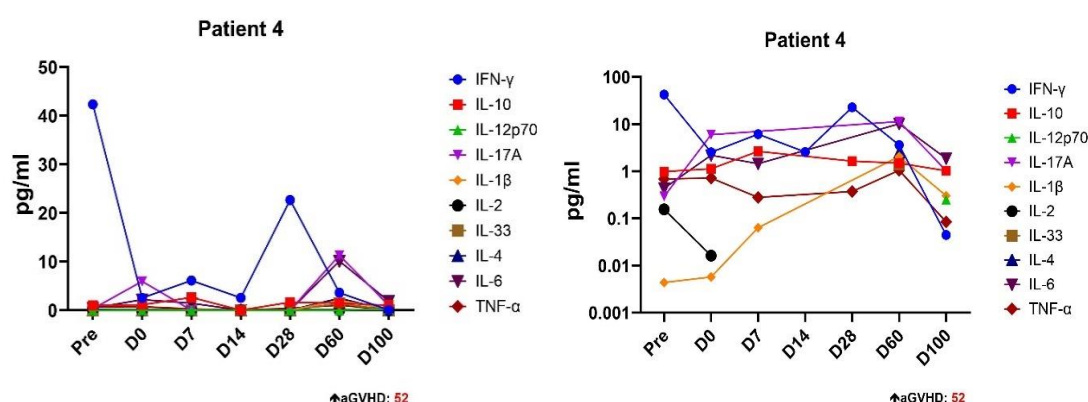


Figure 208: Patient 4 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 4 had IPSS-M moderate-high risk therapy-related MDS with a loss of 20q and mutated *ASXL1*, *DNMT3A*, *TET2*, *PHF6* and *TP53*. Steroid-refractory grade III acute GVHD developed at day 54. A markedly raised IFN γ level of 42.31 pg/ml was noted at the pre-transplant time-point, with a secondary peak of 22.65 pg/ml at day 28, the time-point immediately preceding

development of significant acute gastrointestinal GVHD. Increased IL-1 β , IL-6 and IL-17A were also seen around the onset of GVHD. No further complications occurred at a follow up of 281 days.

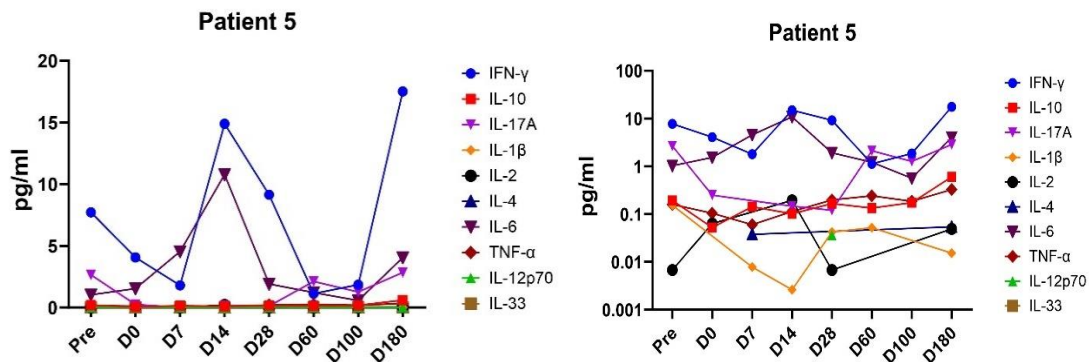


Figure 209: Patient 5 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 5 had favourable risk AML with a normal karyotype and biallelic bZIP *CEBPA* mutations but also a *WT1* mutation. A peak in IFN γ of 17.51 pg/ml was seen at day 180 (median 4.86 pg/ml) which was 8 weeks post-DLI, and at which time a minor rash developed, but was not confirmed as acute GVHD. No complications had developed subsequently at a follow up of 534 days.

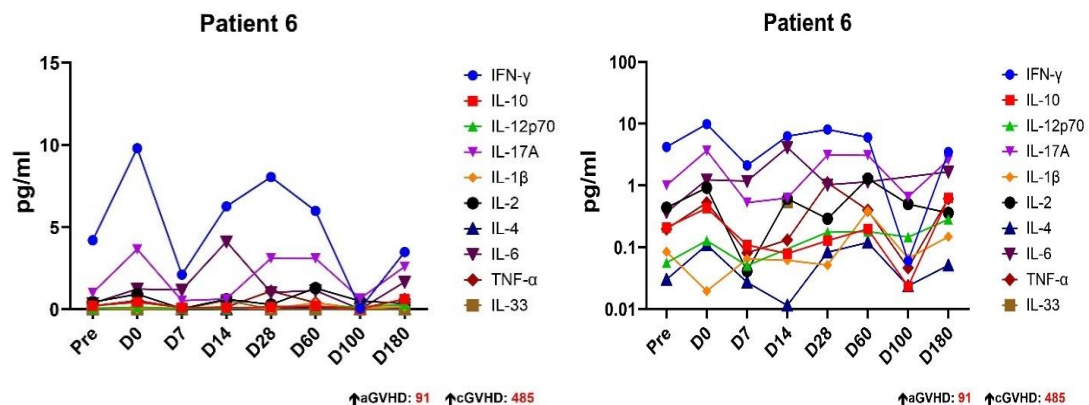


Figure 210: Patient 6 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 6 had IPSS-M high-risk therapy-related MDS with a normal karyotype and mutations in *ASXL1* and *IDH2*. Chronic maintenance steroid use before and throughout transplant, due to pre-morbid sarcoidosis, may have affected the cytokine expression profile and kinetics. IFN γ levels peaked at day 0 and 28 and an additional peak was seen at the time-point preceding acute skin GVHD onset at day 91. Late onset severe and extensive chronic GVHD

developed at day 485 and this occurred spontaneously (no prior DLI). Last follow up was 673 days.

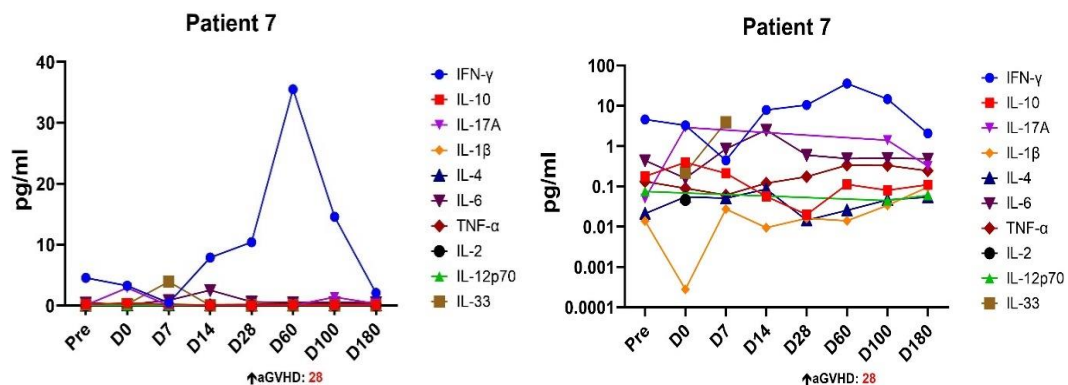


Figure 211: Patient 7 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 7 had adverse risk AML with a normal karyotype and mutations in *ASXL1*, *FLT3* and *IDH2*. A peak IFN γ level of 35.51 pg/ml at day 60 was seen, with a median at that time-point of 4.4 pg/ml. This peak developed after grade II skin and gastrointestinal acute GVHD onset at day 28. IL-10 levels of 0.19 pg/ml, below the median of 0.34 pg/ml were noted at onset of acute GVHD. No further complications had manifested by a follow up of 477 days.

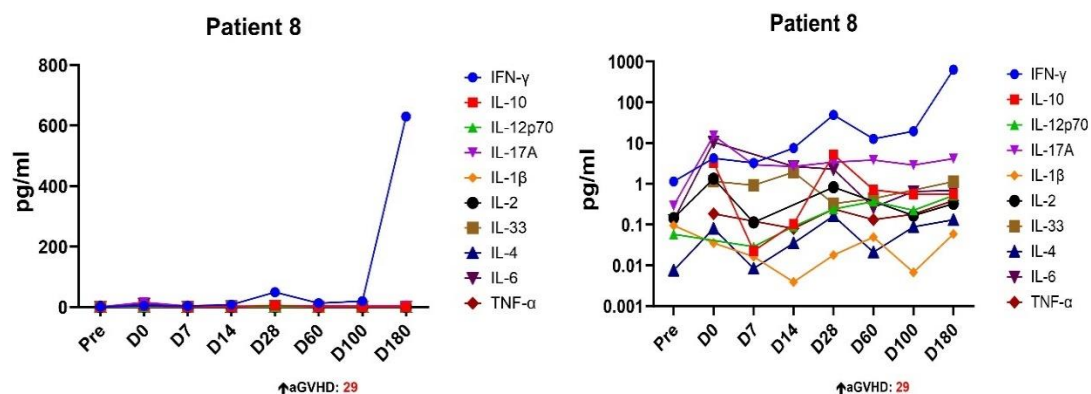


Figure 212: Patient 8 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 8 had CPSS-Mol intermediate-2 CMML with a normal karyotype and mutations in *NRAS* and *SRSF2*. Raised IFN γ levels of 48.96 pg/ml were seen at day 28, median 10.39 pg/ml, which coincided with onset of low-grade acute skin GVHD. A further large spike in IFN γ was seen at day 180 at 629.21 pg/ml (median 4.86 pg/ml), although no clinical complications occurred at this time-point or subsequently up to a follow-up of 491 days.

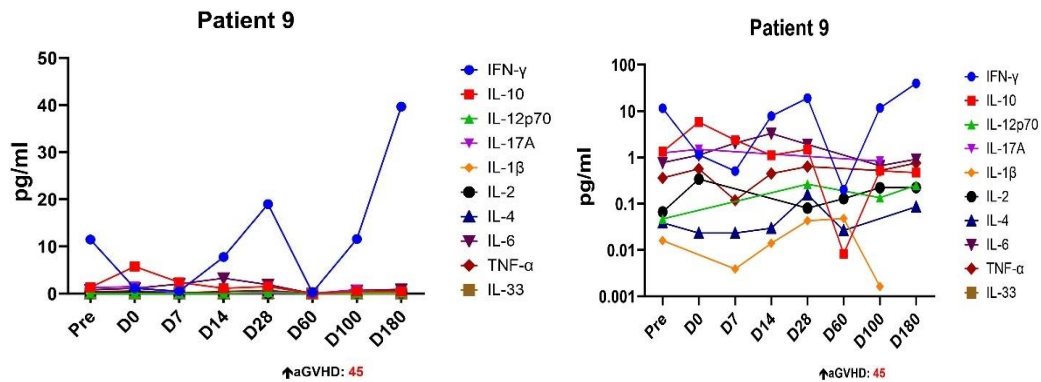


Figure 213: Patient 9 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 9 had IPSS-M very-high risk MDS with monosomy 7 and mutations in *RUNX1* and *BCOR*. An IFN γ peak of 18.98 pg/ml was noted at day 28, prior to grade I acute skin GVHD onset at day 45. A further IFN γ peak was observed at day 180 at 39.66 pg/ml (median 4.86 pg/ml), although no further complications developed by a follow-up of 505 days.

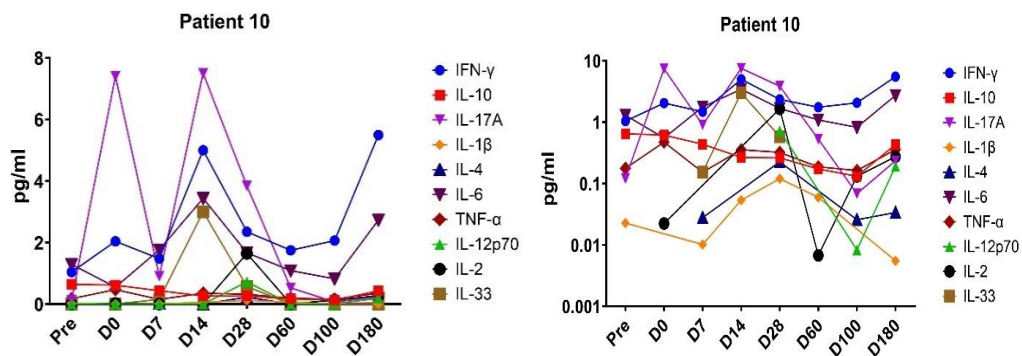


Figure 214: Patient 10 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 10 had intermediate risk AML with a normal karyotype and mutations in *DDX41*, *DNMT3A*, *STAT5B* and *CUX1*. Two peaks of IL-17A were noted at day 0 (7.4 pg/ml, median 5.8 pg/ml) and day 14 (7.49 pg/ml, median 0.61 pg/ml). No transplant complications were noted at a follow-up of 548 days, however prophylactic azacitidine was commenced at 3 months post-transplant due to a suggestion of impending relapse by bone marrow morphology at this time-point. Full donor chimerism was maintained at all assessments.

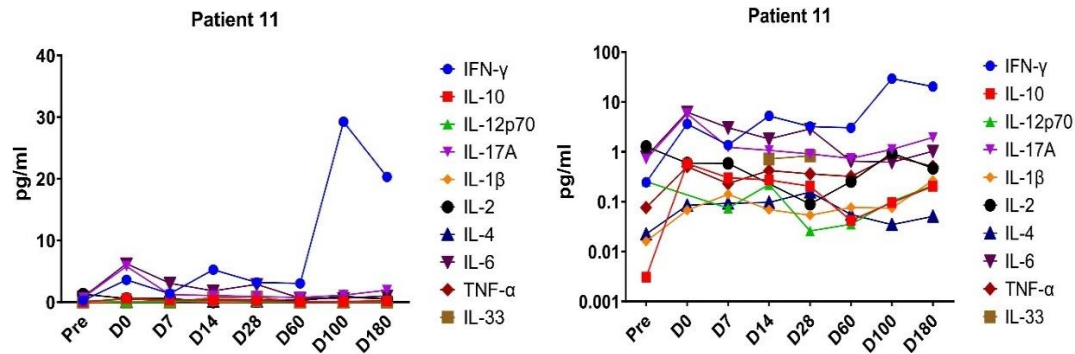


Figure 215: Patient 11 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 11 had intermediate risk AML with a normal karyotype and mutations in *FLT3* and *TET2*. A peak IFN γ level at day 100 was noted at 29.24 pg/ml (median 5.08 pg/ml) but was not associated with any complications up to a follow up of 653 days. Sorafenib maintenance commenced between the day 100 and day 180 time-points.

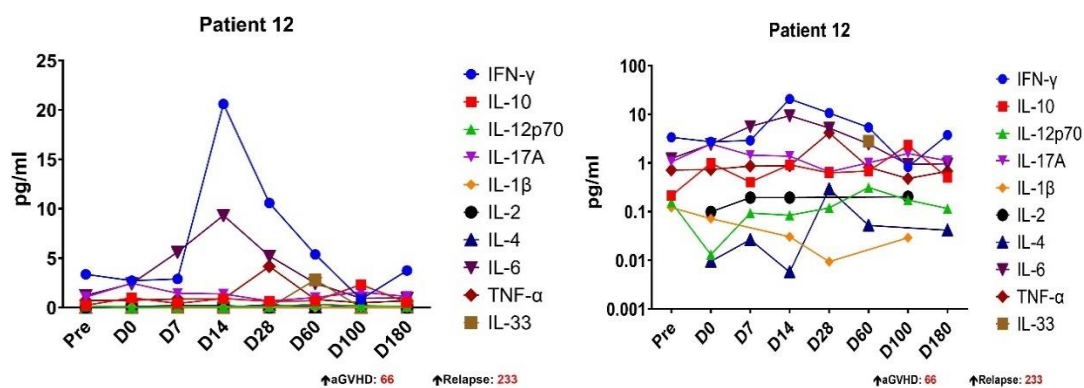


Figure 216: Patient 12 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 12 had intermediate-2 risk myelofibrosis with a normal karyotype and mutations in *JAK2*, *MPL*, *ASXL1* and *CBL*. A peak IFN γ of 20.59 pg/ml occurred at day 14 (median 7.3 pg/ml) alongside IL-6 (9.32 pg/ml, median 2.61 pg/ml) and was followed by the development of grade I skin acute GVHD at day 66. Relapse occurred at day 233, although no significant skewing of cytokine levels was seen at the prior time-point. No further GVHD development occurred up to a follow up of 709 days.

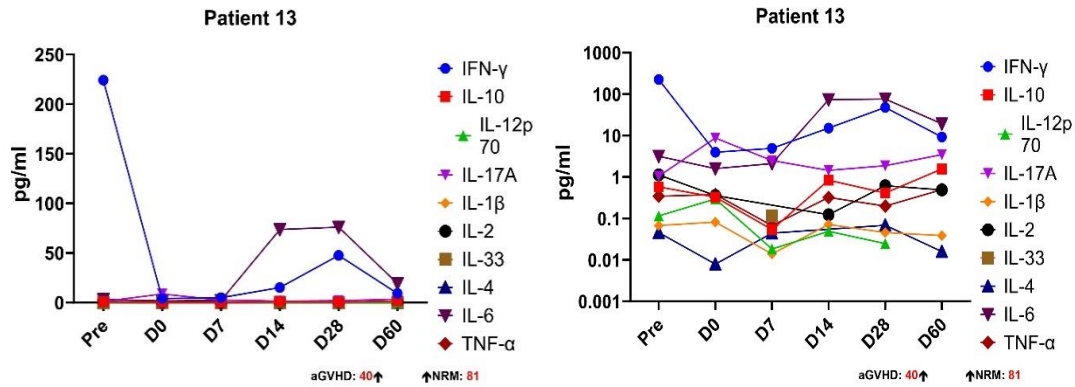


Figure 217: Patient 13 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 13 had intermediate risk AML with a normal karyotype and mutations in *NPM1*, *DNMT3A*, *FLT3* and *TET2*. IFN γ expression was marked in the pre-transplant period, with a level of 224.16 pg/ml, median 4.38 pg/ml. A smaller peak occurred at day 28 at 47.47 pg/ml (median 10.39 pg/ml) in addition to a flare in IL-6 at 76 pg/ml (median 1.09 pg/ml), both of which preceded onset of grade II gastrointestinal acute GVHD at day 40 and a non-relapse mortality event at day 81 due to disseminated and cerebral toxoplasmosis.

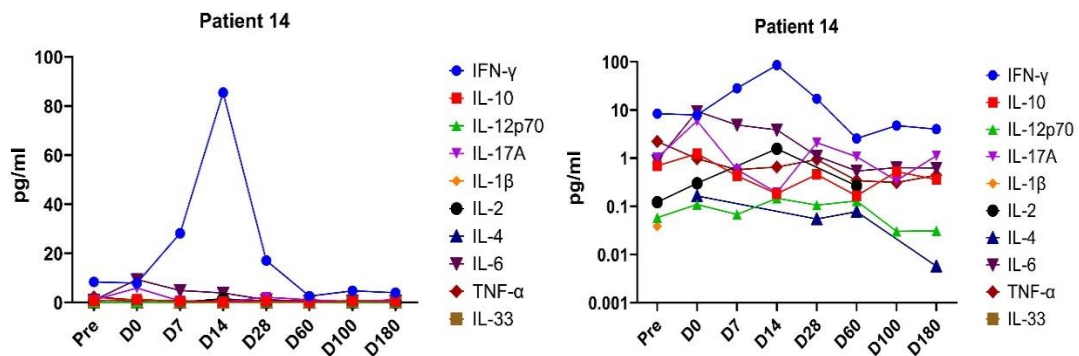


Figure 218: Patient 14 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 14 had adverse risk AML with trisomy 8 and mutations in *ASXL1*, *EZH2* and *U2AF1*. A marked IFN γ level of 85.42 pg/ml was recorded at day 14 (median 7.3 pg/ml) but was not associated with any subsequent complications up to a follow up of 695 days.

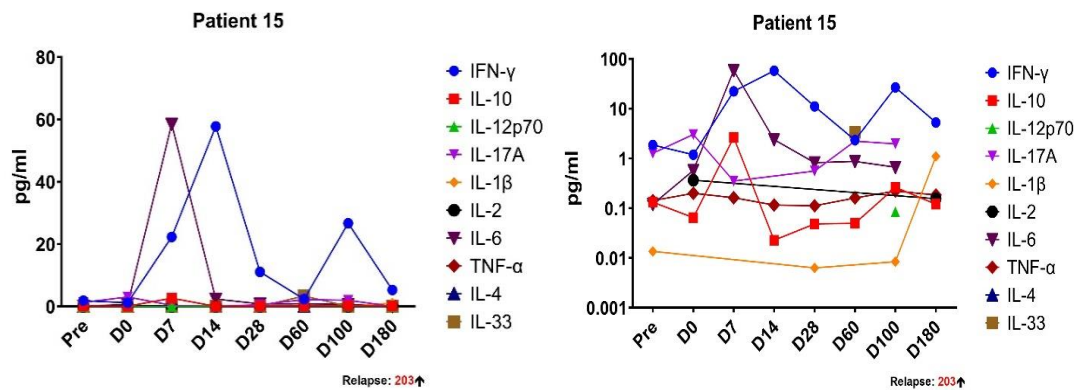


Figure 219: Patient 15 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 15 had IPSS-M high-risk MDS with a failed karyotype and mutations in *RUNX1* (germline), *BCOR*, *DNMT3A*, *GATA2*, *ZRSR2* and *SF3B1*. Two distinct IFN γ peaks were noted at day 14 and day 100 in addition to marked IL-6 secretion at day 7 at 58.25 pg/ml (median 2.03 pg/ml). Relapse occurred at day 203, which was preceded by a rise in IL-1 β to 1.09 pg/ml at day 180 (median 0.04 pg/ml).

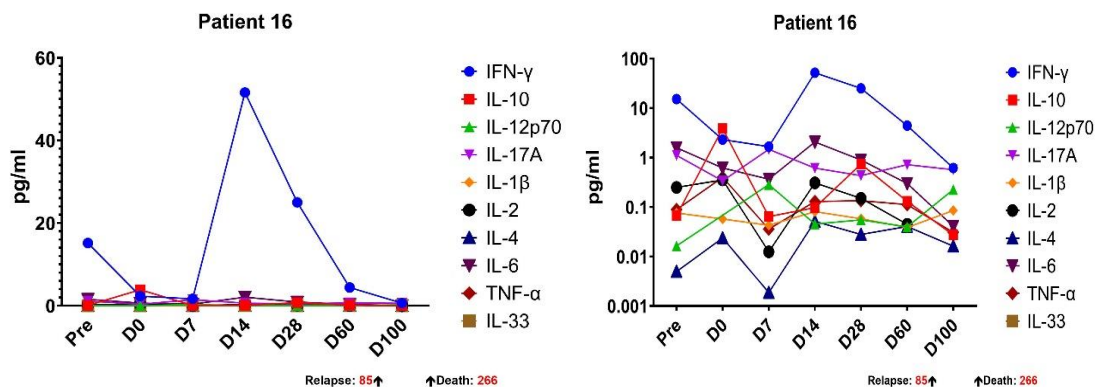


Figure 220: Patient 16 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 16 had adverse risk AML with monosomy 9 and mutations in *SRSF2*, *ASXL1* and *RUNX1*. Raised IFN γ levels pre-transplant were identified at 15.17 pg/ml (median 4.38 pg/ml). A flare in IFN γ secretion was seen at day 14 (51.57 pg/ml, median 7.3 pg/ml). Relapse occurred at day 85 and was followed by relapse-related death at day 266.

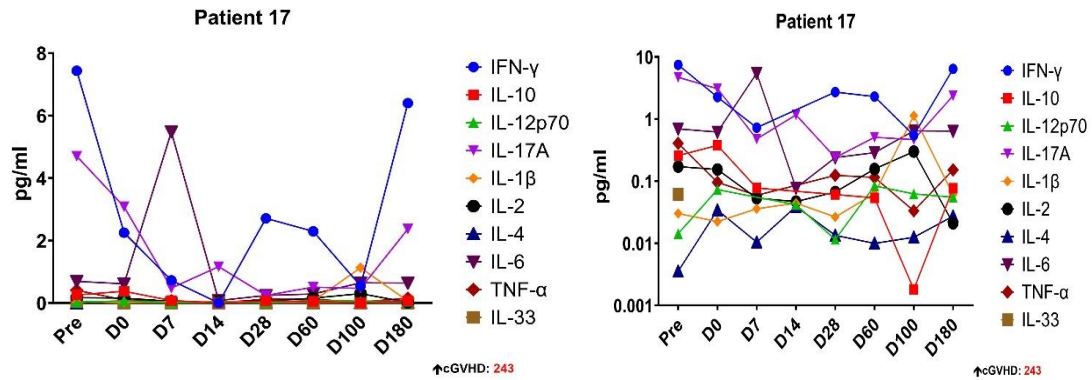


Figure 221: Patient 17 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 17 had intermediate risk AML with -Y and mutations in *NRAS*, *TET2* and *DDX41*. A secondary peak in IFN γ was seen at day 180 at 6.39 pg/ml (median 4.86 pg/ml) in addition to a smaller rise in IL-17A of 2.36 pg/ml (median 0.89 pg/ml) prior to the onset of moderate and limited chronic GVHD at day 243.

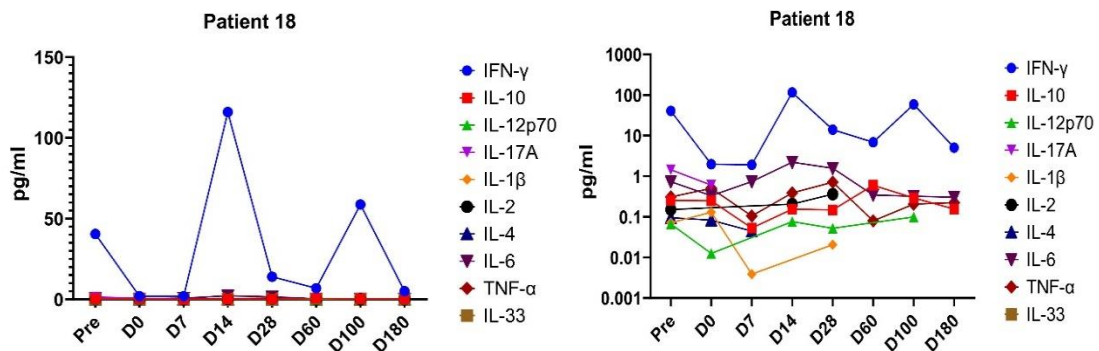


Figure 222: Patient 18 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 18 had adverse risk AML with 11q⁺ and mutations in *FLT3*, *IDH2*, *DNMT3A* and *ASXL1*. Three IFN γ peaks were noted at the pre-transplant time-point (40.49 pg/ml, median 4.38 pg/ml), day 14 (115.92 pg/ml, median 7.3 pg/ml) and day 100 (58.72 pg/ml, median 5.08 pg/ml). No adverse events occurred by a follow-up of 380 days and maintenance midostaurin was commenced between the day 100 and day 180 time-points.

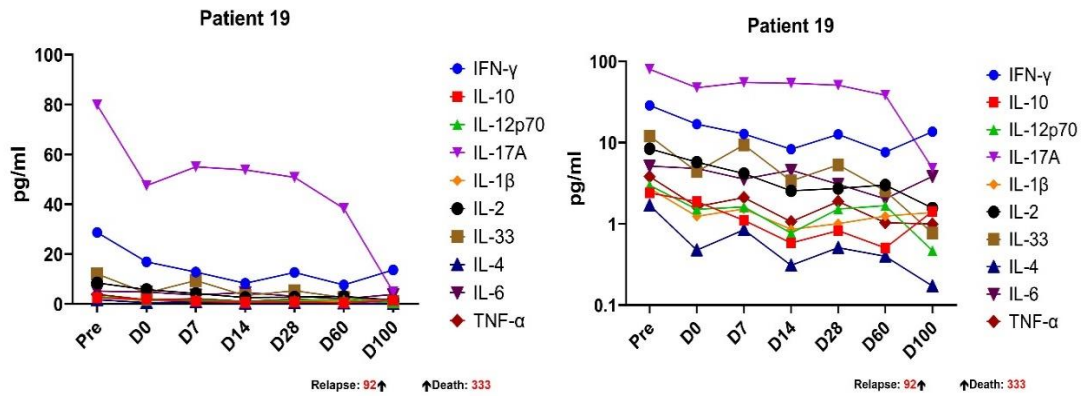


Figure 223: Patient 19 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 19 had intermediate risk AML with trisomy 8, a subclone with an additional X chromosome, an additional MYC signal on FISH and a *WT1* mutation. Marked IL-17A secretion was noted from the pre-transplant time-point up to day 100. Additionally, elevated IFN γ secretion was seen at all time-points. Relapse occurred at day 92, followed by relapse-related death at day 333. This patient was the only participant who demonstrated quantifiable levels of all 10 cytokines included in the panel at all examined time-points.

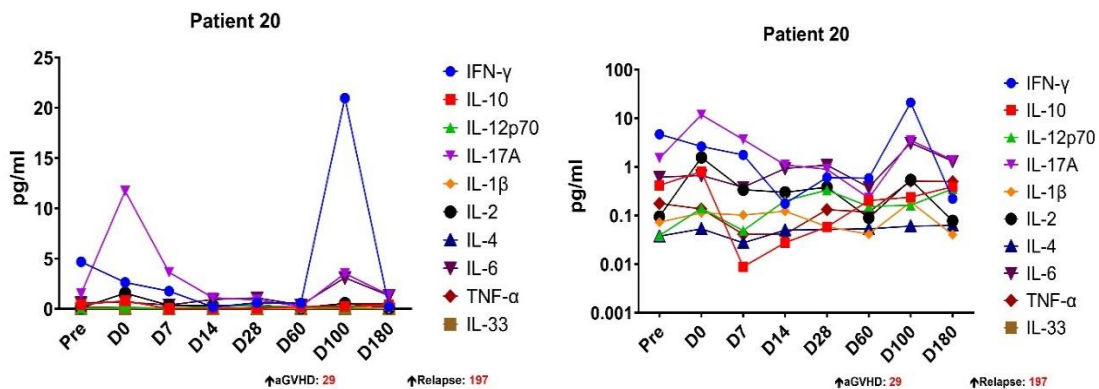


Figure 224: Patient 20 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 20 had adverse risk AML with a DEK::NUP214 translocation and a *FLT3* mutation. Grade II gastrointestinal and skin acute GVHD occurred at day 29 and was preceded by a flare in IL-17A secretion at day 0 (11.71 pg/ml, median 5.8 pg/ml). Relapse occurred at day 197. Maintenance with midostaurin was commenced at 3 months but was stopped shortly after due to poor tolerance. A peak IFN γ level of 20.96 pg/ml was seen at day 100 (median 5.08 pg/ml). Following relapse, salvage therapy with gilteritinib and sequential DLI was commenced with no further complications by day 562.

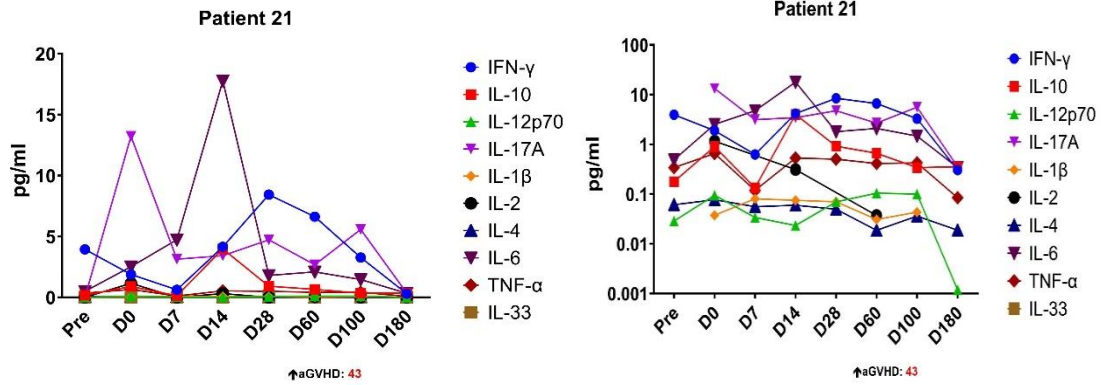


Figure 225: Patient 21 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 21 had high-risk myelofibrosis with mutations in *MPL* and *U2AF1*. Peaks in IL-17A at day 0 (13.22 pg/ml, median 5.8 pg/ml), IL-6 at day 14 (17.78 pg/ml, median 2.61 pg/ml) and IL-10 at day 14 (3.97 pg/ml, median 0.13 pg/ml) preceded liver and gastrointestinal steroid-refractory grade III acute GVHD onset at day 43. No further complications occurred by last follow-up at 352 days.

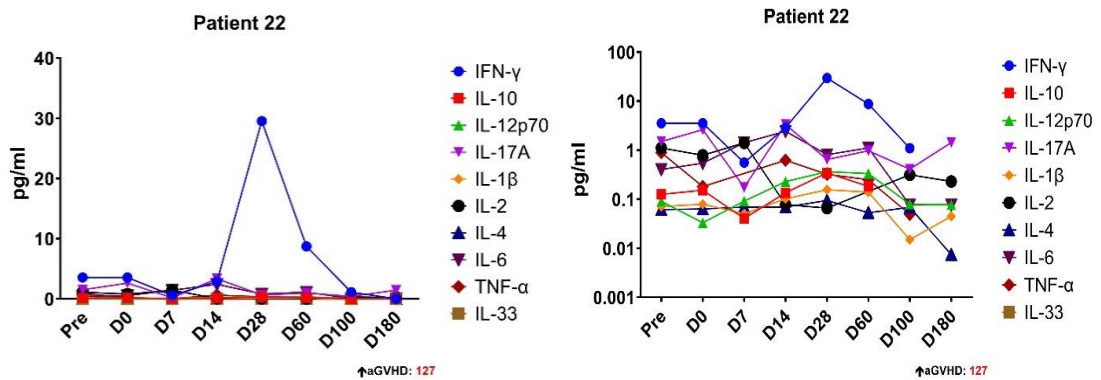


Figure 226: Patient 22 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 22 had intermediate-1 risk myelofibrosis with an *ASXL1* mutation. A peak IFN γ level was noted at day 28 (29.51 pg/ml, median 10.39 pg/ml). Grade II acute GVHD of the liver and gastrointestinal tract occurred at day 127 and was preceded by a drop in IL-10 secretion at the day 100 time-point. No further complications occurred by last follow-up at 576 days.

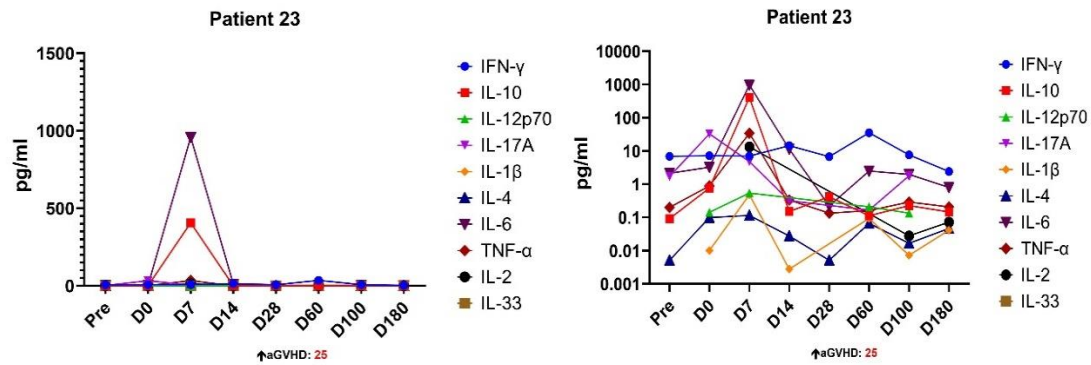


Figure 227: Patient 23 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 23 had CPSS-Mol high-risk CMML with a normal karyotype and mutations in *ASXL1*, *CSF3R*, *SRSF2* and *RUNX1*. Marked IL-6 secretion was noted at day 7 (959.08 pg/ml, median 2.03 pg/ml) in addition to IL-10 secretion (406.38 pg/ml, median 0.21 pg/ml), IL-1 β (0.48 pg/ml, median 0.03 pg/ml), IL-2 (13.26 pg/ml, median 0 pg/ml) and TNF α (33.93 pg/ml, median 0.12 pg/ml). Grade II upper gastrointestinal acute GVHD subsequently developed at day 25. No further complications developed by last follow-up at 477 days.

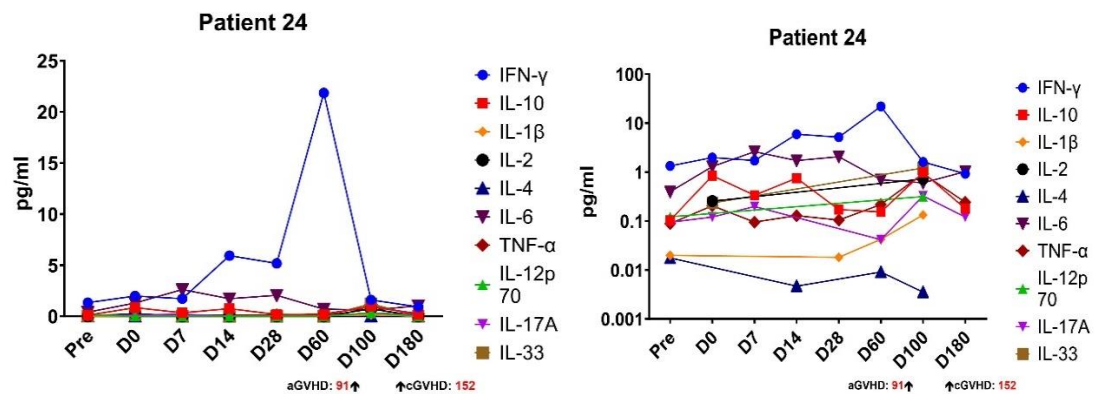


Figure 228: Patient 24 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 24 had CPSS-Mol high-risk CMML with trisomy 8 and mutations in *ASXL1*, *EZH2*, *RUNX1*, *TET2*, *KRAS* and *NRAS*. A peak in IFN γ secretion was seen at day 60 (21.85 pg/ml, median 4.4 pg/ml), which preceded onset of steroid-refractory grade II acute GVHD of the skin and liver at day 91 and subsequently moderate and limited chronic GVHD onset at day 152. Increased IL-1 β secretion occurred at day 100 prior to chronic GVHD onset (0.13 pg/ml, median 0.03 pg/ml). No further complications occurred at a follow-up of 547 days.

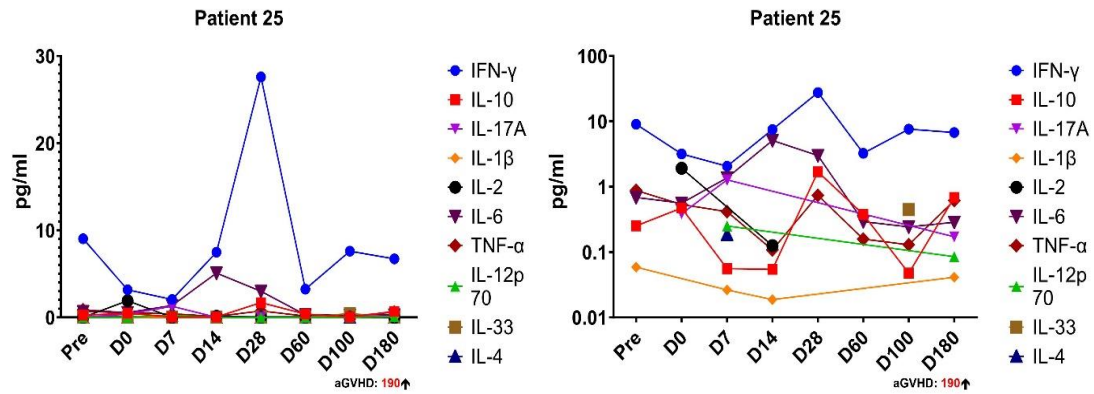


Figure 229: Patient 25 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 25 had intermediate-1 risk myelofibrosis with a normal karyotype and mutations in *CALR* and *TP53*. Peak IFN γ secretion occurred at day 28 (27.59 pg/ml, median 10.39 pg/ml). Grade II acute GVHD of liver and skin occurred at day 190 and no further complications until last follow-up at 387 days.

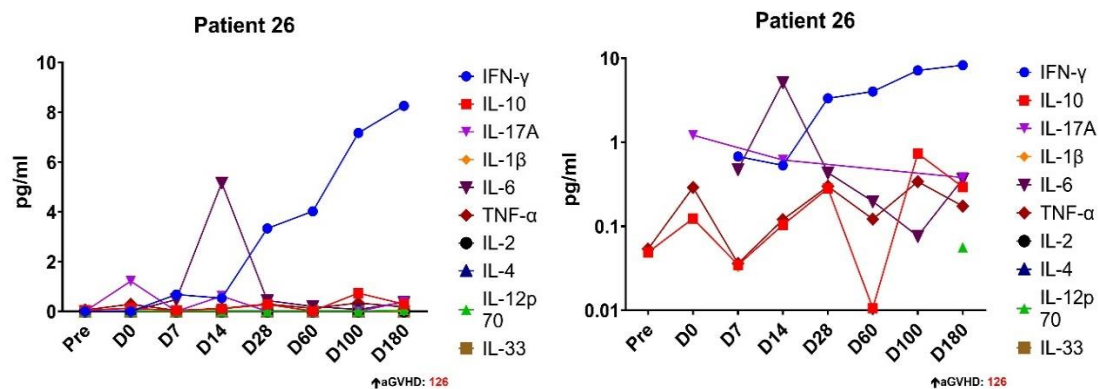


Figure 230: Patient 26 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 26 had IPSS-M high-risk MDS with a normal karyotype and mutations in *BCOR*, *DNMT3A*, *STAG2* and *U2AF1*. Low cytokine secretion of several cytokines was noted. A progressive increase in IFN γ from day 28 preceded the development of grade II acute GVHD of skin at day 126. No further complications occurred by last follow-up at 421 days.

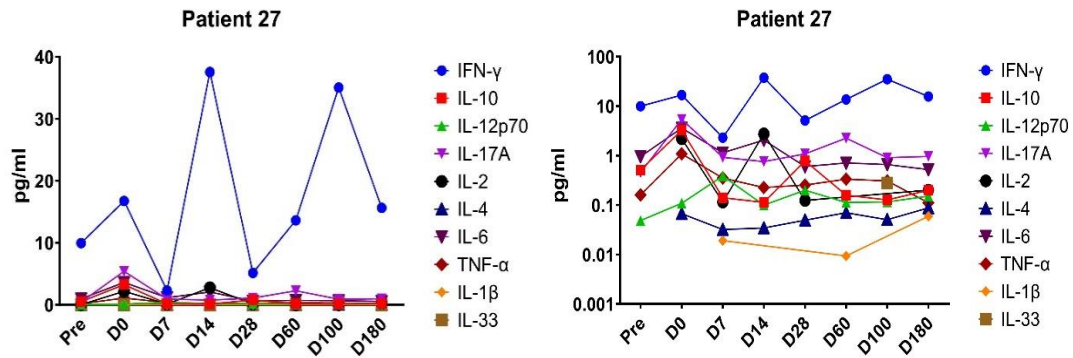


Figure 231: Patient 27 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 27 had an atypical myeloproliferative neoplasm with a $t(13;14)(q12;q32)$ with activation of the *FLT3* gene. Several moderate IFN γ peaks were noted, primarily at day 0, 14 and 100. No complications had occurred by last follow-up at 373 days.

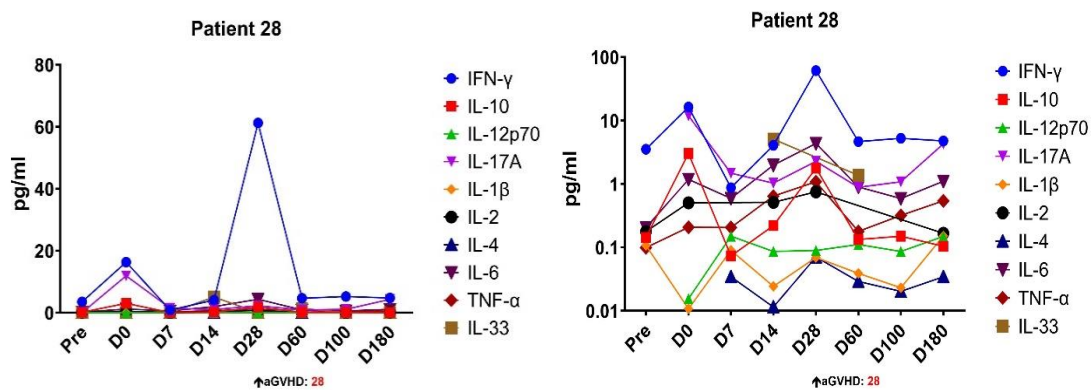


Figure 232: Patient 28 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 28 had adverse risk AML with a complex karyotype and a *TP53* mutation. A peak in IFN γ secretion occurred at the onset of acute lower gastrointestinal GVHD at day 28 (61.23 pg/ml, median 10.39 pg/ml). No further complications occurred by a follow-up of 652 days.

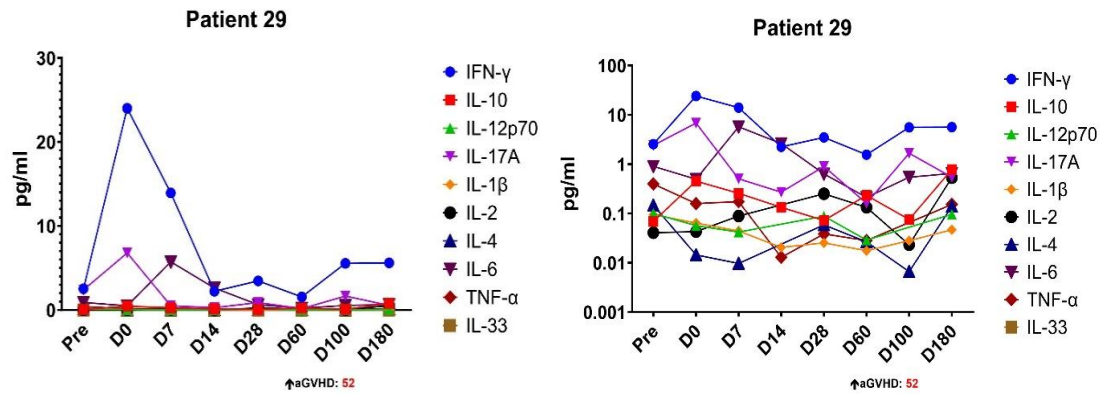


Figure 233: Patient 29 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 29 had intermediate-2 risk myelofibrosis with mutations in *CALR*, *ASXL1* and *U2AF1*. Peak IFN γ secretion occurred at the day zero time-point (23.99 pg/ml, median 3.24 pg/ml). Grade II skin and gastrointestinal acute GVHD occurred at day 52 and no further complications occurred up to a last follow-up of 512 days.

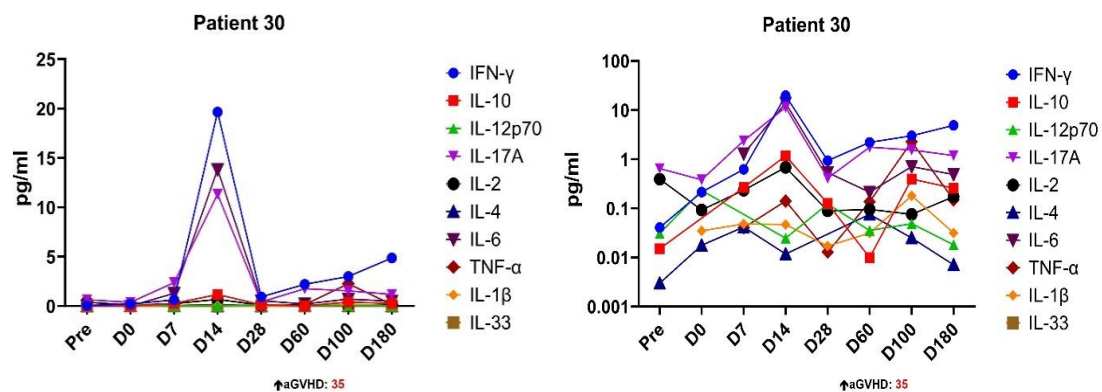


Figure 234: Patient 30 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 30 had favourable risk AML with a normal karyotype and mutations in *NPM1*, *IDH2* and *NRAS*. Increased secretion of IFN γ (19.64 pg/ml, median 7.3 pg/ml), IL-6 (13.82 pg/ml, median 2.61 pg/ml) and IL-17A (11.33 pg/ml, median 0.61 pg/ml) was seen at day 14 and occurred before onset of grade I acute GVHD of the skin at day 35. No further complications were noted by last follow-up at 499 days.

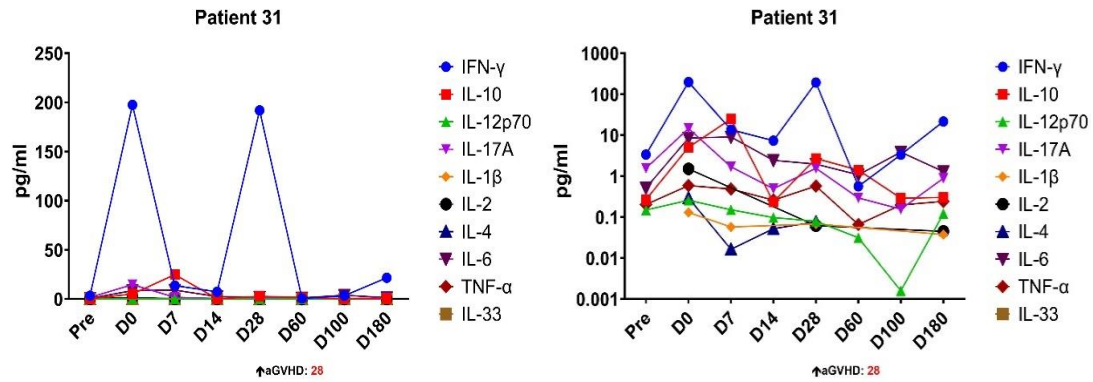


Figure 235: Patient 31 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 31 had favourable risk AML with a normal karyotype and mutations in *NPM1* and *KRAS*. Two distinct peaks of IFN γ secretion were seen at day 0 (197.42 pg/ml, median 3.24 pg/ml) and day 28 (191.44 pg/ml, median 10.39 pg/ml) with grade III acute GVHD of the gastrointestinal tract, liver and skin at day 28. No further complications occurred by last follow-up of 716 days.

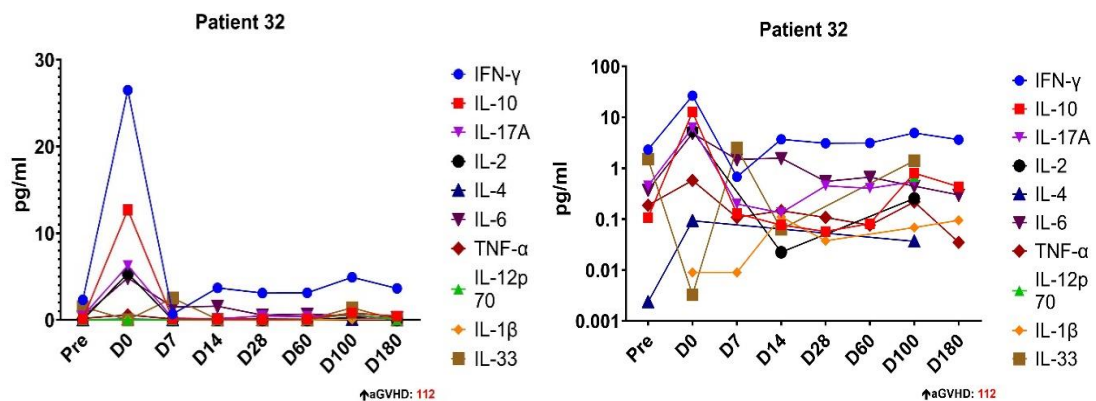


Figure 236: Patient 32 - linear and logarithmic trends in 10-cytokine expression at available time-points.

Patient 32 had IPSS-M high-risk MDS with a *KMT2A* PTD and a mutation in *ETV6*. An early peak in IFN γ (26.48 pg/ml, median 3.24 pg/ml) and IL-10 (12.67 pg/ml, median 0.8 pg/ml) was seen with the development of grade II acute gastrointestinal and liver GVHD at day 112 and no further complications by last follow-up at day 561.

Individual cytokine correlation with static variables and transplant outcomes

Interferon-gamma

No significant difference in IFN γ secretion was seen between disease subtypes or donor age. Female recipients had a higher day 28 level as compared to men ($p=0.01$) (Fig. 237). IFN γ levels did not differ significantly between donor source, ABO mismatch and graft cryopreservation but an increase in day 28 secretion was seen in those who received tacrolimus as compared to ciclosporin ($p=0.03$) (Fig. 238). The IFN γ levels varied most between survivors and those who died at the pre-transplant time-point and at day 28. A significant difference based on the pre-transplant IFN γ level was seen ($p=0.001$) (Fig. 239). No difference in median IFN γ levels was seen in those who relapsed as compared to those who did not. Day 14 IFN γ levels were lower in those who developed grade II-IV acute GVHD ($p=0.003$) (Fig. 240).

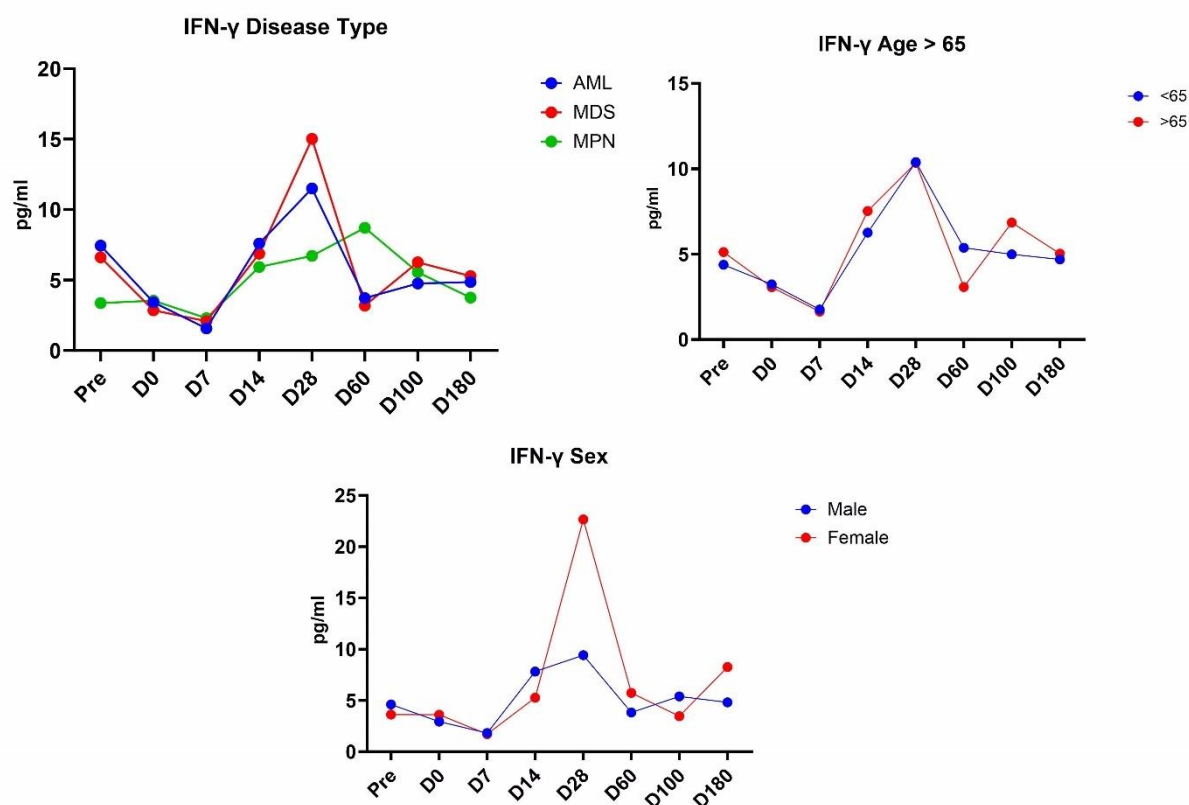


Figure 237: Median IFN γ secretion at serial time-points; disease subtype, age group and recipient sex.

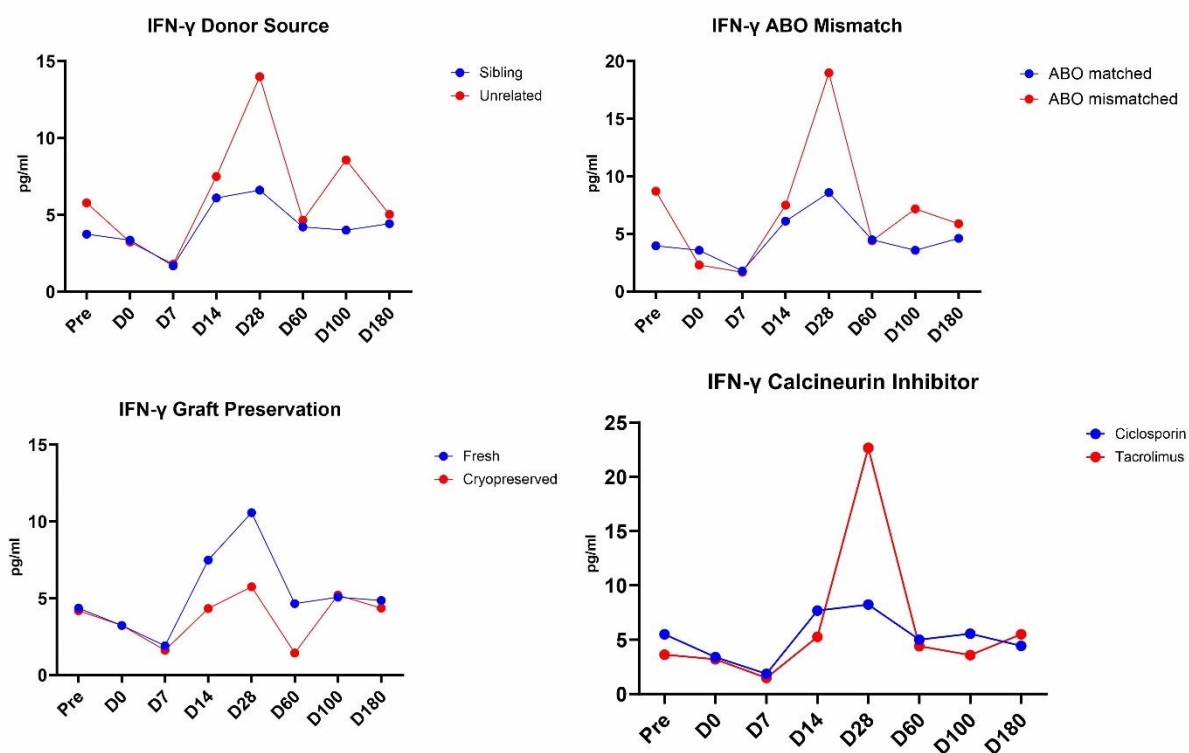


Figure 238: Median IFN γ secretion at serial time-points; donor source, graft preservation, ABO mismatch status and calcineurin inhibitor use.

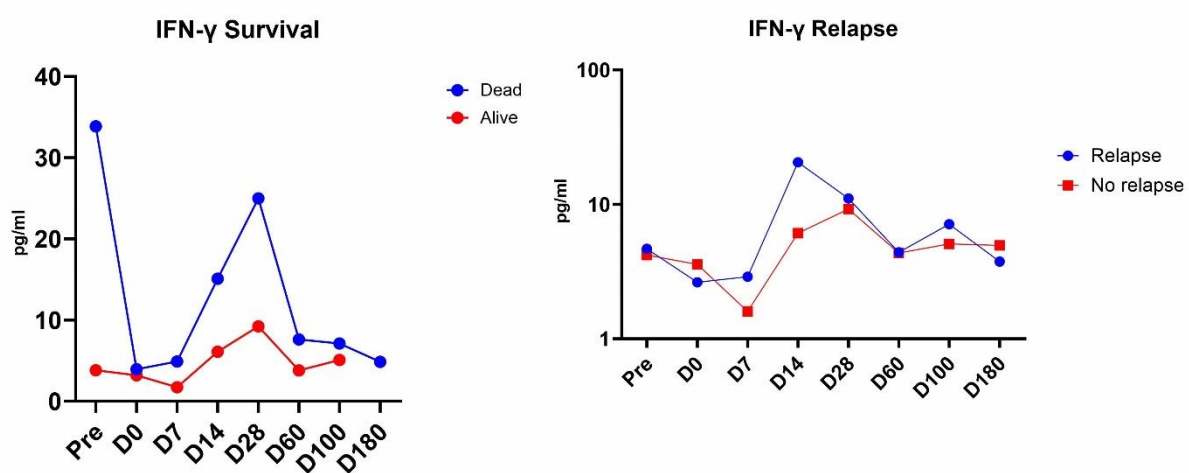


Figure 239: Median IFN γ secretion at serial time-points; relapse and overall survival.

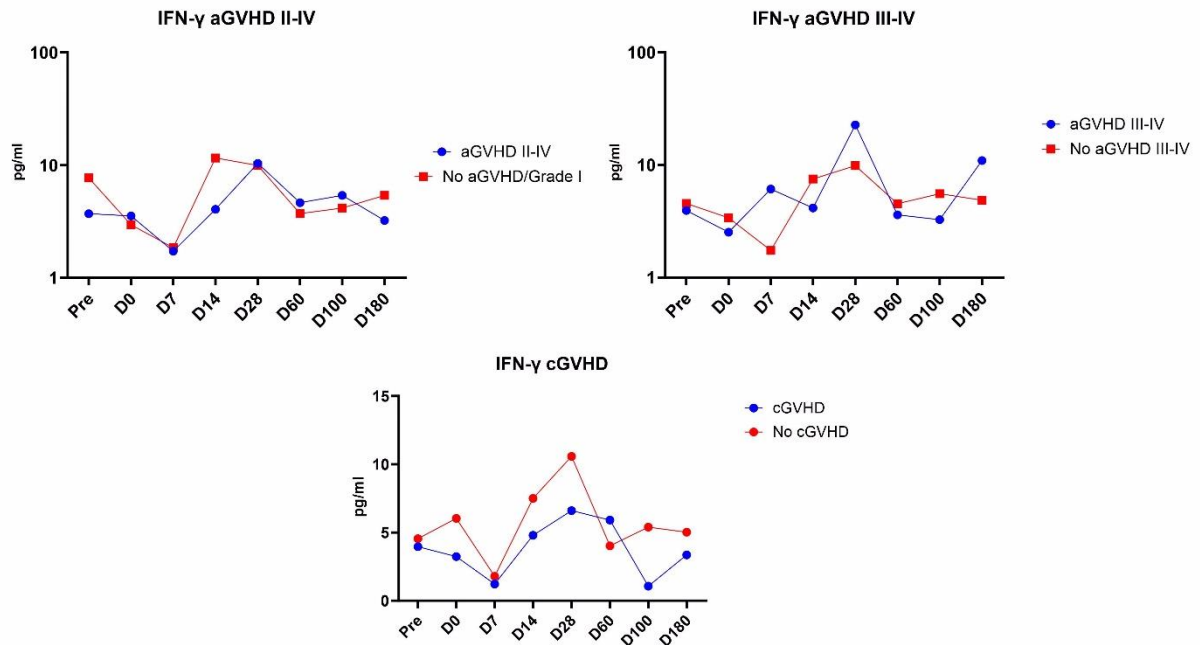


Figure 240: Median IFN γ secretion at serial time-points; grade II-IV aGVHD, grade III-IV aGVHD and cGVHD.

IL-1 β levels did not vary significantly by disease subtype, recipient age or sex (Fig. 241). Levels similarly did not significantly differ by donor source, graft preservation, ABO mismatch status or calcineurin choice (Fig. 242). Median IL-1 β levels diverged at the day 180 time-point between survivors and those who died, although no significant difference in medians was seen (Fig. 243). Increased levels were seen at the pre-transplant time-point in those who subsequently relapsed ($p=0.03$). No difference in secretion was seen between those who developed grade II-IV acute GVHD compared to those who developed grade I disease or no disease, however the pre-transplant IL-1 β level was associated with subsequent grade III-IV acute GVHD onset ($p=0.04$). Day 100 secretion was higher in those who developed chronic GVHD ($p=0.05$) (Fig. 244).

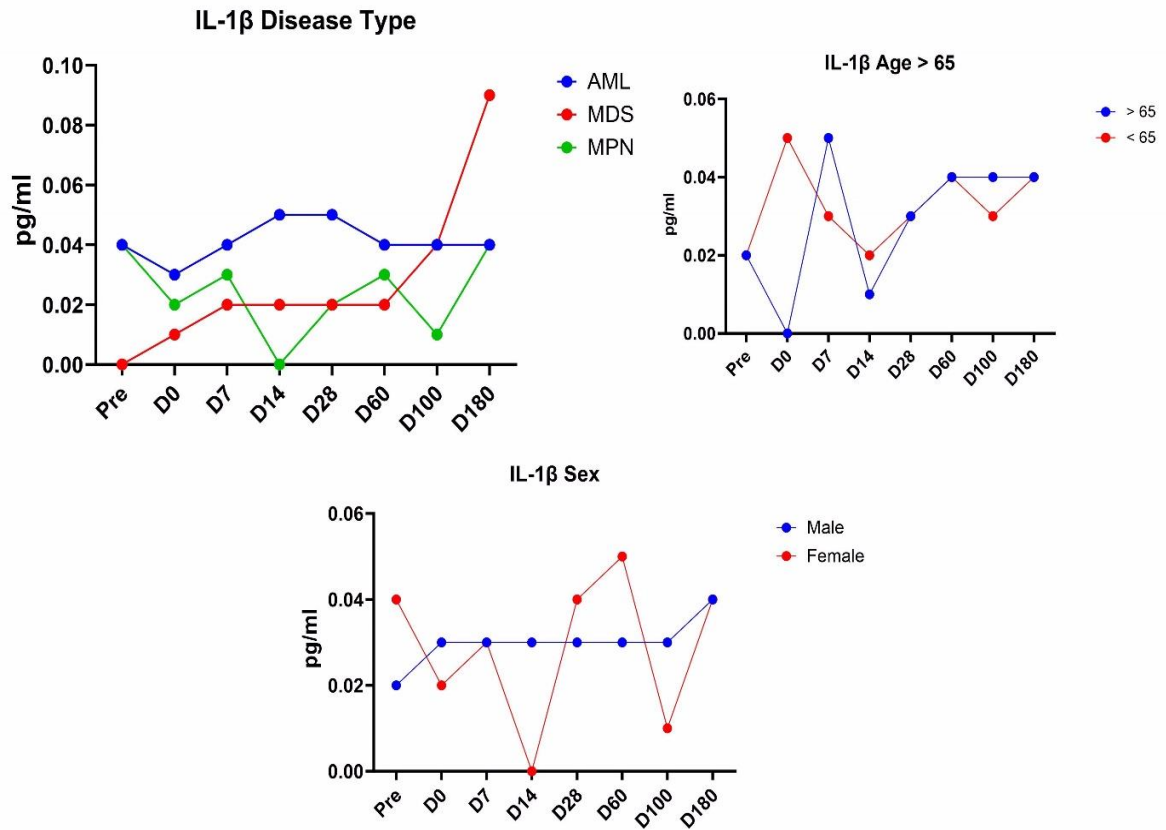


Figure 241: Median IL-1 β secretion at serial time-points; disease subtype, age and recipient sex.

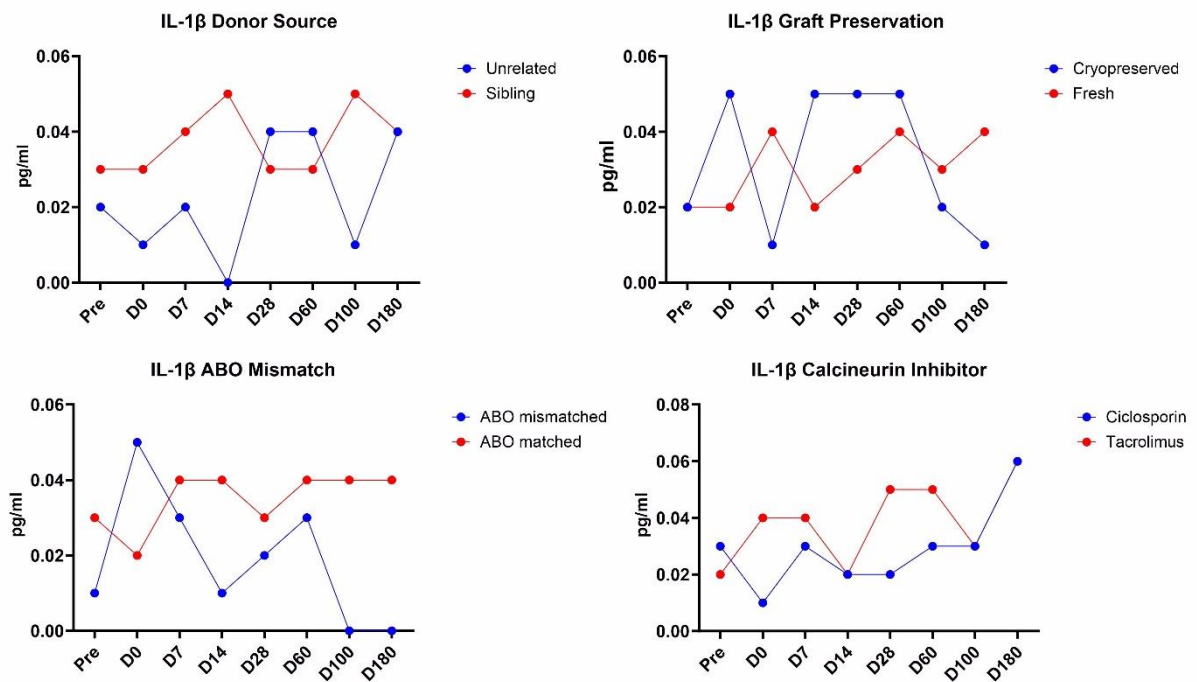


Figure 242: Median IL-1 β secretion at serial time-points; donor source, graft preservation, ABO mismatch status and calcineurin inhibitor choice.

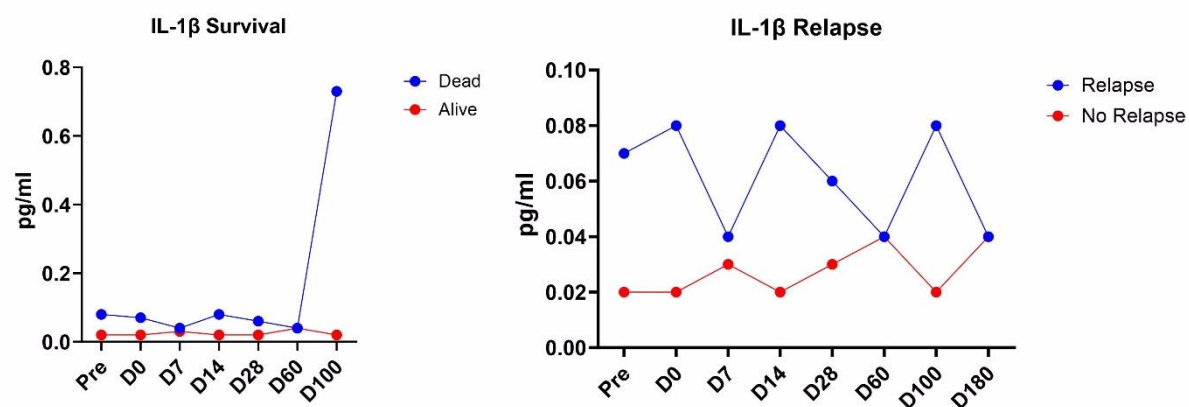


Figure 243: Median IL-1 β secretion at serial time-points; overall survival and relapse.

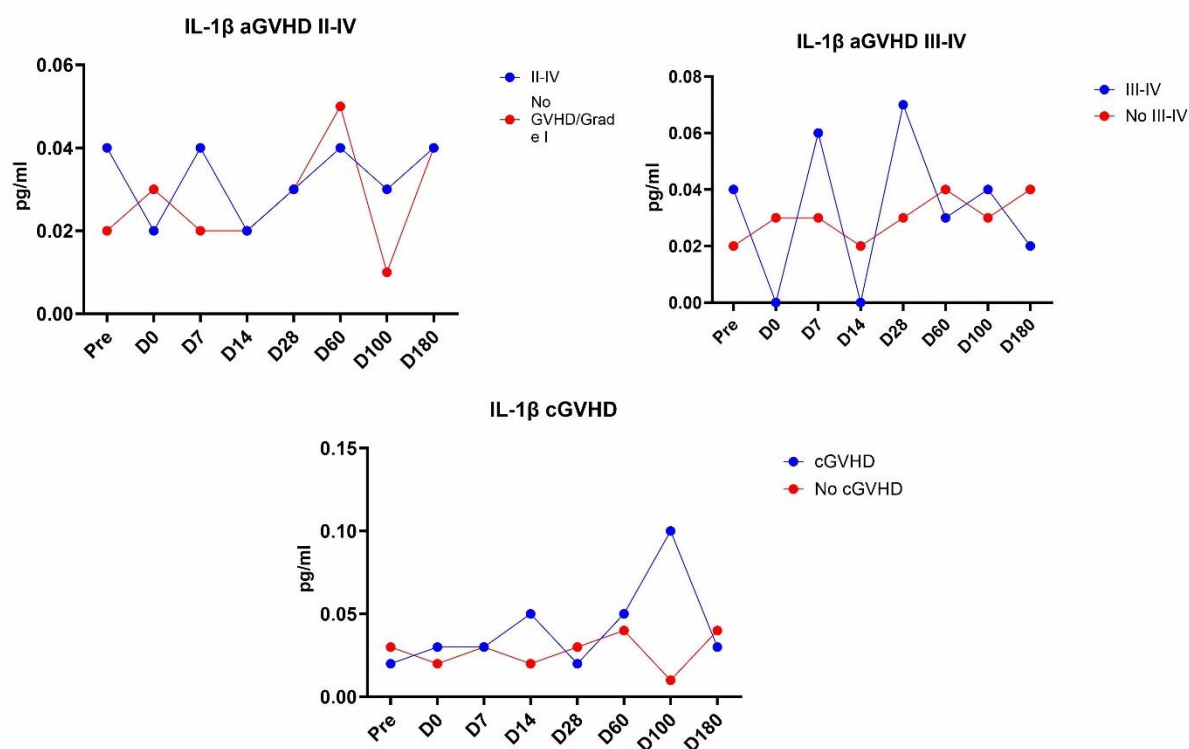


Figure 244: Median IL-1 β levels at serial time-points; grade II-IV acute GVHD, grade III-IV and cGVHD.

IL-2 secretion was similar across disease subtypes, recipient age and sex (Fig. 245). No significant difference was seen based on graft source, graft preservation, ABO mismatch status or calcineurin choice (Fig. 246). IL-2 levels were consistently higher in those who died and reached significance as compared to survivors at the day 28 and day 60 time-points ($p=0.03$ and $p=0.02$ respectively) (Fig. 247). Secretion was similar in those who relapsed as compared to those that did not. Day 28 IL-2 levels were lower in those who developed grade II-IV acute GVHD ($p=0.05$) (Fig. 248).

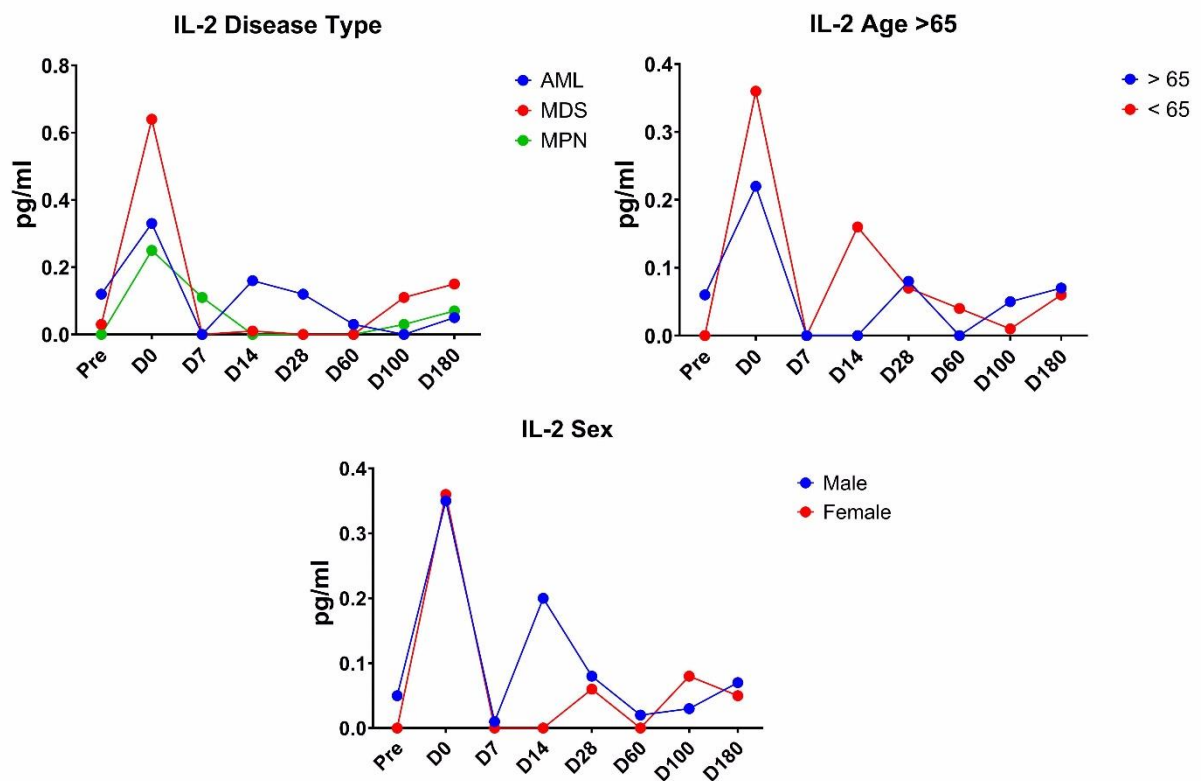


Figure 245: Median IL-2 secretion at serial time-points; disease subtype, age and recipient sex.

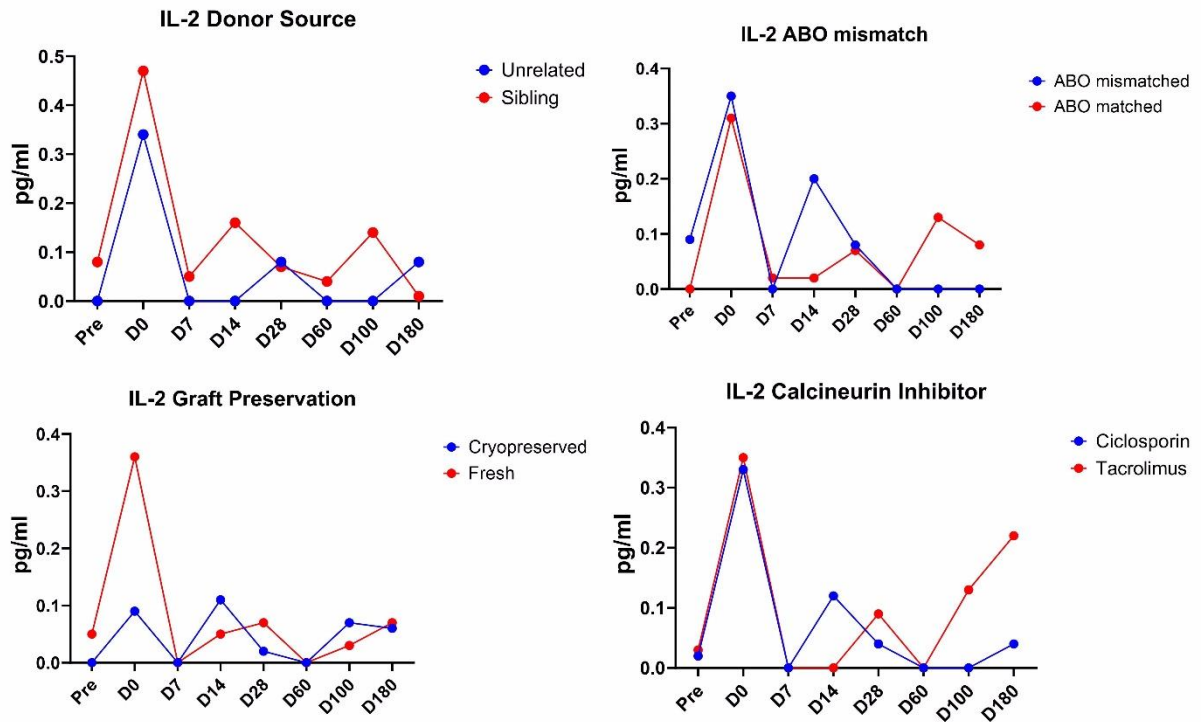


Figure 246: Median IL-2 secretion at serial time-points; donor source, graft preservation, ABO mismatch status and calcineurin inhibitor choice.

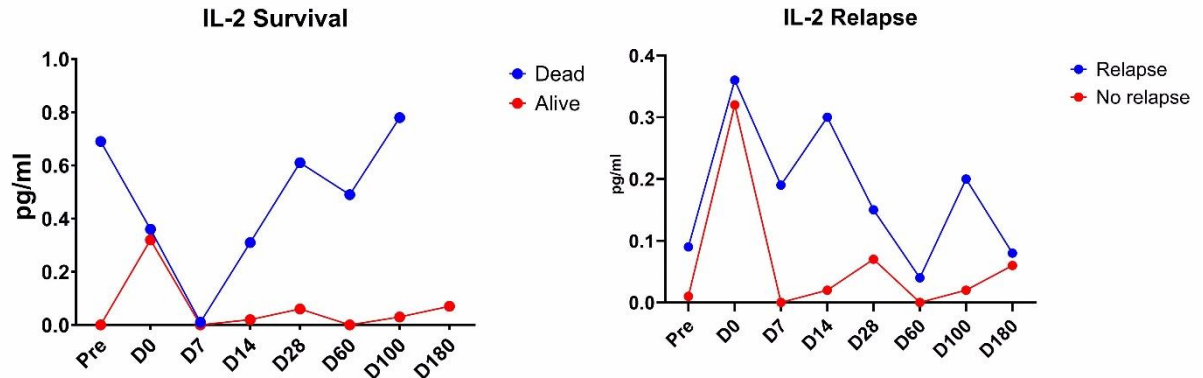


Figure 247: Median IL-2 secretion at serial time-points; overall survival and relapse.

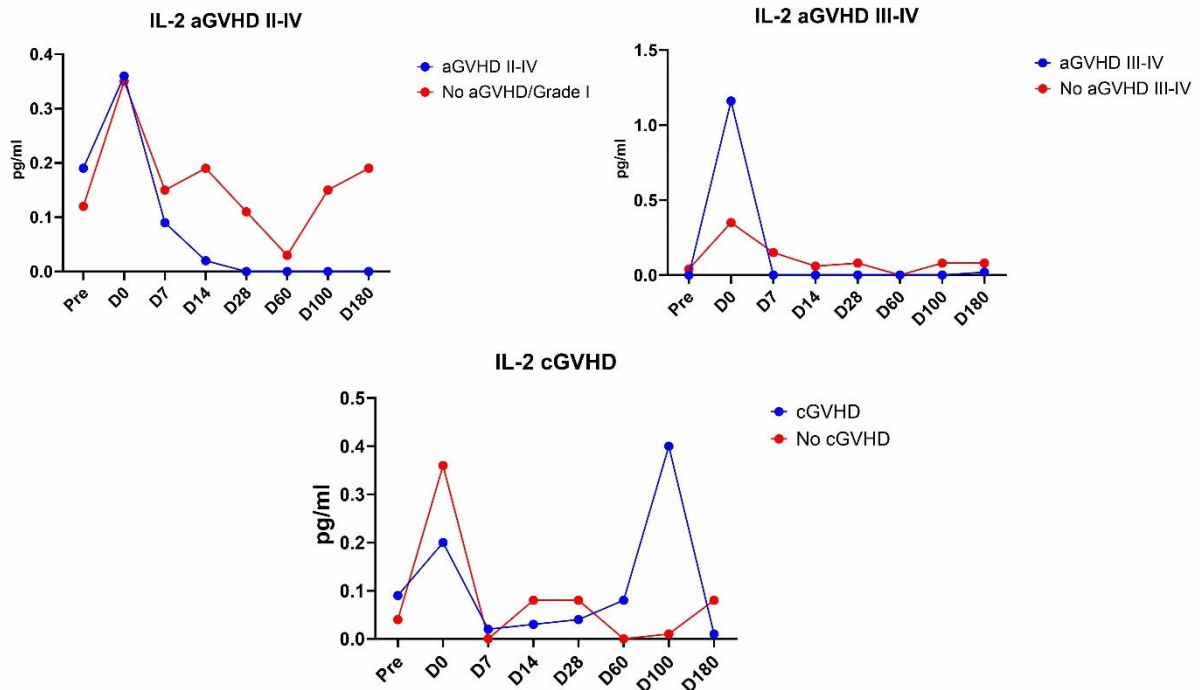


Figure 248: Median IL-2 levels at serial time-points; grade II-IV acute GVHD, grade III-IV and cGVHD.

IL-4 levels did not differ significantly by disease subtype, age profile or recipient sex (Fig. 249). Donor source, graft cryopreservation and ABO mismatch similarly did not show a difference, however day 28 IL-4 was higher in those received tacrolimus as acute GVHD prophylaxis ($p=0.006$) (Fig. 250). IL-4 levels based on survival were most divergent at the day 100 time-point but no significant difference in medians was seen (Fig. 251). IL-4 secretion was similar in those who relapsed and those who did not. No difference in grade II-IV acute GVHD, grade III-IV acute GVHD or chronic GVHD was seen (Fig. 252).

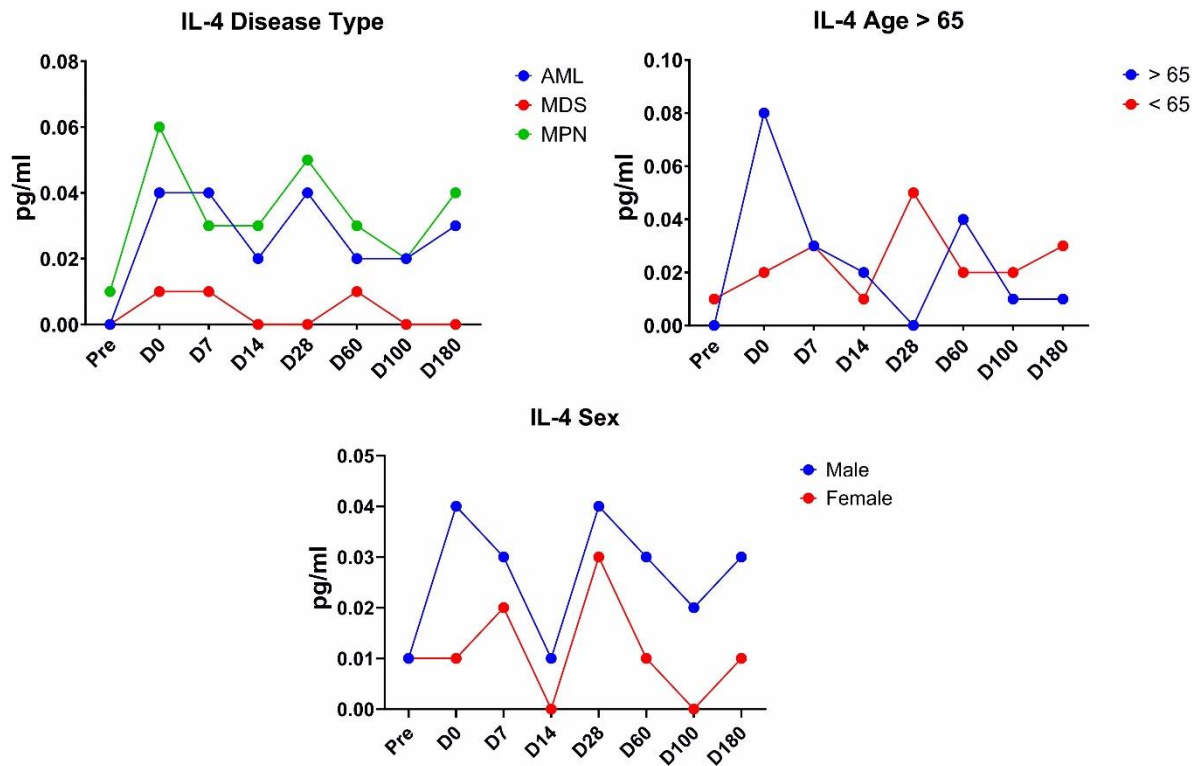


Figure 249: Median IL-4 levels at serial time-points; disease subtype, recipient age and sex.

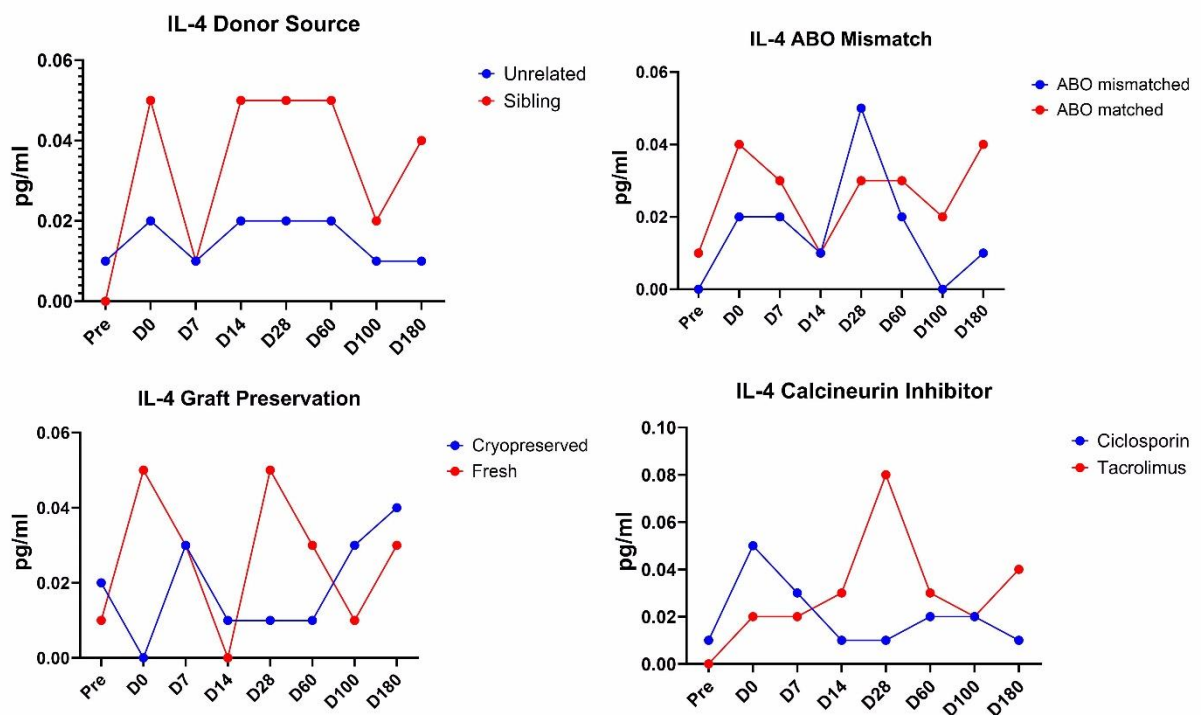


Figure 250: Median IL-4 levels at serial time-points; donor source, graft preservation, ABO mismatch status and calcineurin inhibitor choice.

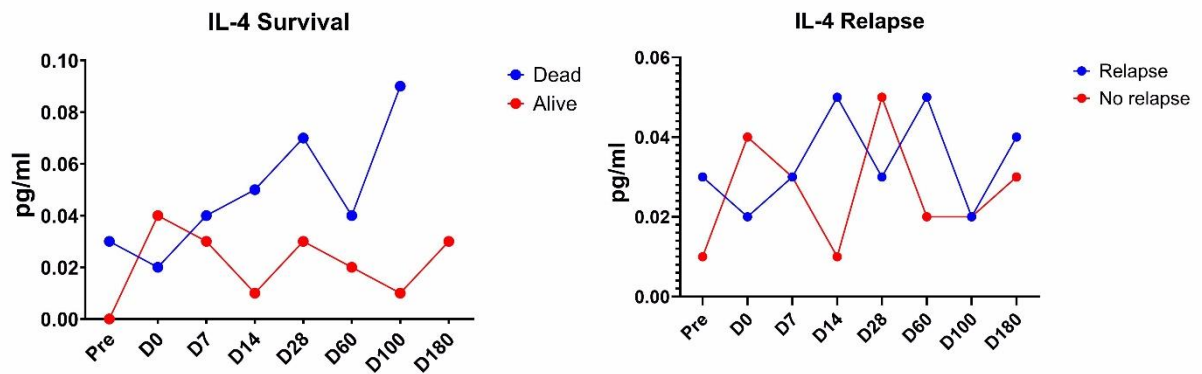


Figure 251: Median IL-4 levels at serial time-points; overall survival and relapse.

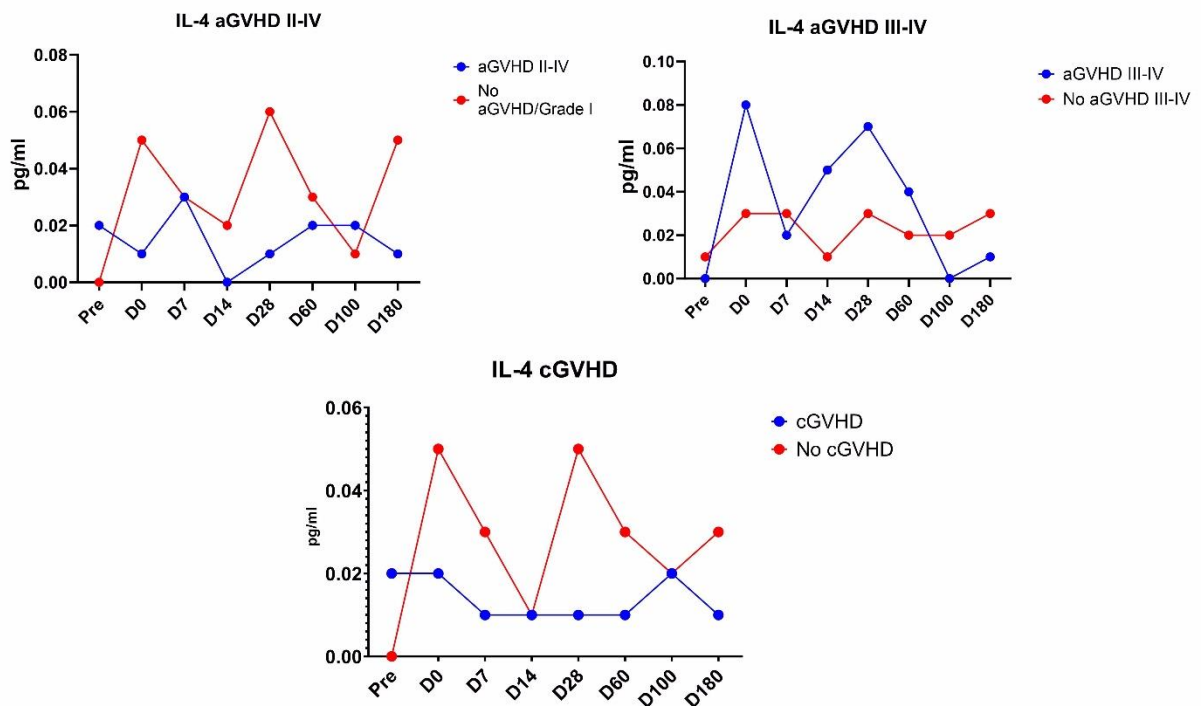


Figure 252: Median IL-4 levels at serial time-points; grade II-IV acute GVHD, grade III-IV, cGVHD.

IL-6 secretion was similar across disease subtypes, age groups and recipient sex (Fig. 253). No significantly different levels were seen based on donor source, graft preservation, ABO mismatch status or calcineurin choice (Fig. 254). IL-6 levels varied most significantly at the pre-transplant time-point, with a significant difference between those who died and those who did not ($p=0.0002$) (Fig. 255). Levels were not significantly different in those who relapsed as compared to those who did not. Levels in those with grade II-IV acute GVHD were similar to

those who developed grade I disease or no disease, but in those who developed grade III-IV disease there was an association with higher levels at day 60 and 100 ($p=0.04$ and $p=0.01$) (Fig. 256). No difference was seen in those who developed chronic GVHD.

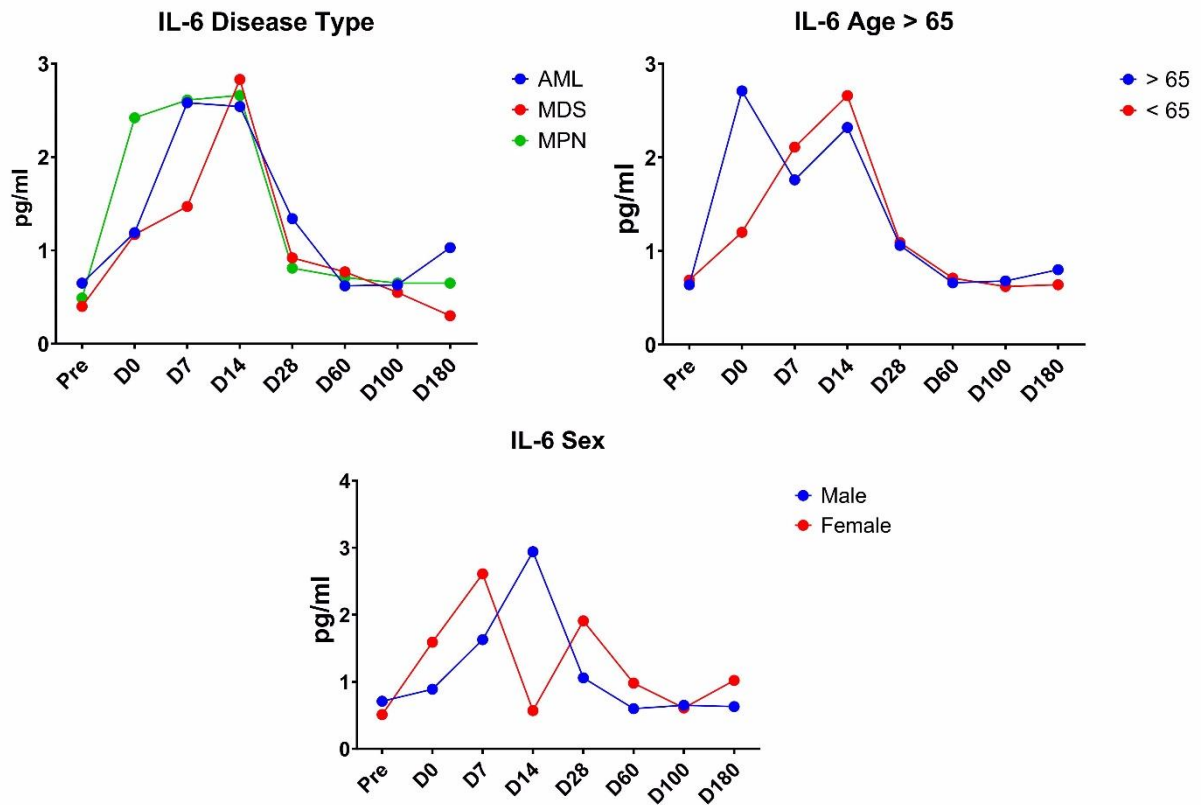


Figure 253: Median IL-6 levels at serial time-points; disease subtype, recipient age and sex.

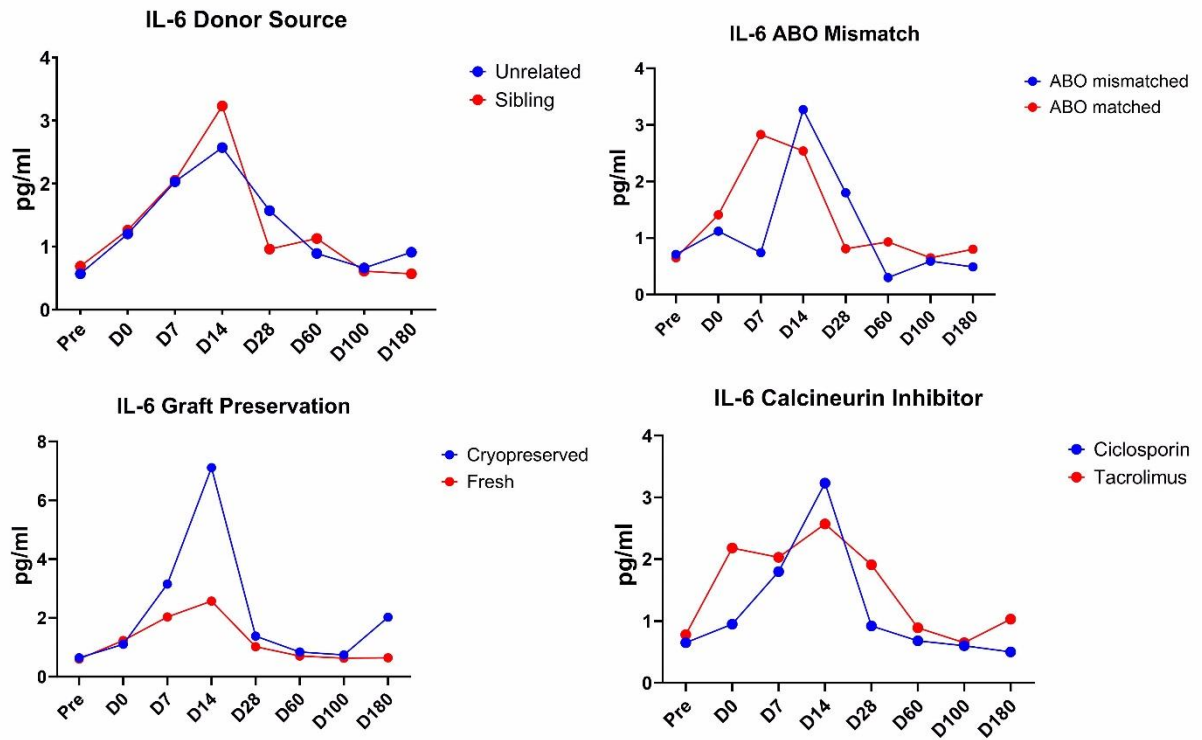


Figure 254: Median IL-6 levels at serial time-points; donor source, graft cryopreservation, ABO mismatch status and calcineurin inhibitor choice.

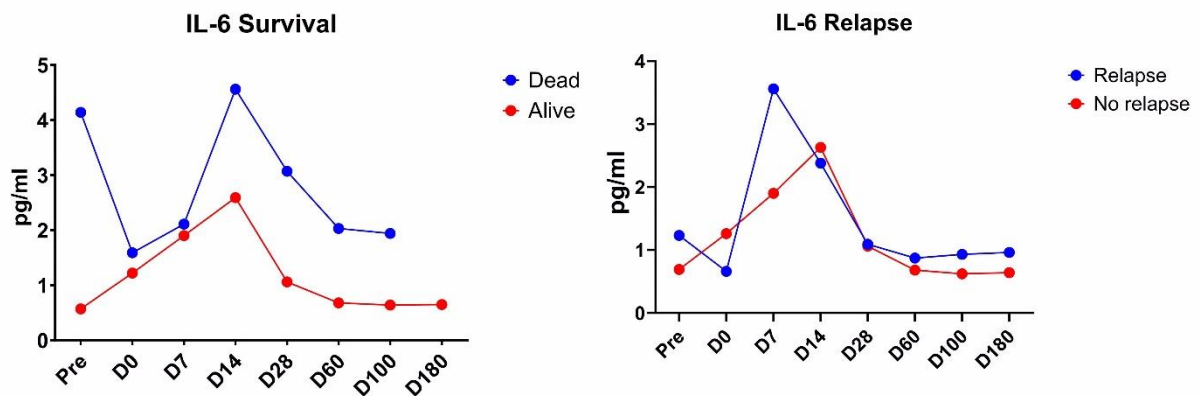


Figure 255: Median IL-6 levels at serial time-points; disease subtype, recipient age and sex.

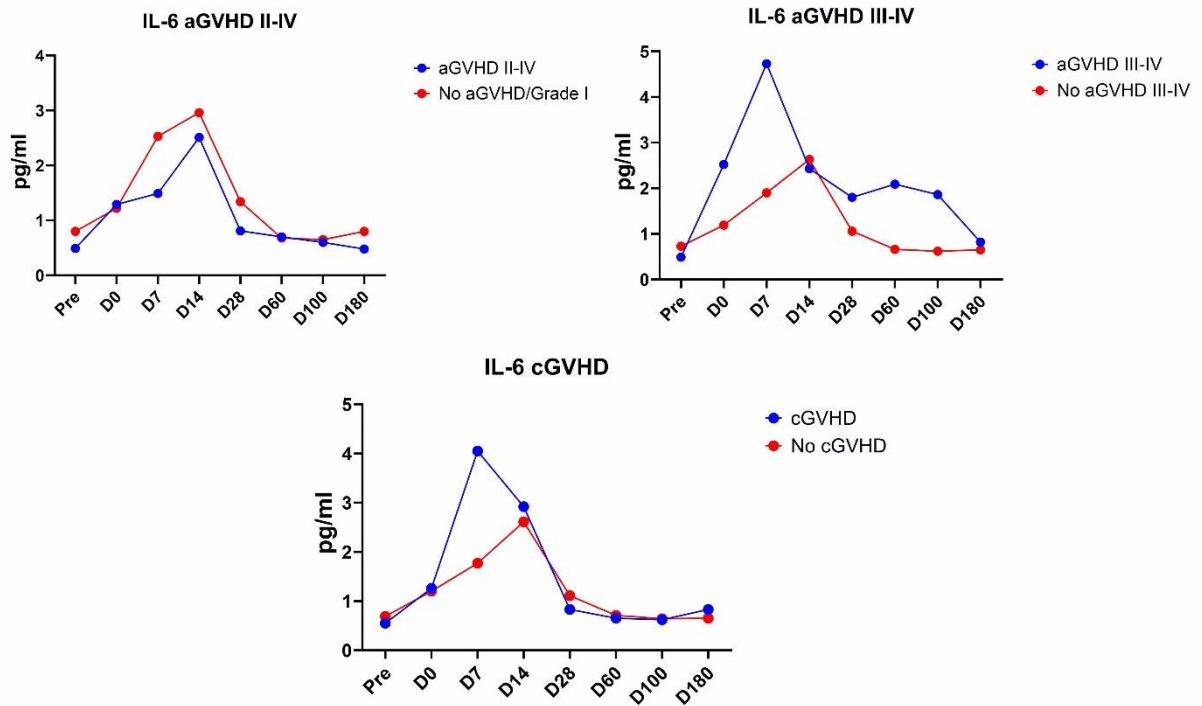


Figure 256: Median IL-6 levels at serial time-points; grade II-IV acute GVHD, grade III-IV, cGVHD.

IL-10 levels were similar in disease subtypes, recipient age group and sex (Fig. 257). Levels were also unaffected by donor source, graft preservation, ABO mismatch or calcineurin inhibitor choice (Fig. 258). IL-10 levels were higher in those who died except for the day 7 time-point, but no significant difference was seen between the two groups (Fig. 259). No difference was seen between those who relapsed and those that did not. No difference was seen between those who developed grade II-IV acute GVHD compared to those who developed grade I or no GVHD, however at the day 28 and day 60 time-points, higher IL-10 secretion was seen in those who developed grade III-IV disease ($p=0.01$ and $p=0.007$) (Fig. 260). No difference was seen in the chronic GVHD group.

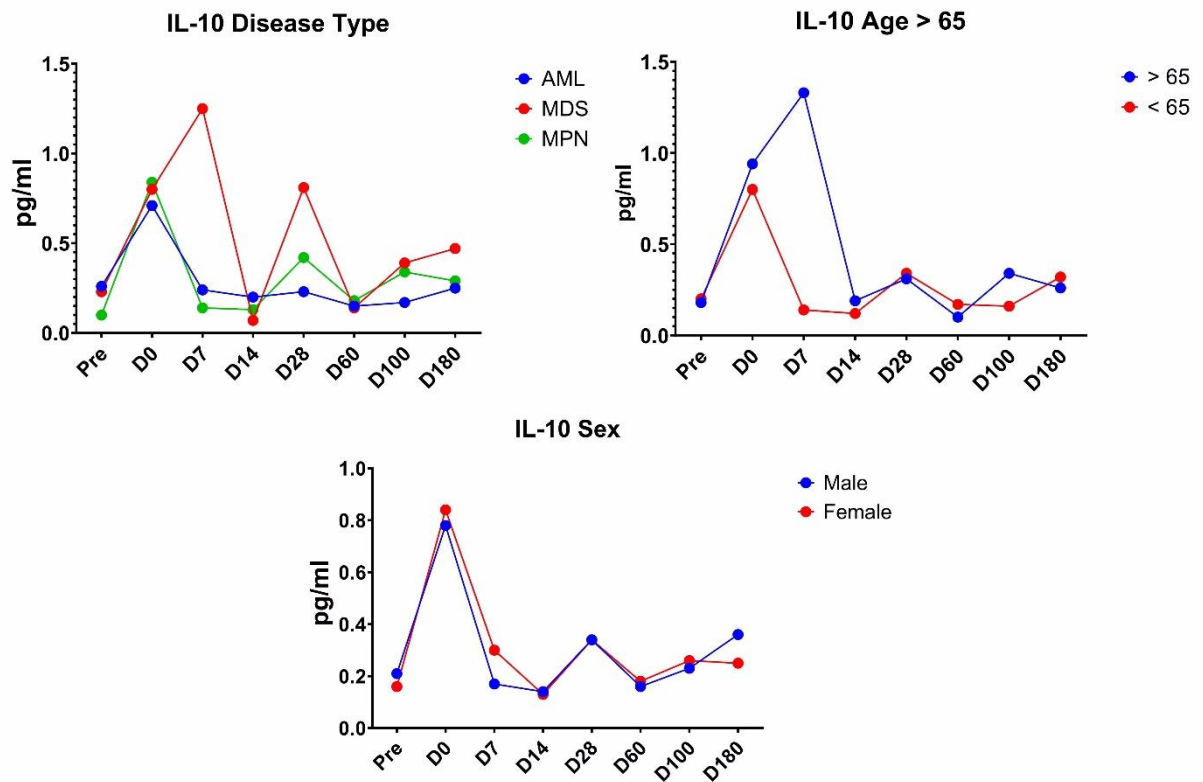


Figure 257: Median IL-10 levels at serial time-points; disease subtype, age group and sex.

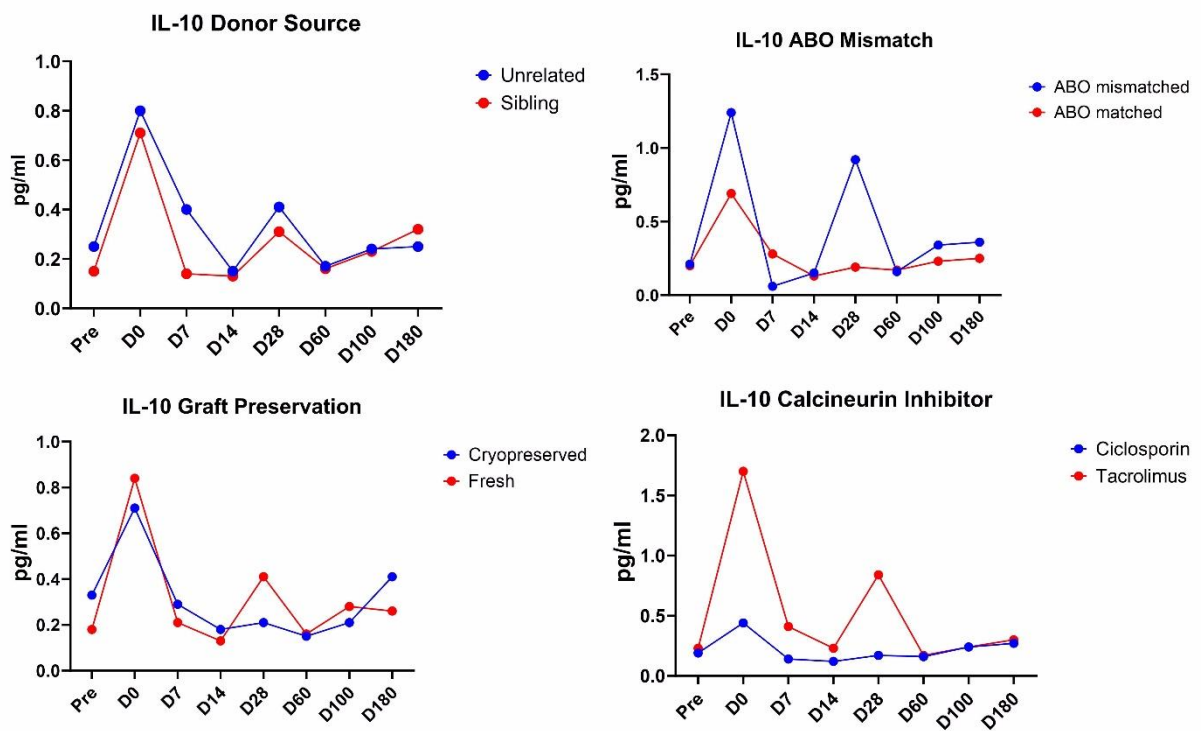


Figure 258: Median IL-10 levels at serial time-points; donor source, graft preservation, ABO mismatch status and calcineurin inhibitor choice.

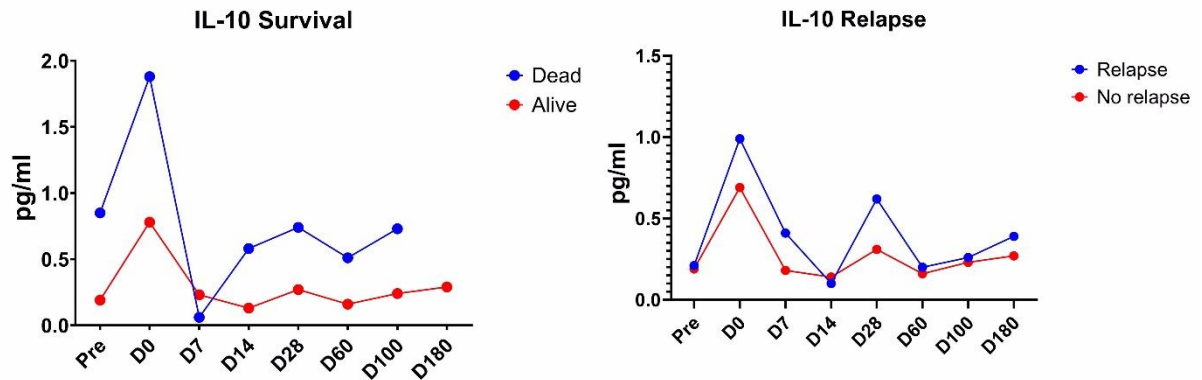


Figure 259: Median IL-10 levels at serial time-points; overall survival and relapse.

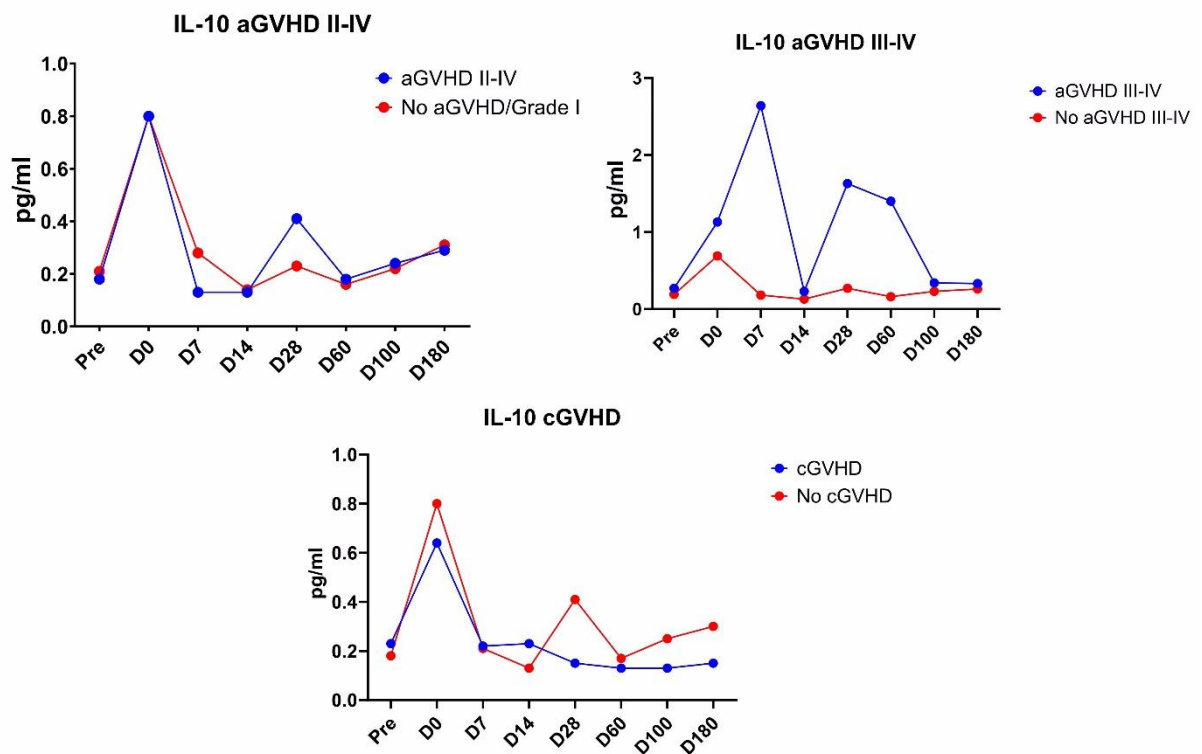


Figure 260: Median IL-10 levels at serial time-points; grade II-IV acute GVHD, grade III-IV, cGVHD.

IL-12p70 levels were low at most time-points in the MDS cohort but no significant difference in secretion between disease, age group or sex was seen (Fig. 261). There was no significant difference in secretion based on donor source, graft preservation, ABO mismatch or calcineurin inhibitor choice (Fig. 262). IL-12p70 levels were higher in those who died, with the widest gap at day 0, 7 and 100. The difference at day 100 was statistically significant ($p=0.04$) (Fig. 263). Higher levels at day 100 were seen in those who relapsed ($p=0.03$). Levels were similar across grade II-IV acute GVHD, grade III-IV acute GVHD and chronic GVHD (Fig. 264).

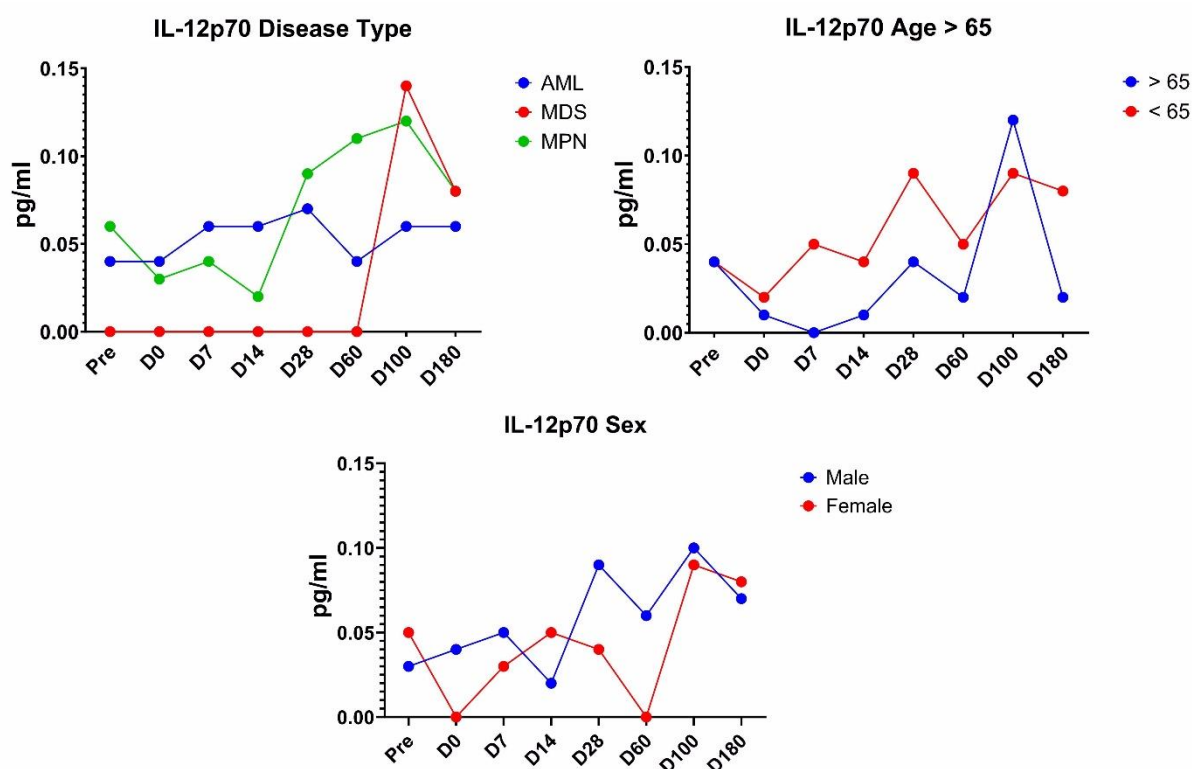


Figure 261: Median IL-12p70 levels at serial time-points; disease subtype, age group and sex.

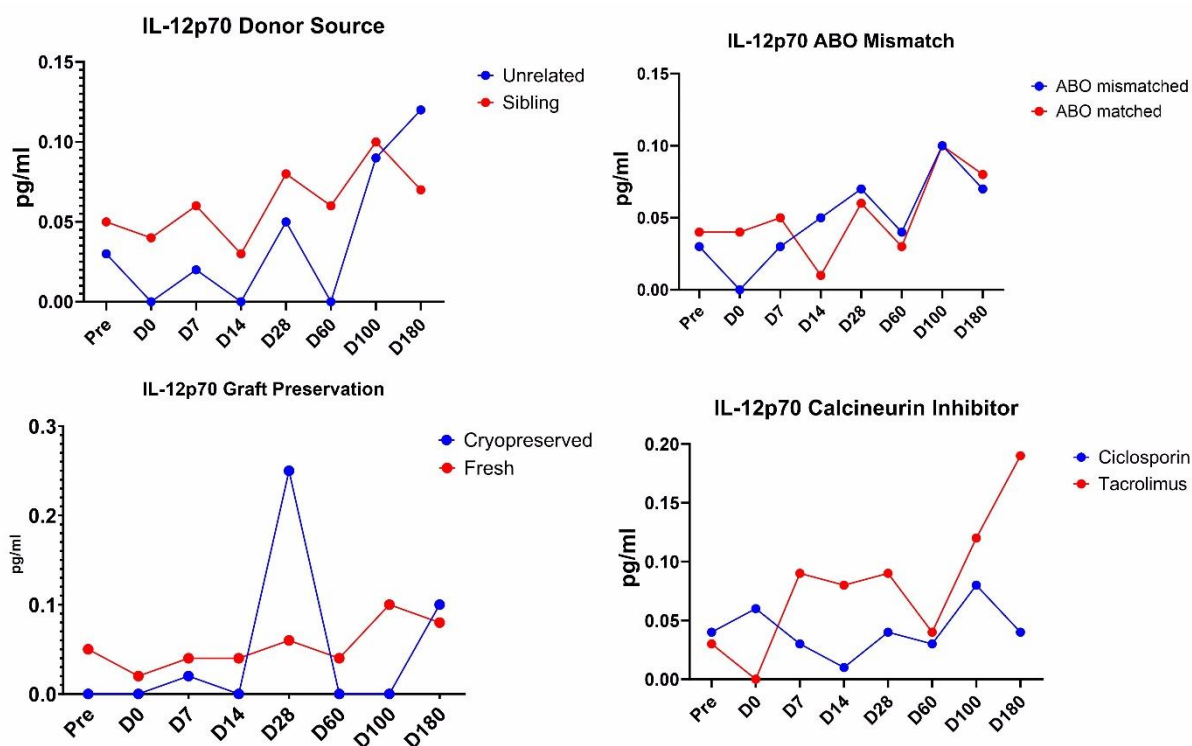


Figure 262: Median IL-12p70 levels at serial time-points; donor source, graft preservation, ABO mismatch status and calcineurin inhibitor choice.

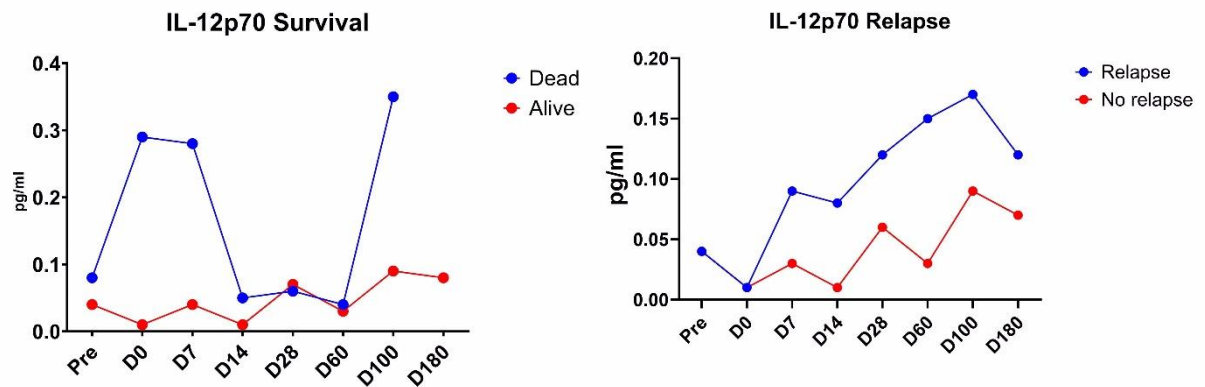


Figure 263: Median IL-12p70 levels at serial time-points; overall survival and relapse.

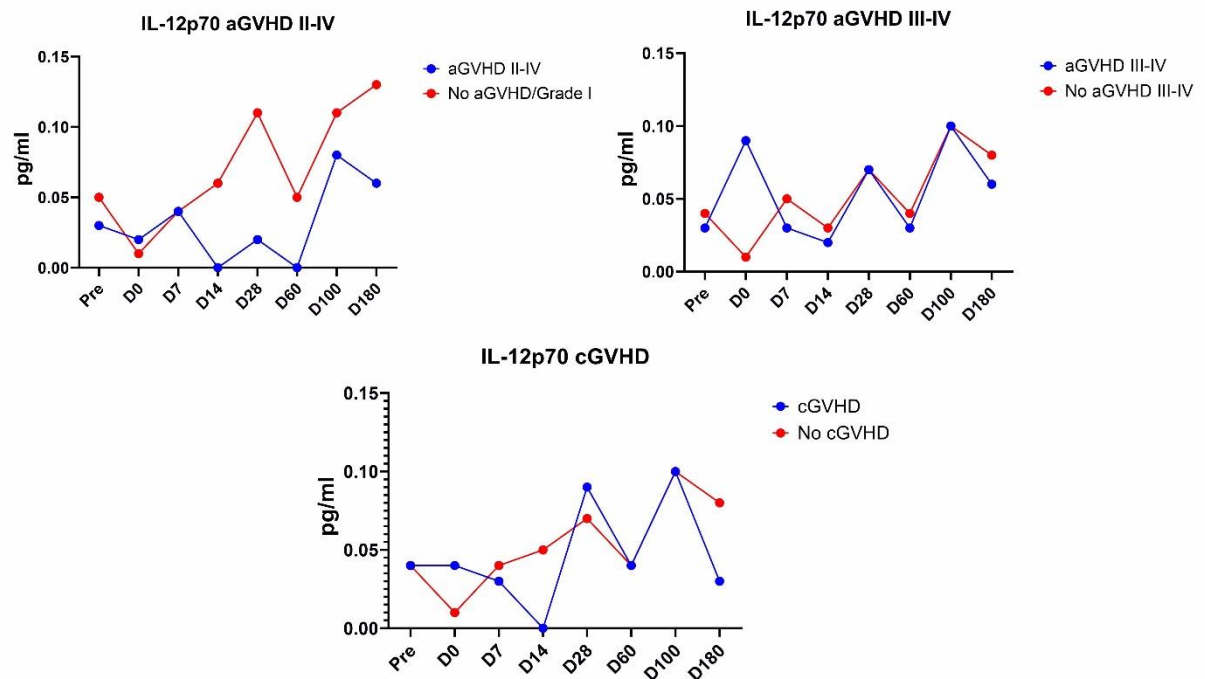


Figure 264: Median IL-12p70 levels at serial time-points; grade II-IV acute GVHD, grade III-IV, cGVHD.

IL-17A levels were similar by disease subtype, age group, sex, donor source, graft preservation, ABO mismatch status and calcineurin inhibitor choice (Fig. 265 and 266). Levels followed a similar pattern based on mortality, but a significant difference was detected at the day 7 time-point ($p=0.04$) (Fig. 267). Levels at day 100 were higher in those who relapsed ($p=0.03$). No difference was seen based on any grade acute GVHD or chronic GVHD (Fig. 268).

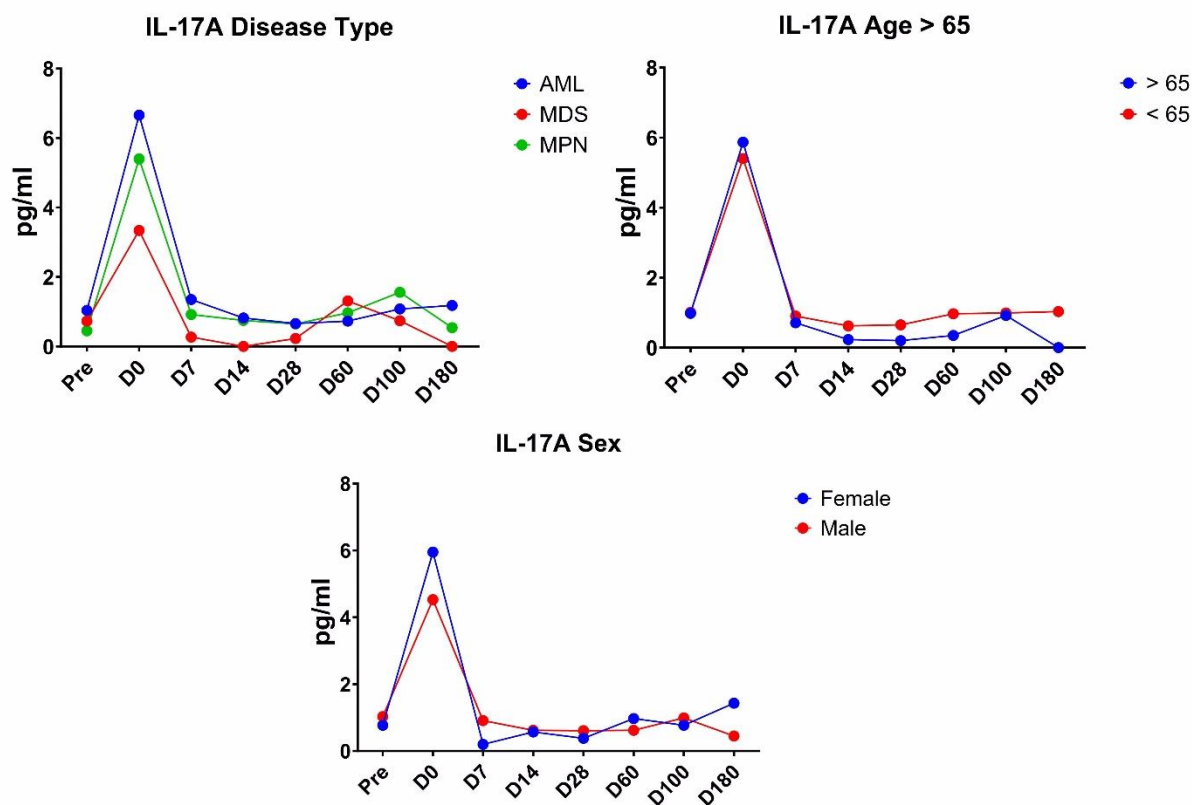


Figure 265: Median IL-17A levels at serial time-points; disease subtype, age group and sex.

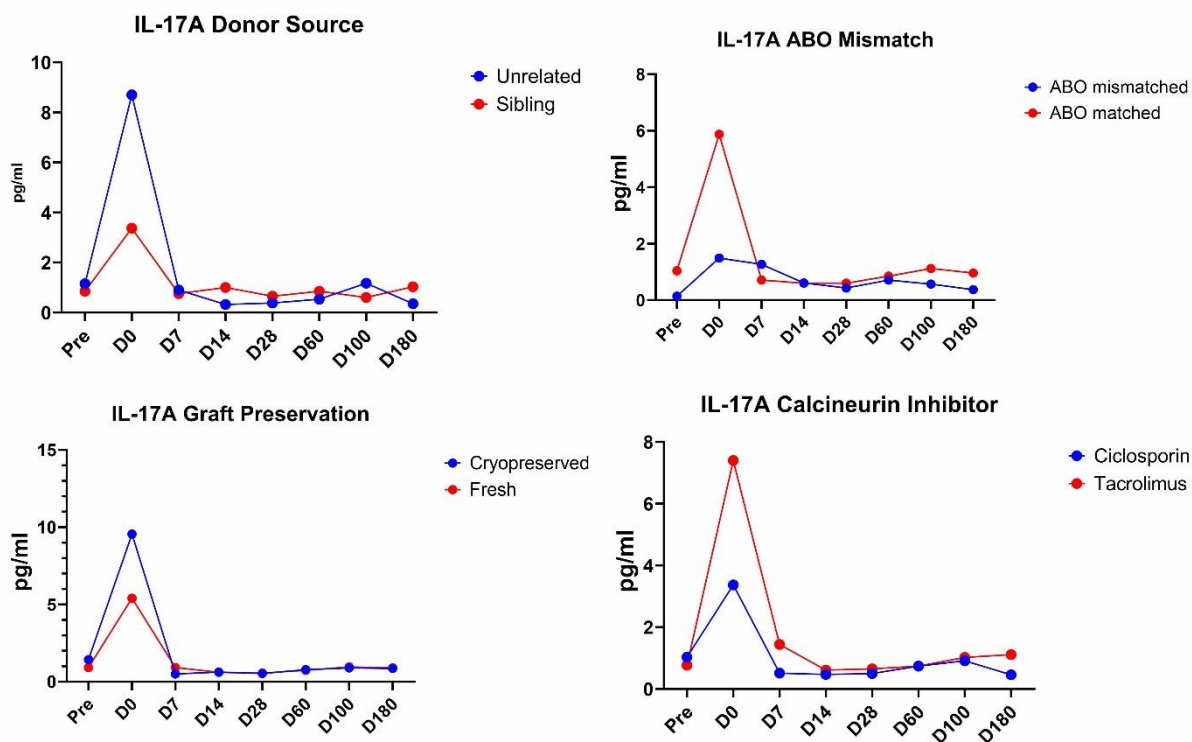


Figure 266: Median IL-17A levels at serial time-points; donor source, graft preservation, ABO mismatch and calcineurin inhibitor choice.

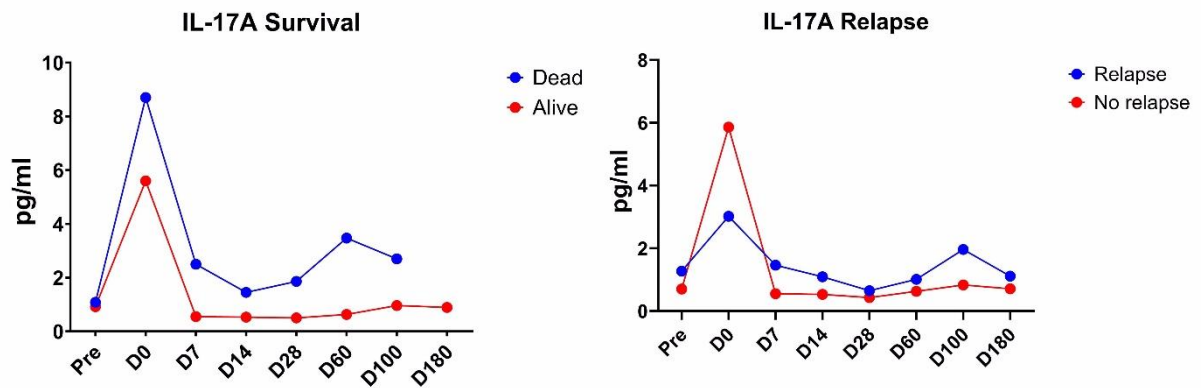


Figure 267: Median IL-17A levels at serial time-points; overall survival and relapse.

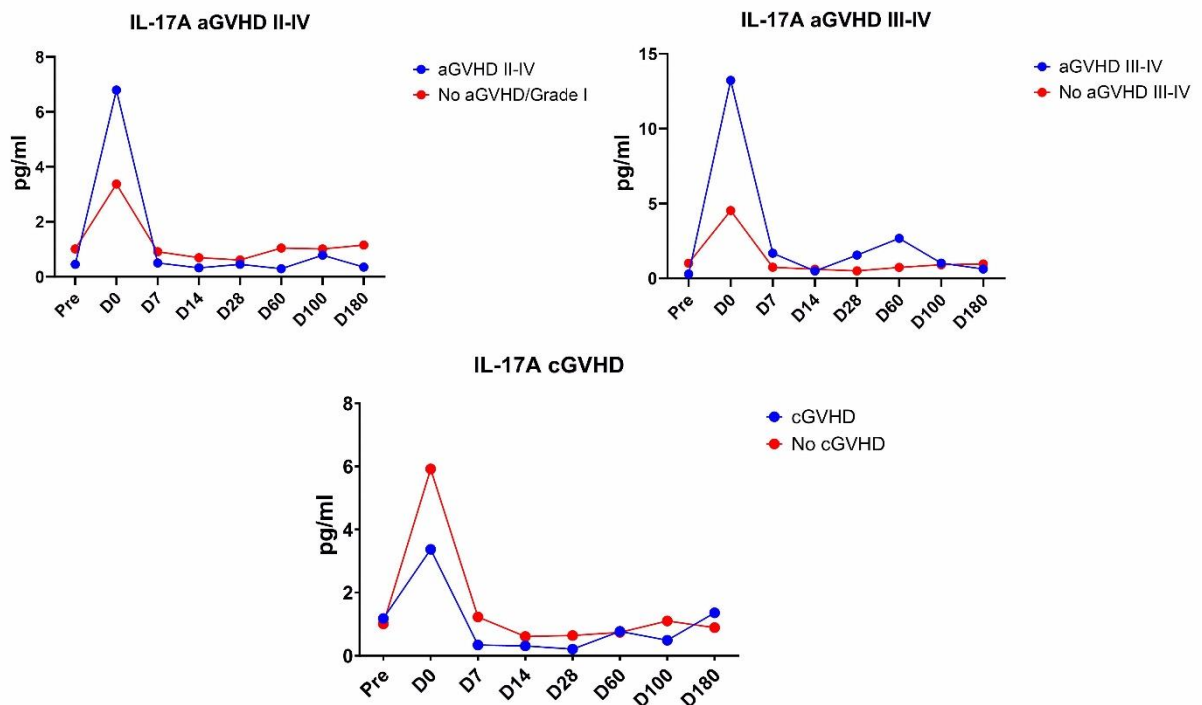


Figure 268: Median IL-17A levels at serial time-points; grade II-IV acute GVHD, grade III-IV, cGVHD.

TNF α levels were similar in each disease subtype, age group and recipient sex (Fig. 269). Additionally, levels did not vary by donor source, graft preservation, ABO mismatch status or calcineurin inhibitor choice (Fig. 270). Levels did not differ significantly between survivors or those who died, nor in relapse (Fig. 271). TNF α levels were similar across all grades of acute

GVHD but at the day 14 time-point higher levels resulted in a lower risk of subsequent chronic GVHD ($p=0.05$) (Fig. 272).

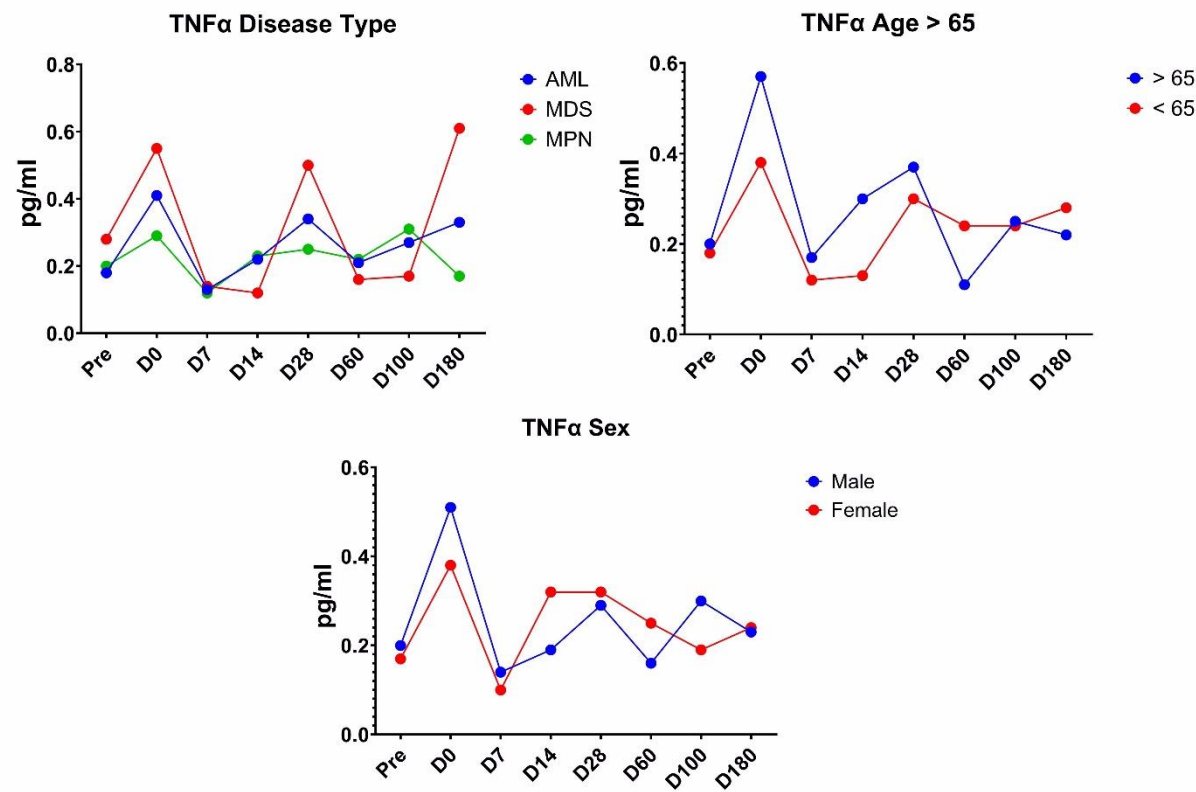


Figure 269: Median TNFα levels at serial time-points; disease subtype, age group and sex.

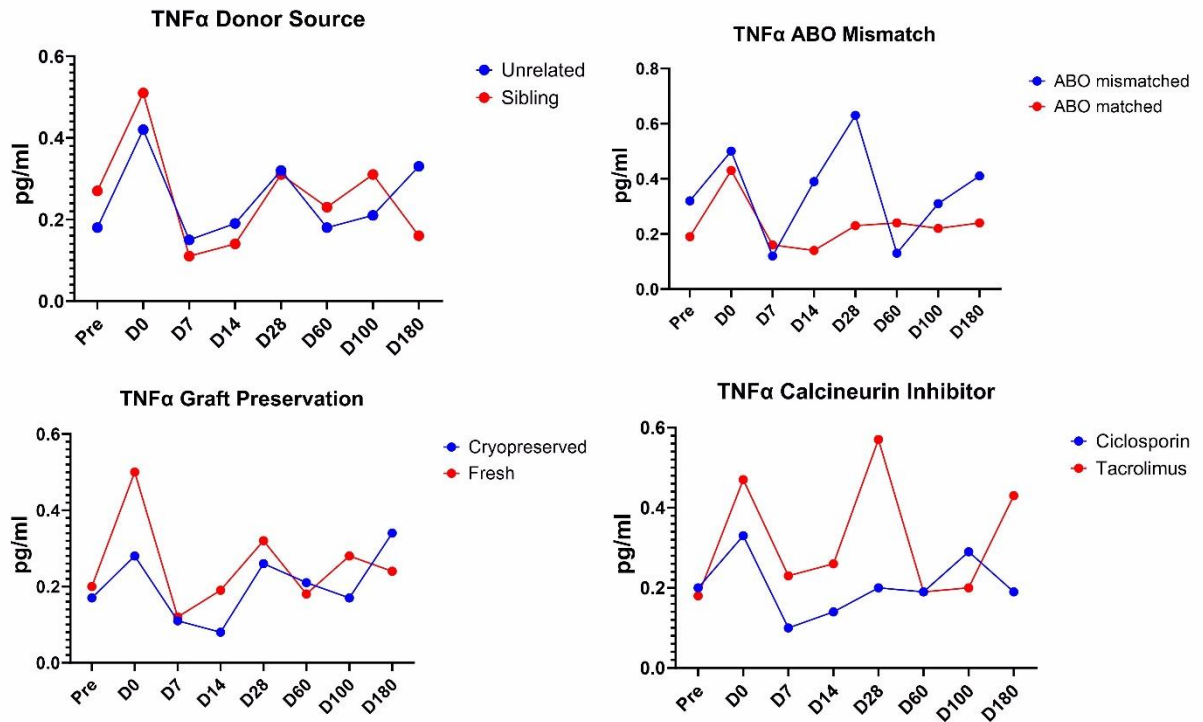


Figure 270: Median TNFα levels at serial time-points; donor source, graft preservation, ABO mismatch status and calcineurin inhibitor choice.

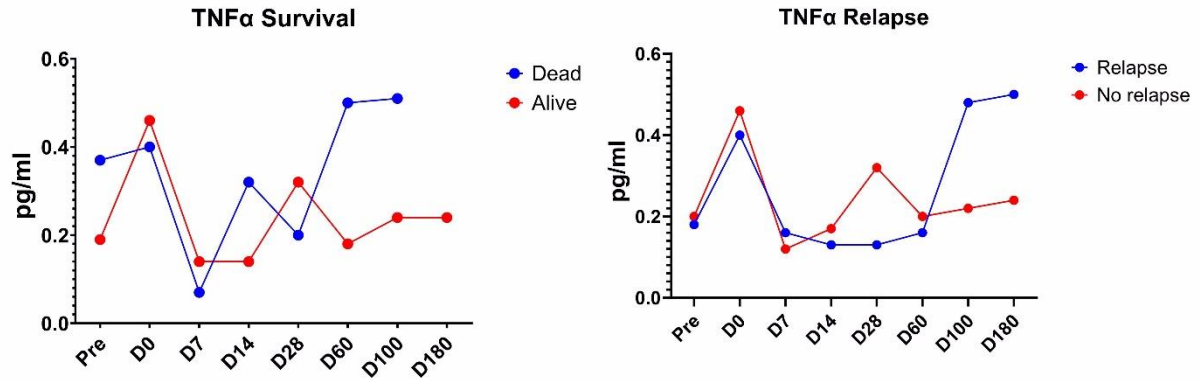


Figure 271: Median TNFα levels at serial time-points; overall survival and relapse.

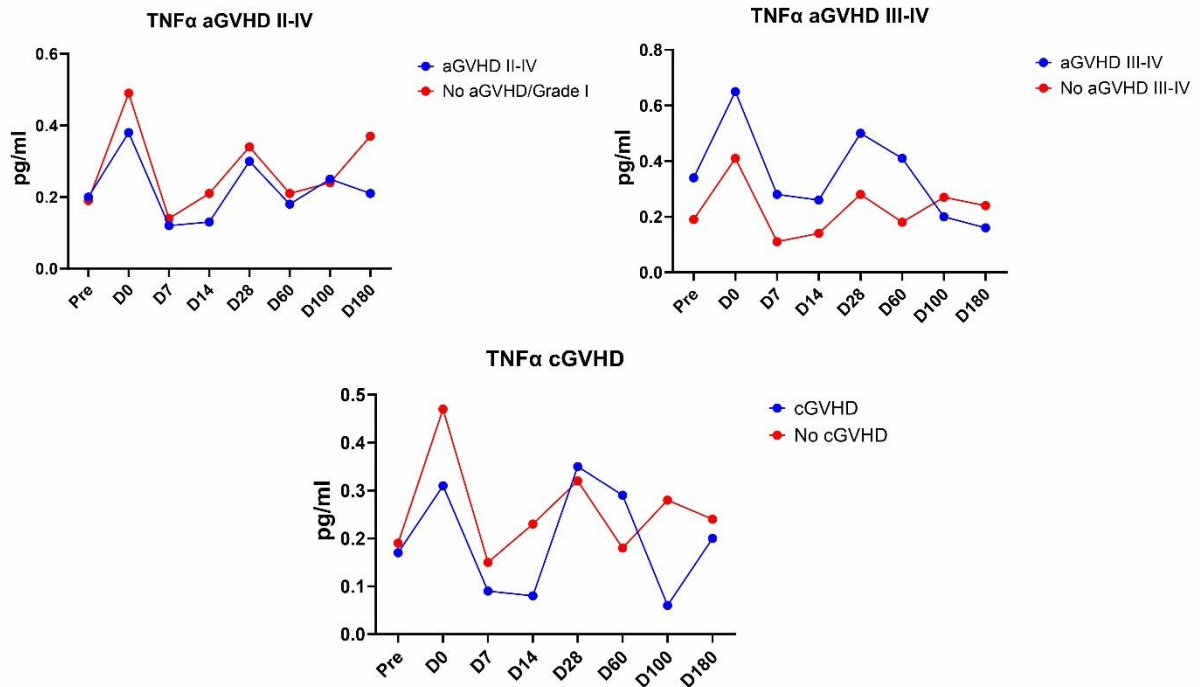


Figure 272: Median TNFα levels at serial time-points; grade II-IV acute GVHD, grade III-IV, cGVHD.

Correlation by time-point

There was no evidence of a strong correlation between cytokine expression and increasing recipient age at transplant. For most cytokines, the inverse was seen, with a negative association apparent with lower levels in older patient groups. The exception to this trend was with IFN γ , where at time-points where a correlation with age was seen, the trend indicated higher secretion in older patients. However, these associations were not statistically significant at any time-point (Fig. 273). At the pre-transplant time-point, increasing age-adjusted HCT-CI score showed a positive correlation with increased secretion of all cytokines, except for IFN γ , where a neutral correlation was seen (Fig. 274). Small variances in cytokine secretion were noted between those who received higher dose ATLG and those who received lower dose, and by extension, transplants performed with unrelated versus sibling donors, however there were no significant correlations (Fig. 275). The EASIX score at the pre-transplant and day 0 time-point did not correlate strongly with corresponding cytokine levels for those time-points (Fig. 276).

Graft T cell content, including CD3⁺, CD4⁺ and CD8⁺ cell levels showed a predominantly negative correlation with cytokine secretion at the 7 and day 14 time-points, with CD4⁺/CD8⁺ levels showing a mixed correlation pattern (Fig. 277 and 278). At day 28 the trend skewed toward a positive correlation, which then reversed again by day 60 (Fig. 279 and 280). *In vivo* T cell and lymphocyte levels at day 100 showed a predominantly neutral relationship, except for a negative correlation between absolute lymphocyte count and CD4 count with IL-6

secretion, and a positive correlation between CD3 and CD8 count with TNF α secretion (Fig. 281). At day 180 there was a negative correlation between IL-10 secretion and recipient absolute lymphocyte count, CD3, CD4 and CD8 count and a positive correlation with IFN γ , IL-1 β , IL-2 and TNF α secretion was seen (Fig. 282).

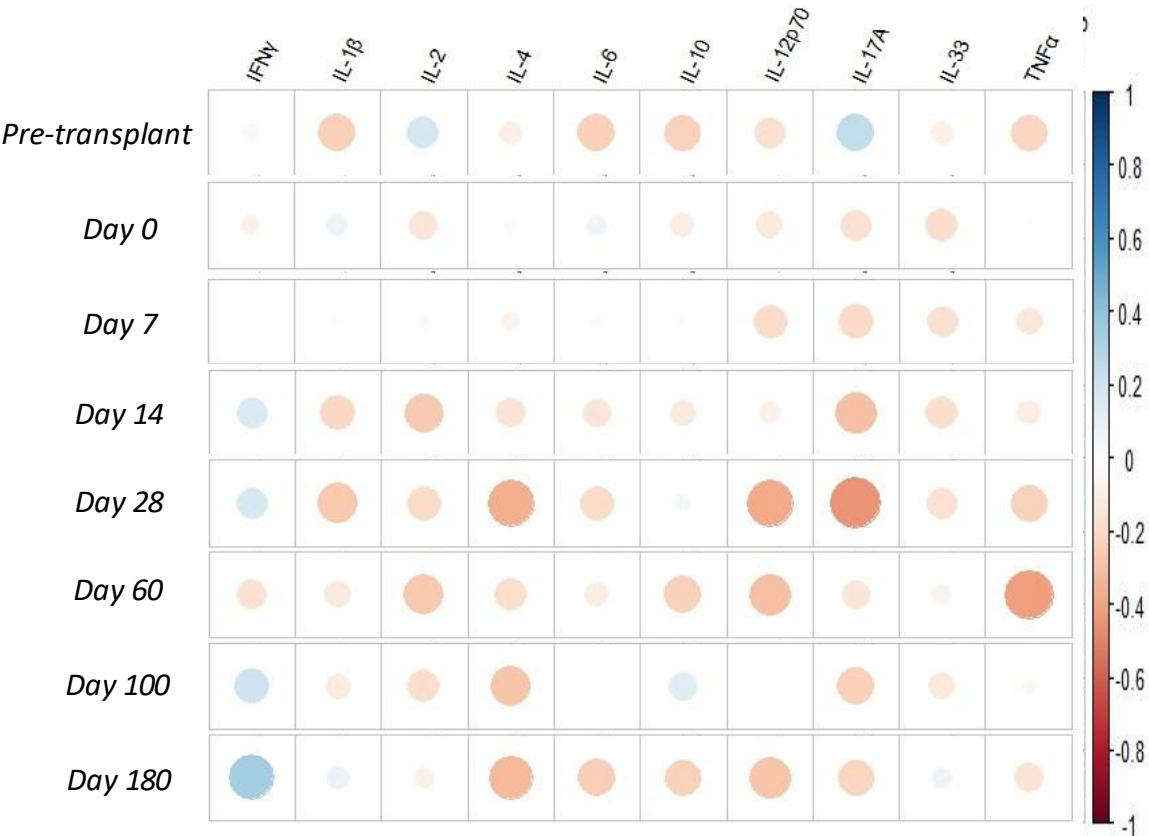


Figure 273: Correlation matrix of recipient age and serial cytokine measurements. Red hues indicate negative correlation and blue indicate positive correlation with increasing age.

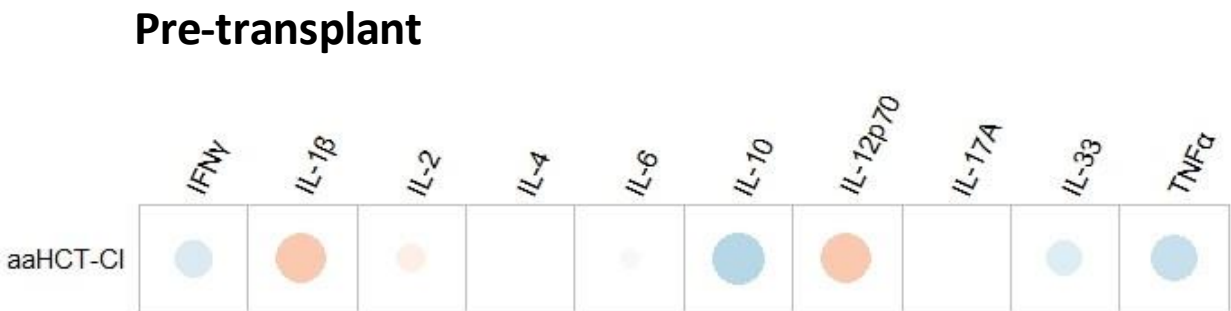


Figure 274: Correlation matrix of age-adjusted HCT-CI score and pre-transplant cytokine measurements. Red hues indicate negative correlation and blue indicate positive correlation with increasing HCT-CI score.

ATLG Dose

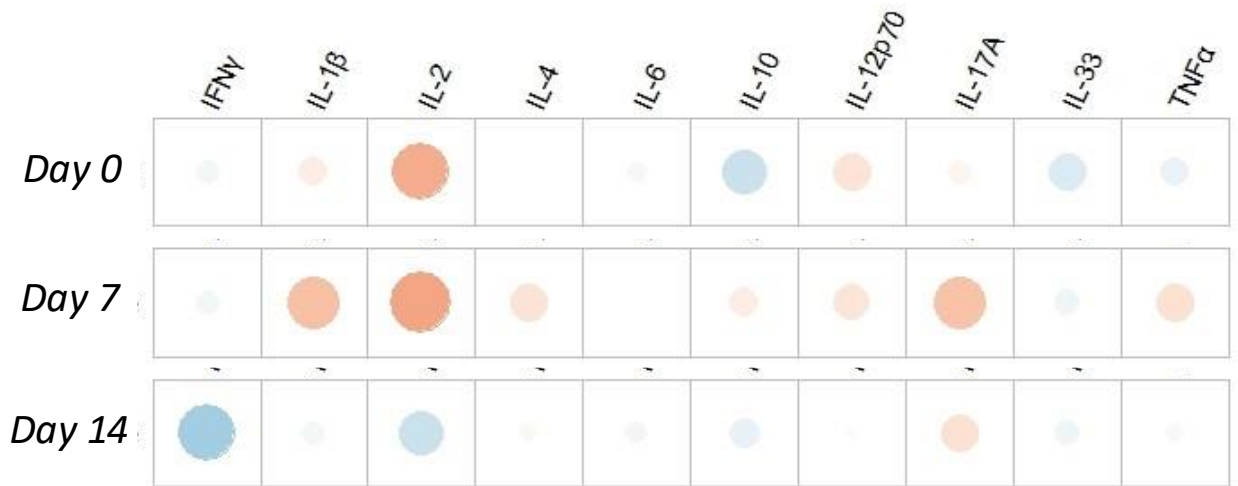


Figure 275: Correlation matrix of ATLG dose and cytokine measurements during drug half-life at day 7, 14 and 28. Red hues indicate negative correlation and blue indicate positive correlation with ATLG dose.

EASIX

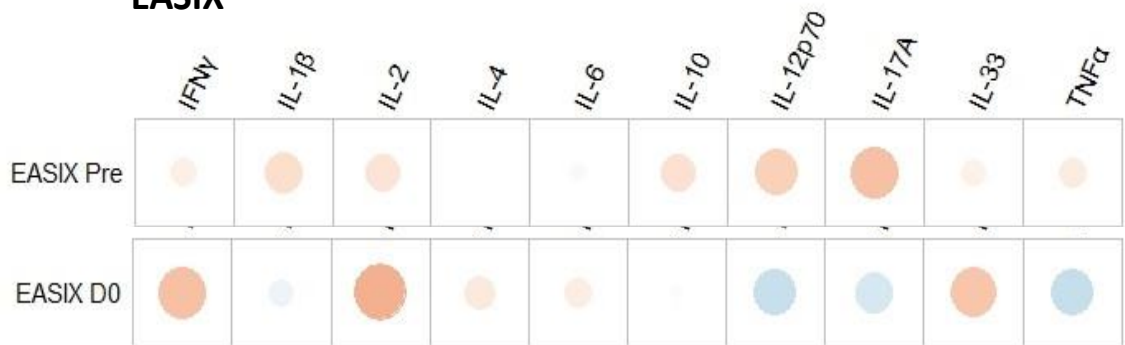


Figure 276: Correlation matrix of EASIX score and cytokine measurements at corresponding time-points of pre-transplant and day 0. Red hues indicate negative correlation and blue indicate positive correlation with EASIX score.

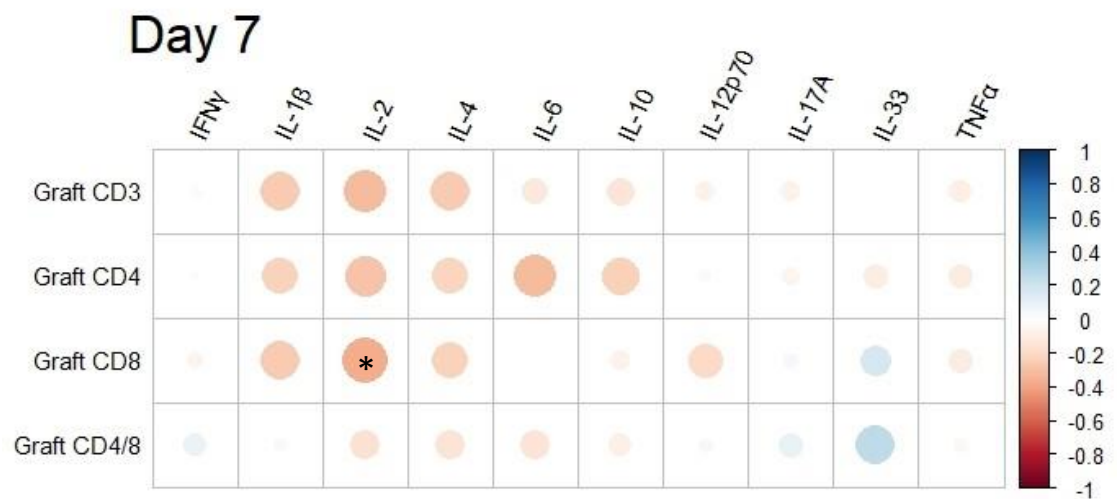


Figure 277: Correlation matrix of graft T cell content and cytokine measurements at day 7. Red hues indicate negative correlation and blue indicate positive correlation with T cell content.

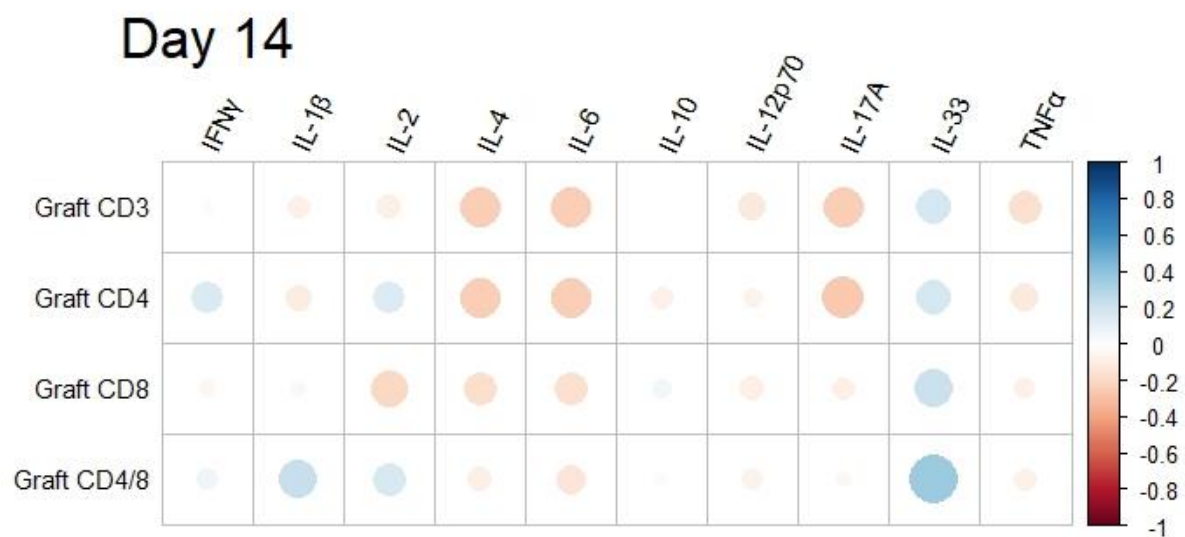


Figure 278: Correlation matrix of graft T cell content and cytokine measurements at day 14. Red hues indicate negative correlation and blue indicate positive correlation with T cell content.

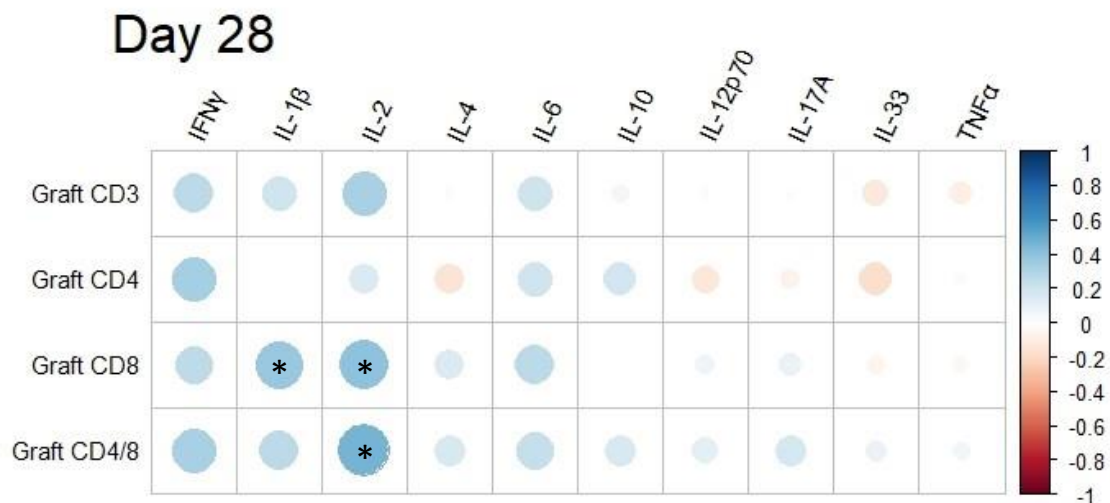


Figure 279: Correlation matrix of graft T cell content and cytokine measurements at day 28. Red hues indicate negative correlation and blue indicate positive correlation with T cell content.

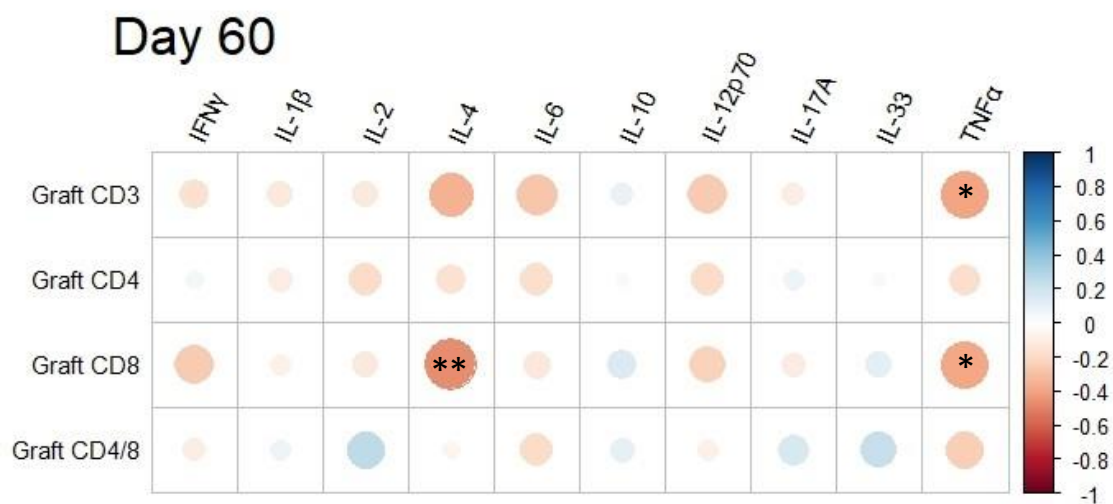


Figure 280: Correlation matrix of graft T cell content and cytokine measurements at day 60. Red hues indicate negative correlation and blue indicate positive correlation with T cell content.

Day 100 T cell subsets



Figure 281: Correlation matrix of in vivo T cell recovery and corresponding cytokine measurements at day 100. Red hues indicate negative correlation and blue indicate positive correlation with T cell recovery.

Day 180 T cell subsets

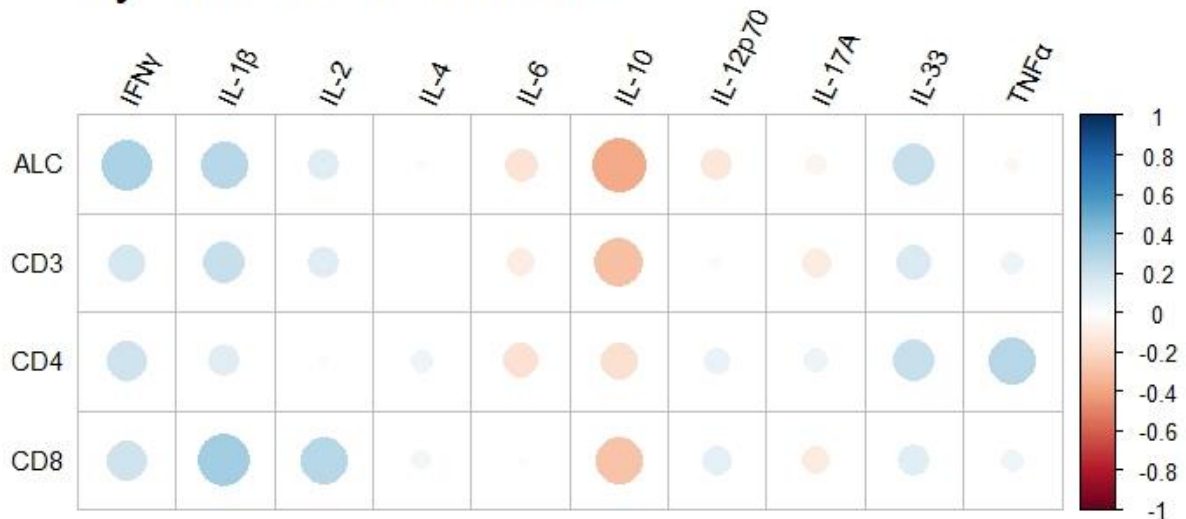


Figure 282: Correlation matrix of in vivo T cell recovery and corresponding cytokine measurements at day 180. Red hues indicate negative correlation and blue indicate positive correlation with T cell recovery.

Cytokine secretion was then correlated with five primary transplant outcomes: acute GVHD grade II-IV, acute GVHD grade III-IV, chronic GVHD, relapse and death. At the pre-transplant time-point, a weak negative correlation between all cytokines and grade II-IV acute GVHD was seen, except IL-6 and IFN γ , where the correlation was neutral. No association was seen for chronic GVHD or high grade acute GVHD. A stronger positive correlation was seen with death and relapse and increasing cytokine levels (*Fig. 283*). At day 0 a similar positive correlation between death or relapse and several cytokines was seen and the relationship between IFN γ and high grade acute GVHD was seen, with a lesser correlation seen with IL-6 at this time-point (*Fig. 284*).

At day 7, many interactions are neutral, however the pattern of increased secretion associating with mortality and relapse continues (*Fig. 285*). At day 14, minor negative correlations are seen with chronic GVHD, notably in IFN γ , TNF α and IL-12p70 (*Fig. 286*). By day 28 a shift in the correlation between GVHD outcomes and IFN γ levels occurs, while the remainder of associations remain broadly static, with a trend towards a protective effect of IL-4 and IL-10 against chronic GVHD (*Fig. 287*). At day 60 the pattern of correlation is like that seen at day 28 (*Fig. 288*). Day 100 recapitulates the negative association between IFN γ and GVHD outcomes and further highlights the positive correlation of death or relapse and increased secretion of several cytokines (*Fig. 289*). At day 180 higher cytokine secretion across most analytes shows a negative correlation with acute GVHD (*Fig. 290*). A summary of significant and trends in correlation is provided in Table 33.

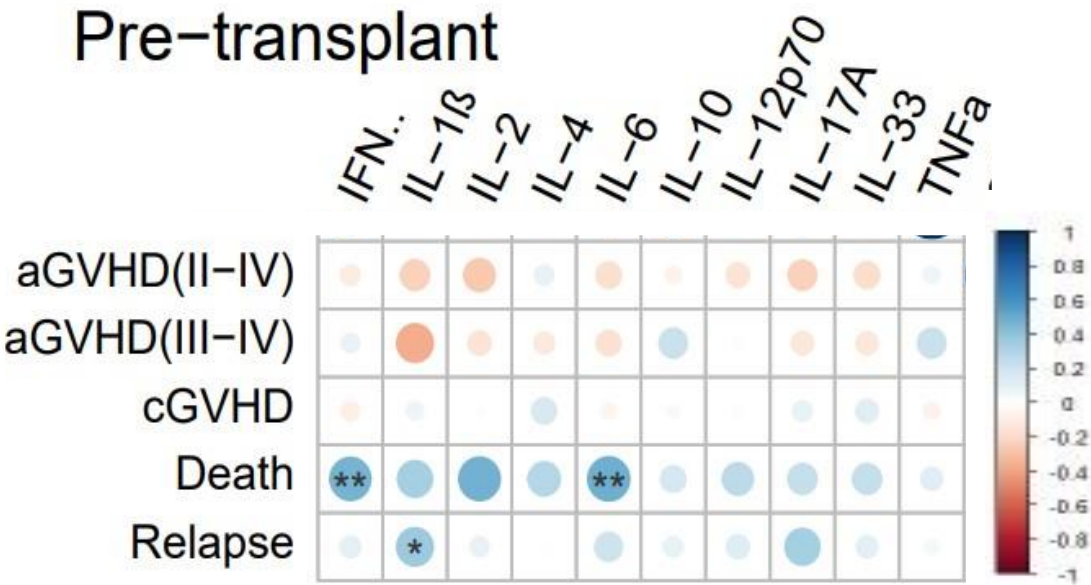


Figure 283: Correlation matrix of acute GVHD grades, chronic GVHD, death and relapse with cytokine secretion at the pre-transplant time-point. Red hues indicate negative correlation and blue indicate positive correlation.

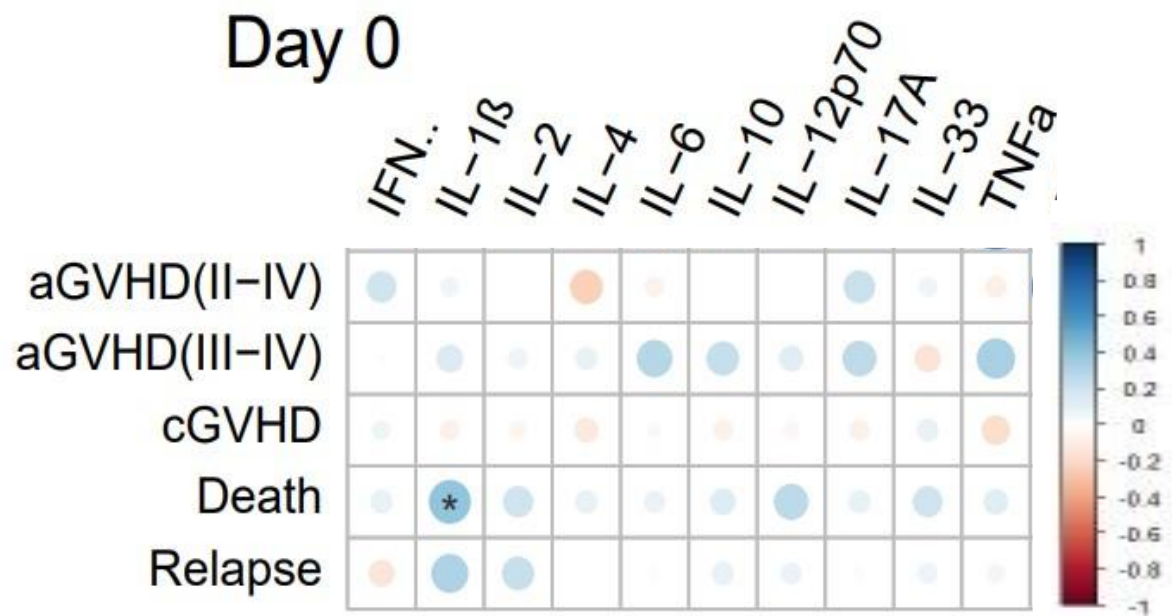


Figure 284: Correlation matrix of acute GVHD grades, chronic GVHD, death and relapse with cytokine secretion at the day 0 time-point. Red hues indicate negative correlation and blue indicate positive correlation.

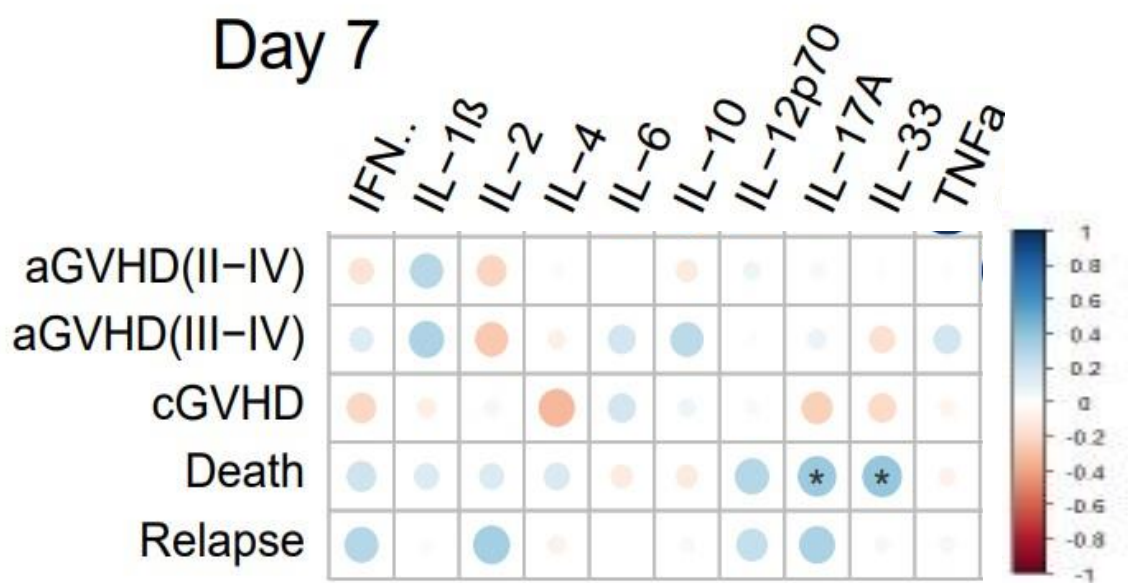


Figure 285: Correlation matrix of acute GVHD grades, chronic GVHD, death and relapse with cytokine secretion at the day 7 time-point. Red hues indicate negative correlation and blue indicate positive correlation.

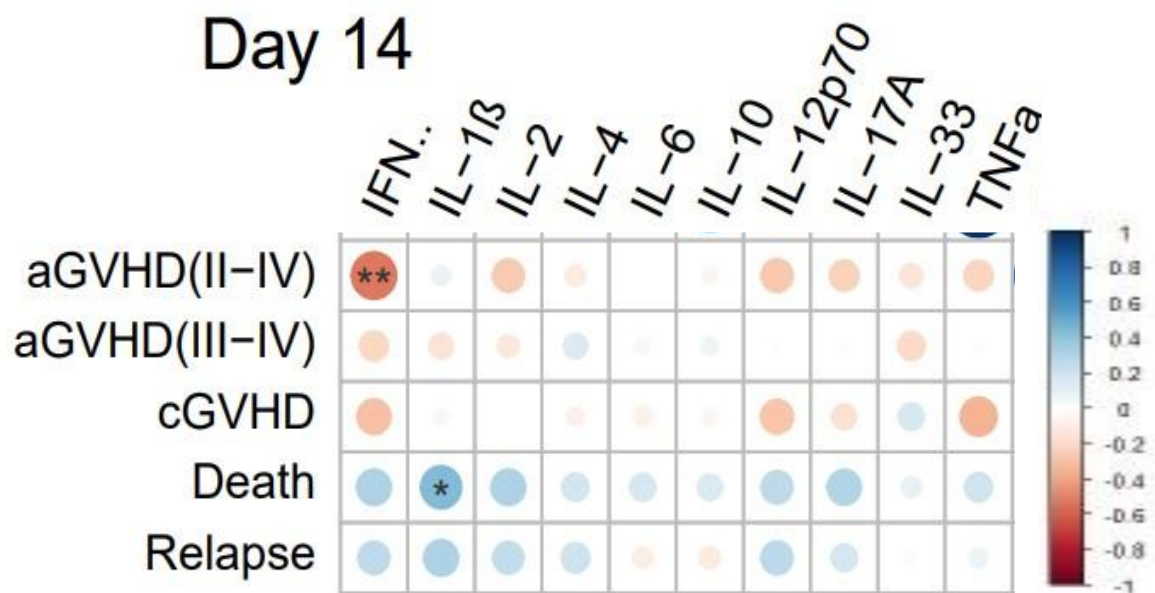


Figure 286: Correlation matrix of acute GVHD grades, chronic GVHD, death and relapse with cytokine secretion at the day 14 time-point. Red hues indicate negative correlation and blue indicate positive correlation.

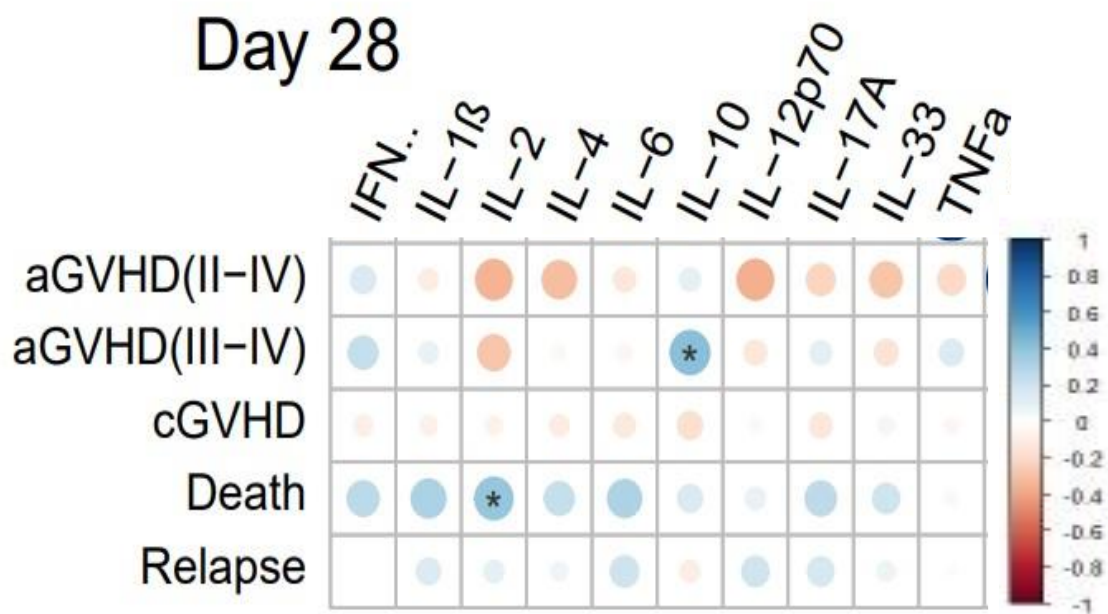


Figure 287: Correlation matrix of acute GVHD grades, chronic GVHD, death and relapse with cytokine secretion at the day 28 time-point. Red hues indicate negative correlation and blue indicate positive correlation.

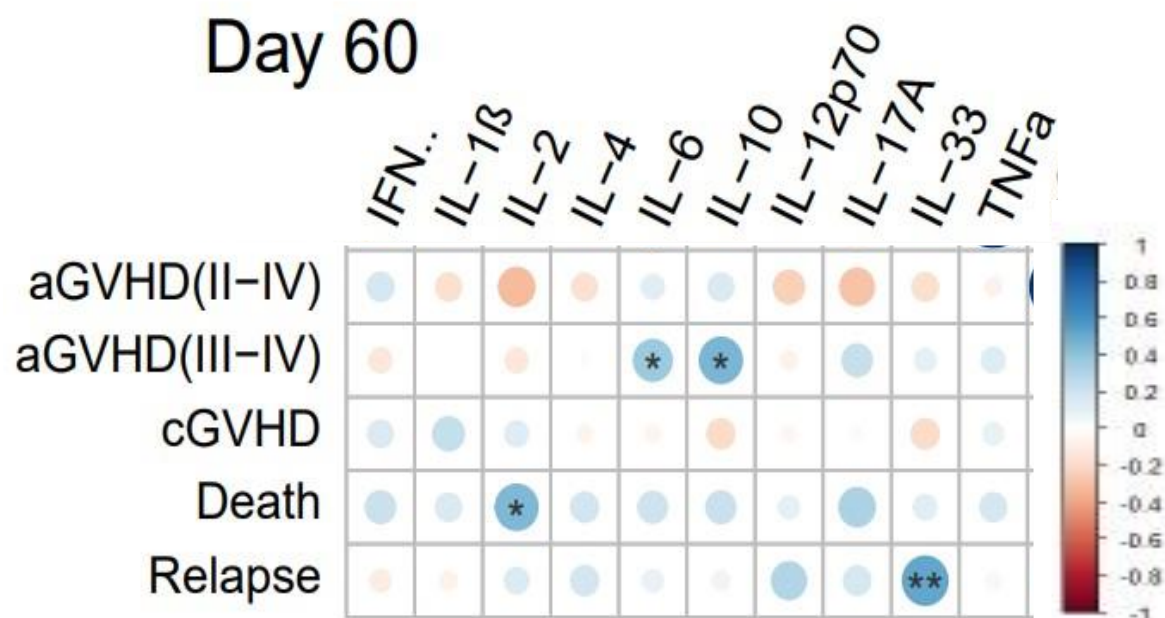


Figure 288: Correlation matrix of acute GVHD grades, chronic GVHD, death and relapse with cytokine secretion at the day 60 time-point. Red hues indicate negative correlation and blue indicate positive correlation.

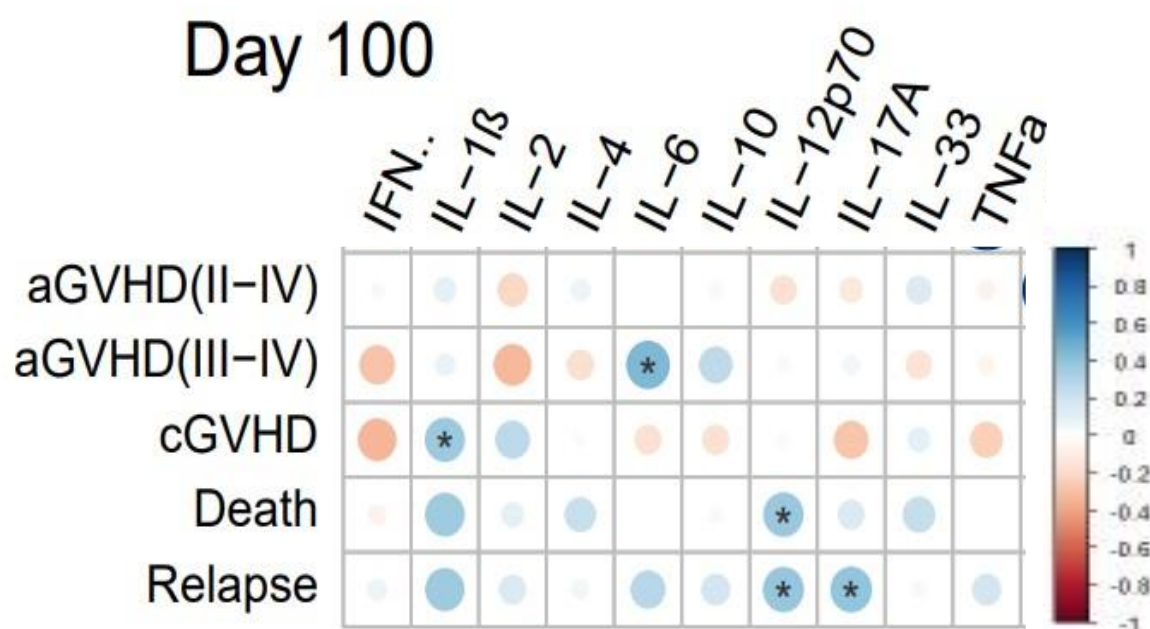


Figure 289: Correlation matrix of acute GVHD grades, chronic GVHD, death and relapse with cytokine secretion at the day 100 time-point. Red hues indicate negative correlation and blue indicate positive correlation.

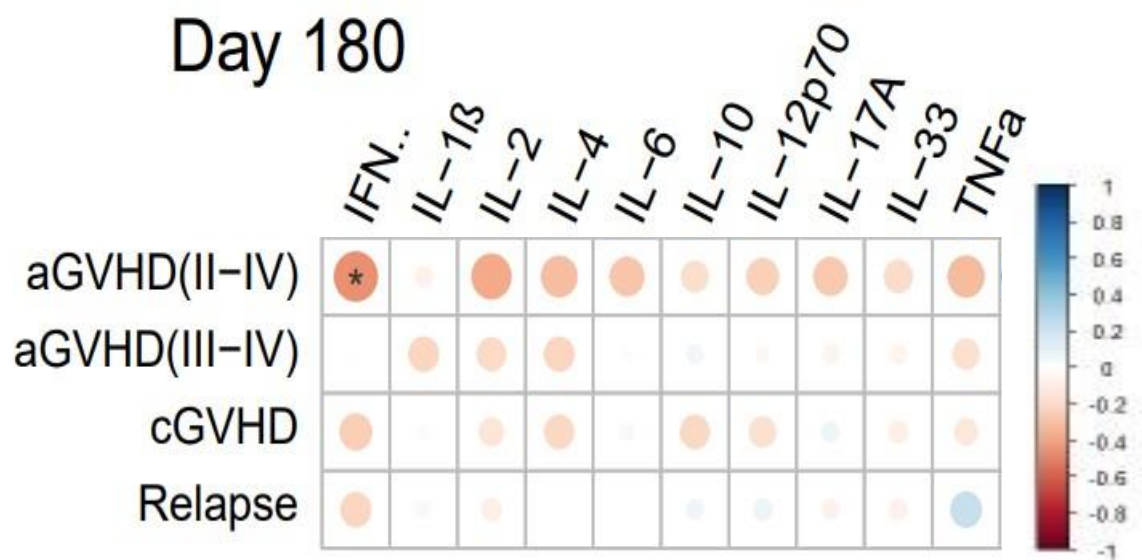


Figure 290: Correlation matrix of acute GVHD grades, chronic GVHD, death and relapse with cytokine secretion at the day 180 time-point. Red hues indicate negative correlation and blue indicate positive correlation.

Table 34: Summary of cytokine clinical correlates

Time	IFN γ	IL-1 β			IL-2		IL-4		IL-6		IL-10		IL-12p70		IL-33		IL-17A		TNF α
Pre	OS*	O S	II/I II	R*	OS	II	OS		OS *	II/I II	OS, III	OS	II	O S	II	R, OS	II	III	
Day 0	II	OS*		R	OS, R		II		III		III	OS		OS		II, III		II I	C
Day 7	C	R	II, III		II, III	R	C		III, C		III	OS, R		O S *	C, III	OS *, R	C	III	
Day 14	II*, III, C	OS , R	OS*		R	II	OS , R	OS, R	OS			OS , R	II, C	III		OS , R	II, C	II, C	
Day 28	III, OS		OS		OS *	II, III	II O S		OS		III*	II	R	O S	II	OS	II	II	
Day 60	II, OS		C		OS *	II	II		III*		III*	R	II	R *	C	OS , III	II	OS	
Day 100	III, C		OS, R	C*	C	III, II	OS		III*	R	III	OS*, R*		OS		R*	C	C	
Day 180	II*	C, R	III		II, III		II, III, C		II		II, C	II, C		II		II		II	R

Table 31: Summary of correlation trends between individual cytokines and outcomes post-transplant. Asterix and darker colour denotes statistical significance, blue indicating a positive correlation, red indicating and negative correlation. OS = overall survival, R = relapse, II = grade II-IV acute GVHD, III = Grade III-IV acute GVHD and C = chronic GVHD.

Cytokines in acute GVHD subtypes

The following section examines whether discrete cytokine expression patterns were seen in patients who developed acute GVHD and if specific organ involvement was associated with those differing patterns. Furthermore, the temporal association between such patterns is examined to identify if cytokine expression occurs before, during or after clinical diagnosis of acute GVHD. Differential cytokine expression profiles were noted in acute GVHD subtypes including skin, gastrointestinal and liver. Peak cytokine secretion was higher in liver and gastrointestinal type disease as compared to skin at several time-points, most notably at day 0 for IL-2, IL-10, TNF α and IL-17A and at day 28 for IL-10 and IFN γ (Fig. 390-302). In skin GVHD, no statistically significant difference was seen in IL1- β , IL2 or IL-4 between patients who developed acute skin GVHD and both those who did not develop any GVHD or those who developed non-skin type GVHD. Pre-transplant IFN γ levels were lower in those who subsequently developed skin GVHD as compared to those who did not ($p=0.03$). Pre-transplant and day 60 IL-6 levels were lower in those who developed skin GVHD (pre-transplant $p=0.02$, day 60 $p=0.01$). Higher late IL-10 levels were seen in those who developed skin GVHD at day 180 ($p=0.03$), however, most cases of acute GVHD had occurred before this time-point. Both early and late IL-12p70 levels were higher in those who developed skin GVHD (pre-transplant $p=0.01$, day 180 $p=0.04$). IL-17A levels were lower in those with subsequent skin GVHD at the day 0 time-point ($p=0.04$) and again at day 60 ($p=0.03$). Late TNF α levels were increased in those with skin GVHD ($p=0.01$).

In gastrointestinal (GI) acute GVHD levels of IL-2 and IL-4 did not correlate with incidence. IFN γ levels at day 0 were higher in those who developed GI GVHD compared to non-GI GVHD cases and those who did not develop any GVHD ($p=0.03$). Conversely, at the day 180 time-point, lower IFN γ levels were seen in those patients ($p=0.01$). Early IL-1 β levels were higher in those with GI GVHD at both day 0 ($p=0.05$) and day 7 ($p=0.04$). Day 60 IL-6 levels showed a trend towards increase in GI GVHD ($p=0.07$) and for IL-10, levels were lower at day 180 ($p=0.07$). IL-17A showed a strong association with subsequent GI GVHD at the day 0 time-point ($p=0.005$). TNF α levels were lower at day 180 in those who had developed GI GVHD ($p=0.04$). In liver GVHD there was no association seen with IFN γ , IL-1 β , IL-6, IL-10, IL-12p70, IL-17A or TNF α levels. At day 0, excess IL-2 secretion was noted in those with subsequent liver GVHD as compared to non-liver GVHD cases and those who did not develop any GVHD ($p=0.01$). This association reversed at day 28, with low levels seen in those who developed GI GVHD ($p=0.02$). IL-4 levels were low in those who developed liver GVHD at the day 60 ($p=0.01$) and day 180 time-points ($p=0.01$) (Figures 303 – 308, Table 34).

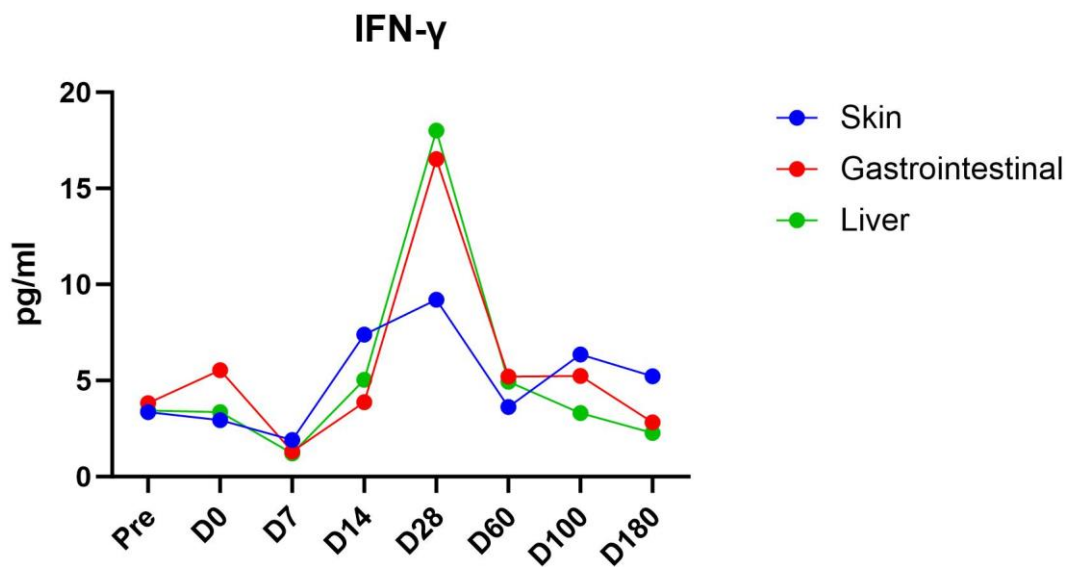


Figure 291: IFN γ secretion at serial time-points in skin, gastrointestinal and liver acute GVHD. Significantly higher expression of IFN γ is most notable at the day 28 time-point in those who developed gastrointestinal and liver subtypes of acute GVHD as compared to isolated skin disease.

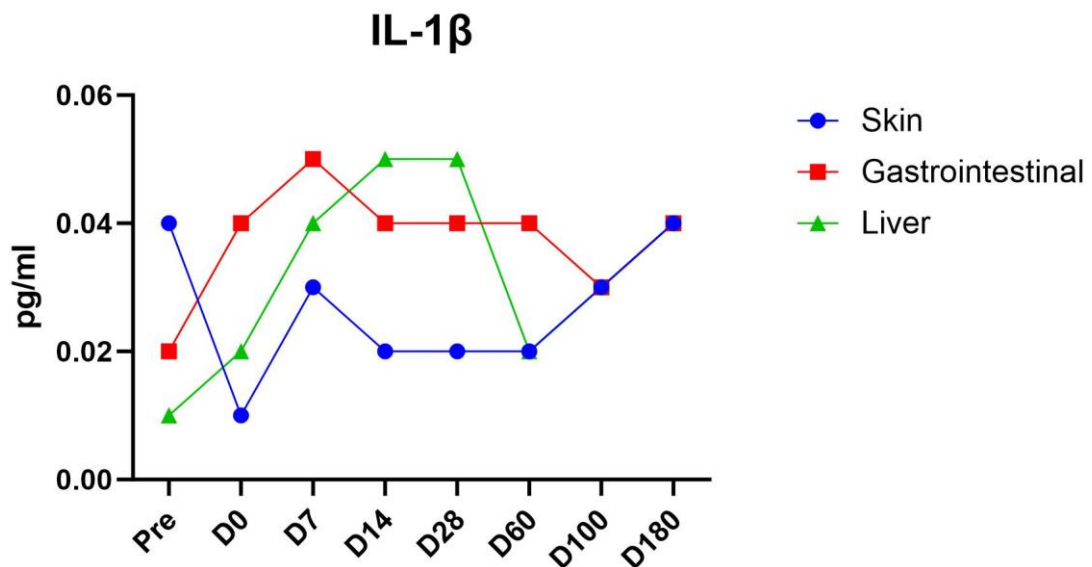


Figure 292: IL-1 β secretion at serial time-points in skin, gastrointestinal and liver acute GVHD. Broadly increased early-transplant expression is seen in those who subsequently developed gastrointestinal and liver acute GVHD as compared to skin only disease.

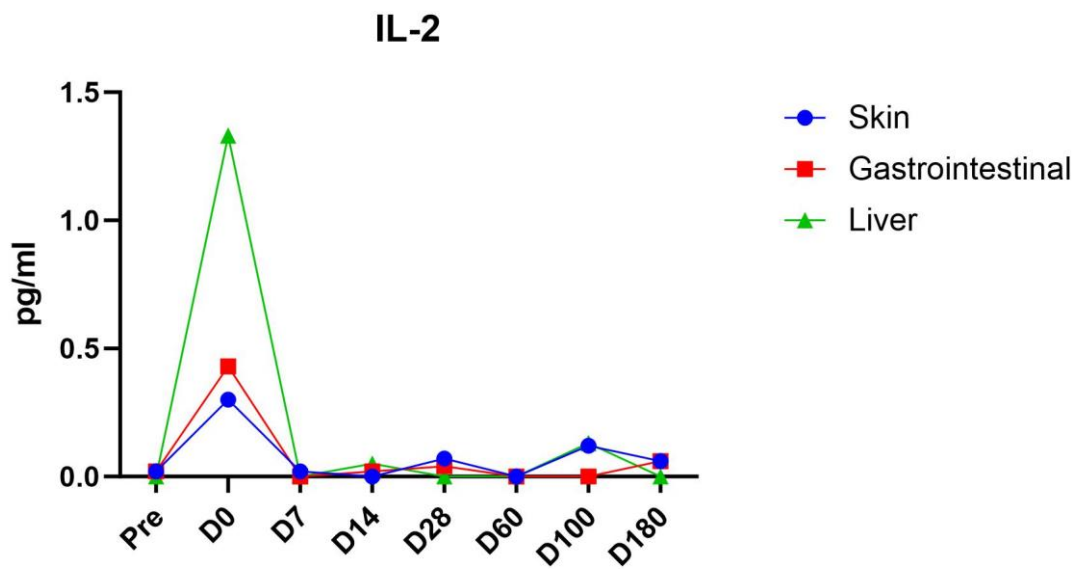


Figure 293: IL-2 secretion at serial time-points in skin, gastrointestinal and liver acute GVHD. A marked increase in early IL-2 levels in those who went on to develop liver GVHD was seen at the day 0 time-point.

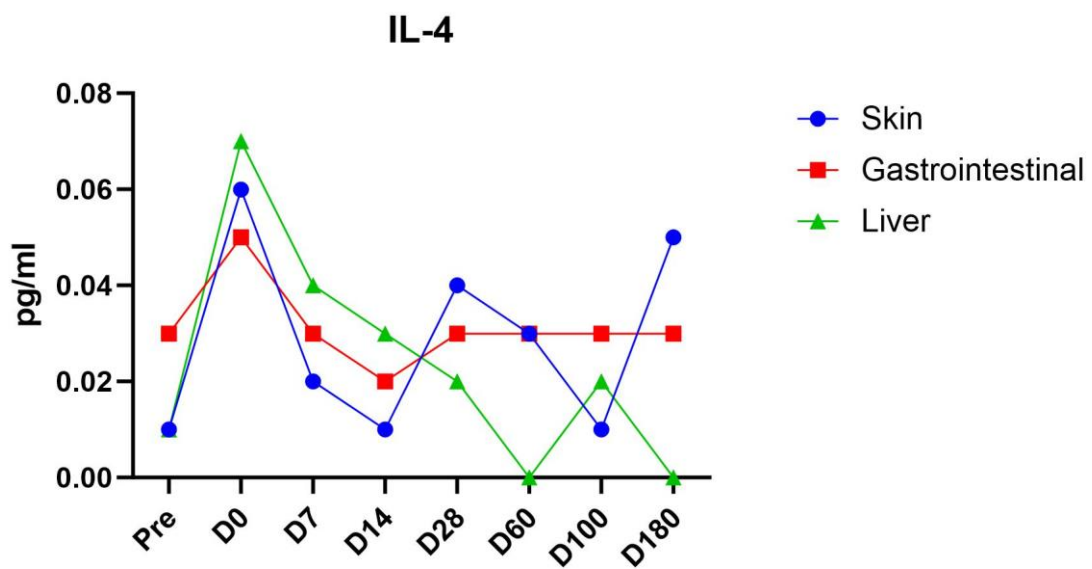


Figure 294: IL-4 secretion at serial time-points in skin, gastrointestinal and liver acute GVHD. Reduced IL-4 expression was noted in those at day 60 who went on to develop liver acute GVHD as compared to other subtypes.

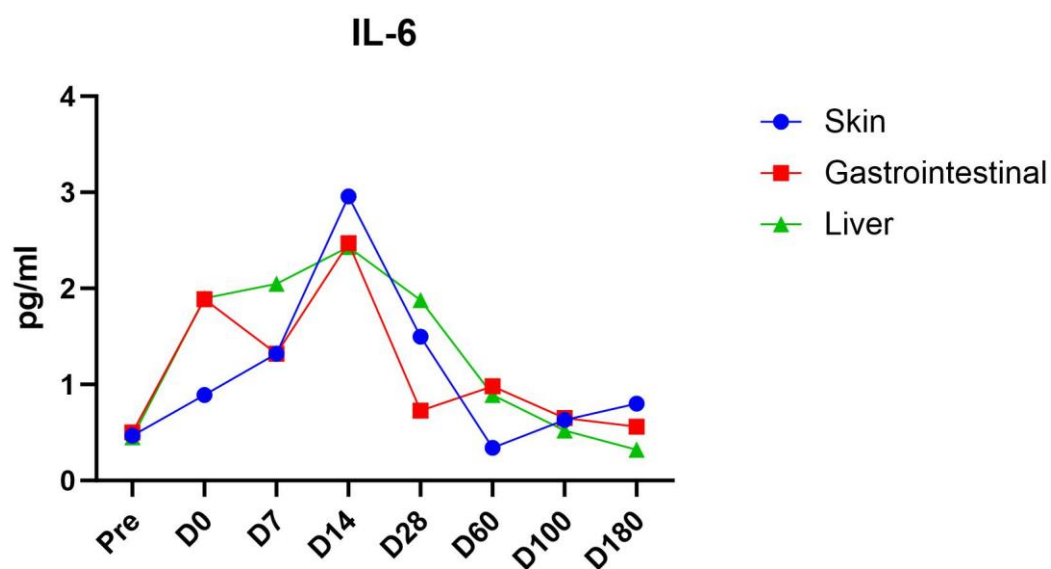


Figure 295: IL-6 secretion at serial time-points in skin, gastrointestinal and liver acute GVHD.

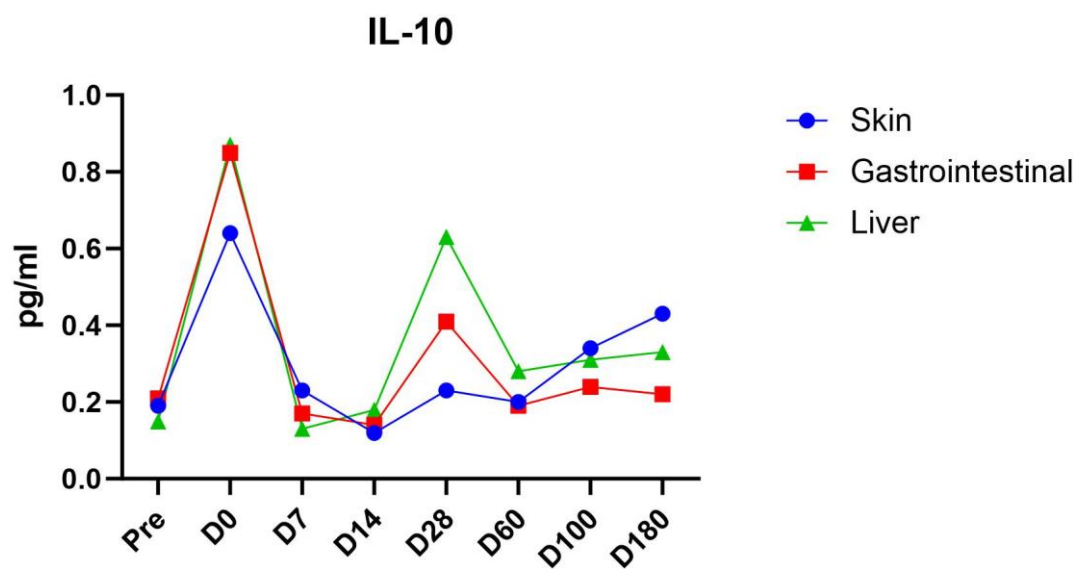


Figure 296: IL-10 secretion at serial time-points in skin, gastrointestinal and liver acute GVHD.

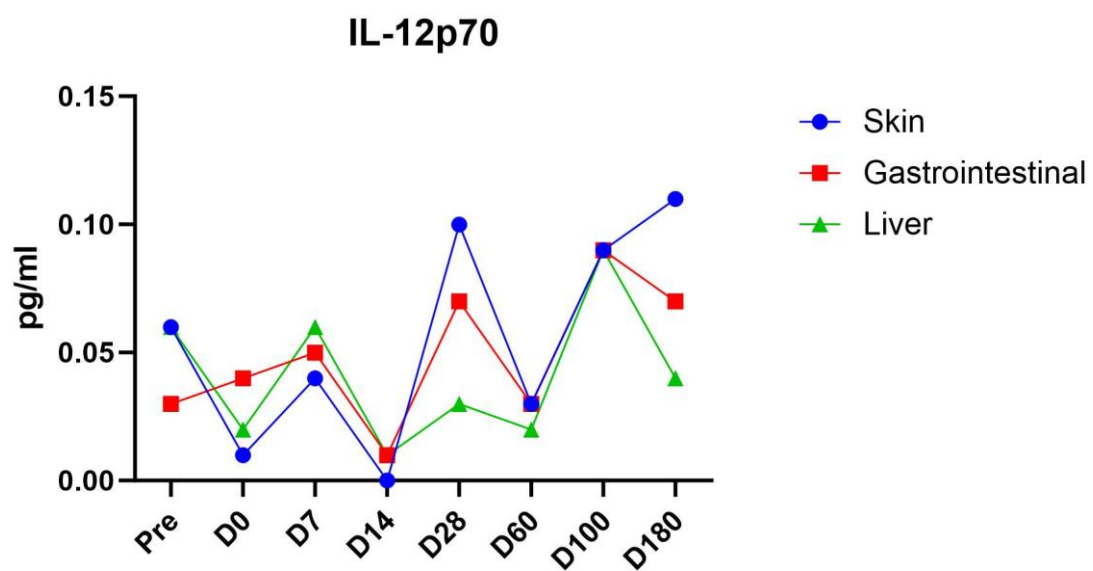


Figure 297: IL-12p70 secretion at serial time-points in skin, gastrointestinal and liver acute GVHD.

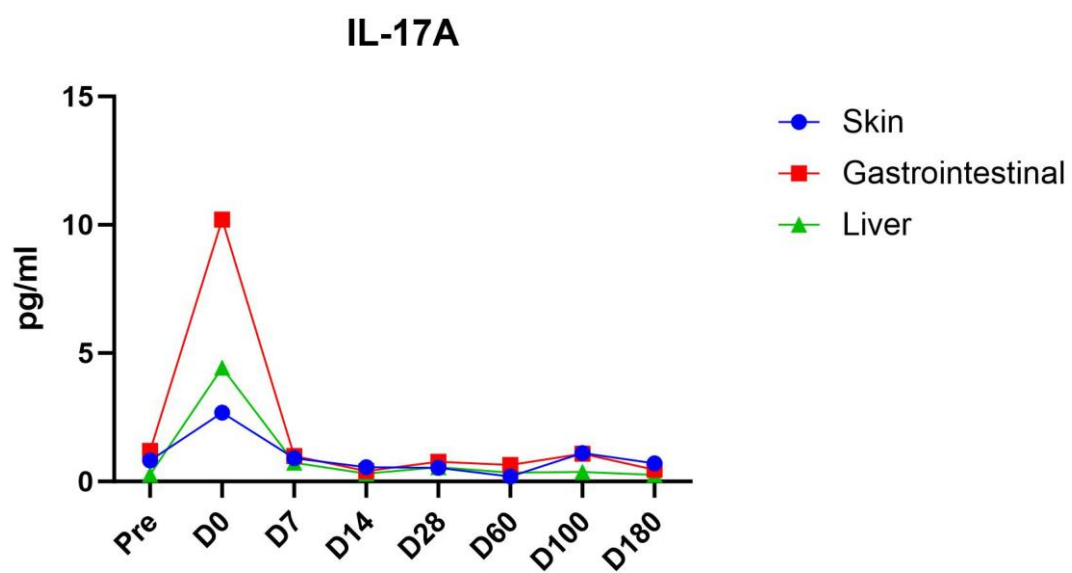


Figure 298: IL-17A secretion at serial time-points in skin, gastrointestinal and liver acute GVHD. A notable increase in expression at day 0 in those who went on to develop acute gastrointestinal GVHD was seen.

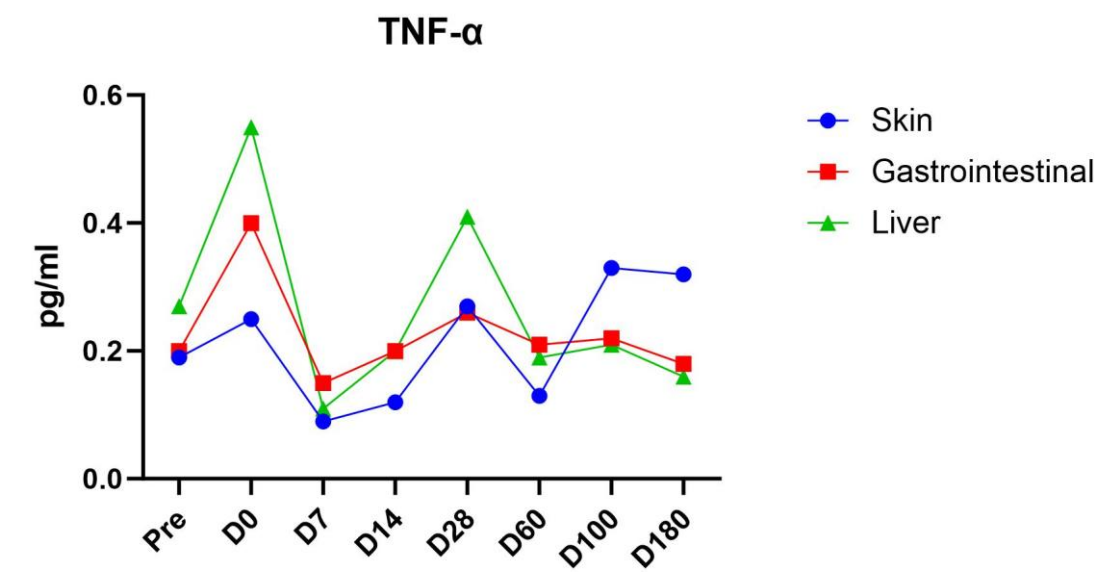


Figure 299: TNF α secretion at serial time-points in skin, gastrointestinal and liver acute GVHD.

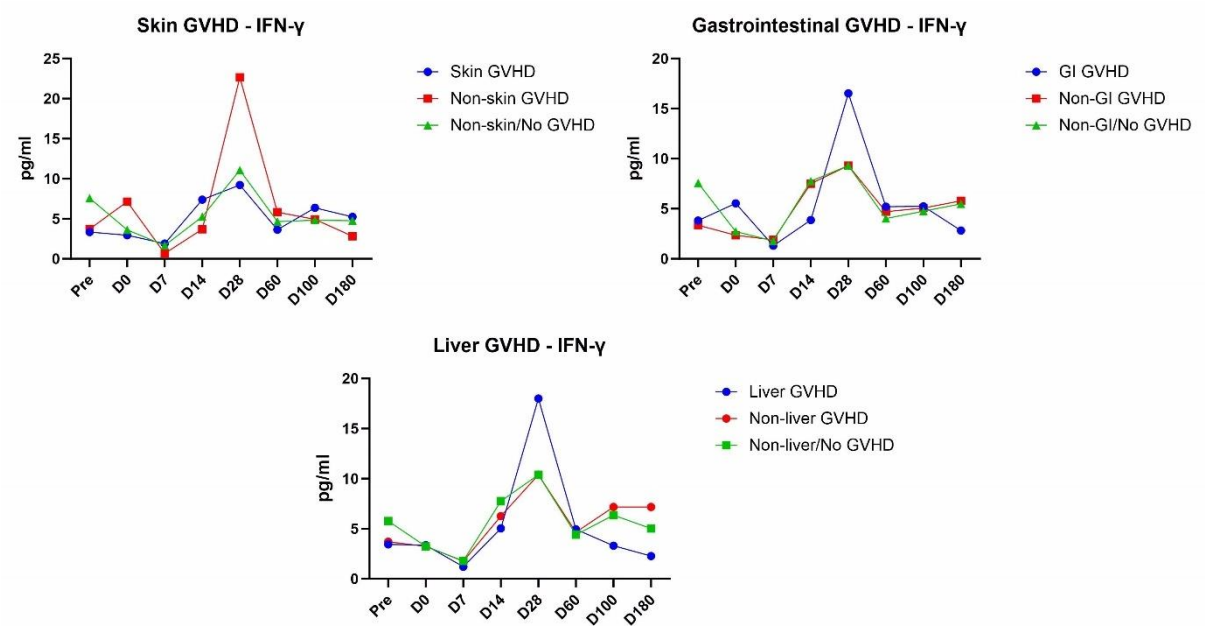


Figure 300: IFN γ secretion at serial time-points in acute GVHD subtype compared to non-subtype acute GVHD and non-GVHD cases: skin, gastrointestinal and liver. Day 28 peaks in gastrointestinal and liver GVHD are noted as compared to skin only disease or no GVHD.

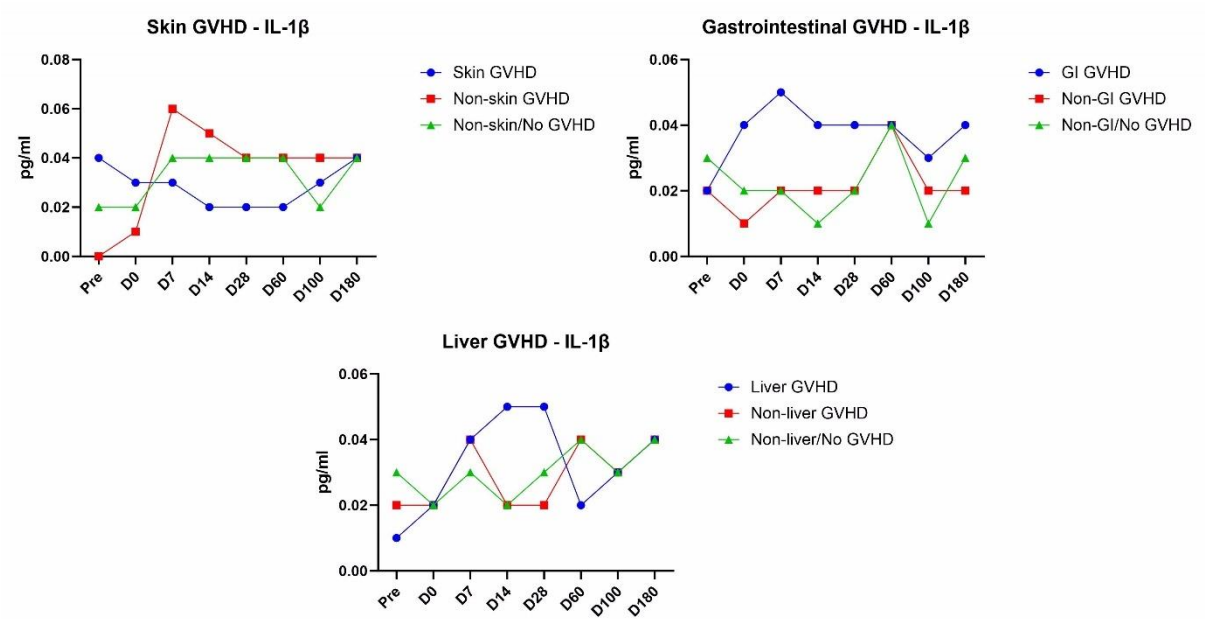


Figure 301: IL-1 β secretion at serial time-points in acute GVHD subtype compared to non-subtype acute GVHD and non-GVHD cases: skin, gastrointestinal and liver. Days 0 and 7 expression of IL-1 β is increased in the gastrointestinal subtype of acute GVHD but not liver or skin.

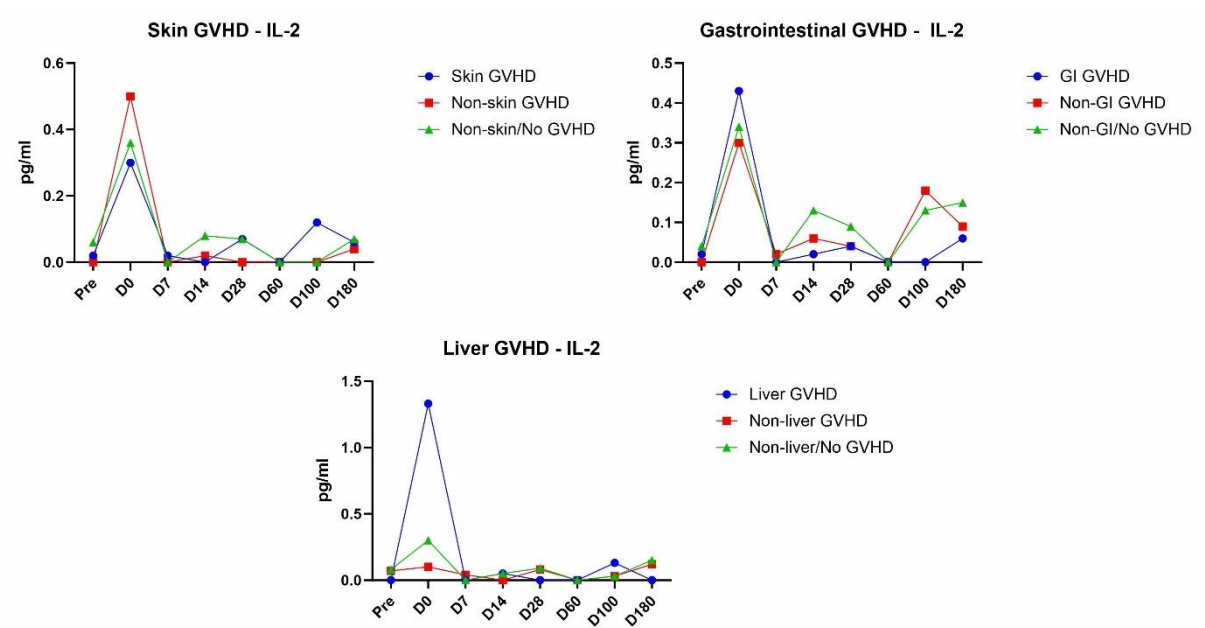


Figure 302: IL-2 secretion at serial time-points in acute GVHD subtype compared to non-subtype acute GVHD and non-GVHD cases: skin, gastrointestinal and liver. Increased IL-1 expression at day 0 is seen in those who subsequently developed acute liver GVHD.

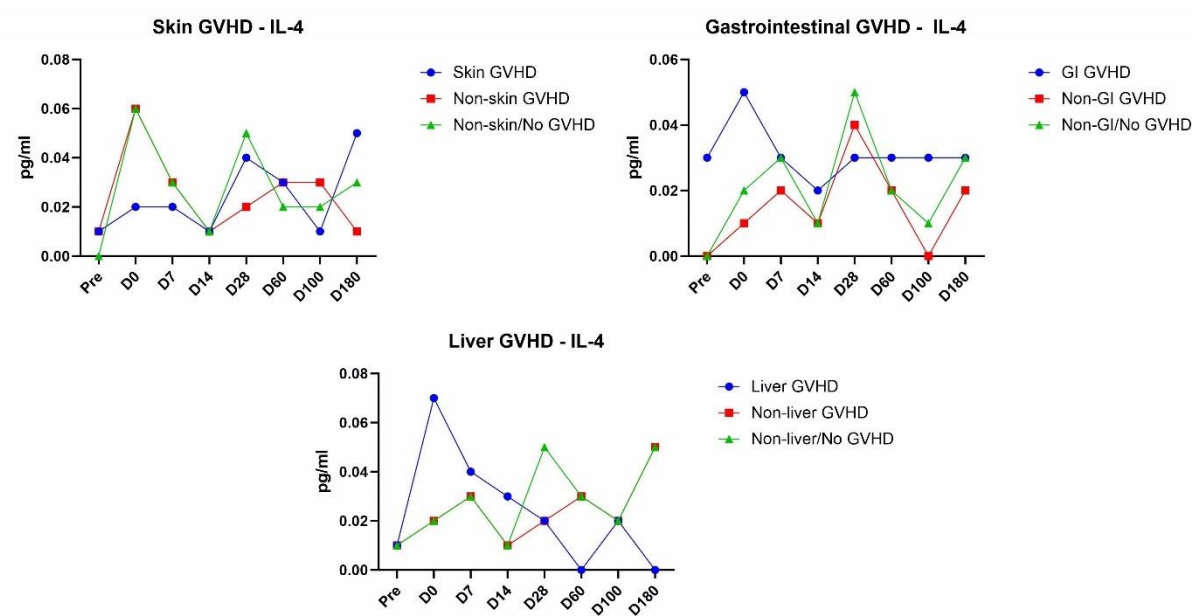


Figure 303: IL-4 secretion at serial time-points in acute GVHD subtype compared to non-subtype acute GVHD and non-GVHD cases: skin, gastrointestinal and liver. Reduced IL-4 expression is seen at day 60 in those who went on to develop liver acute GVHD.

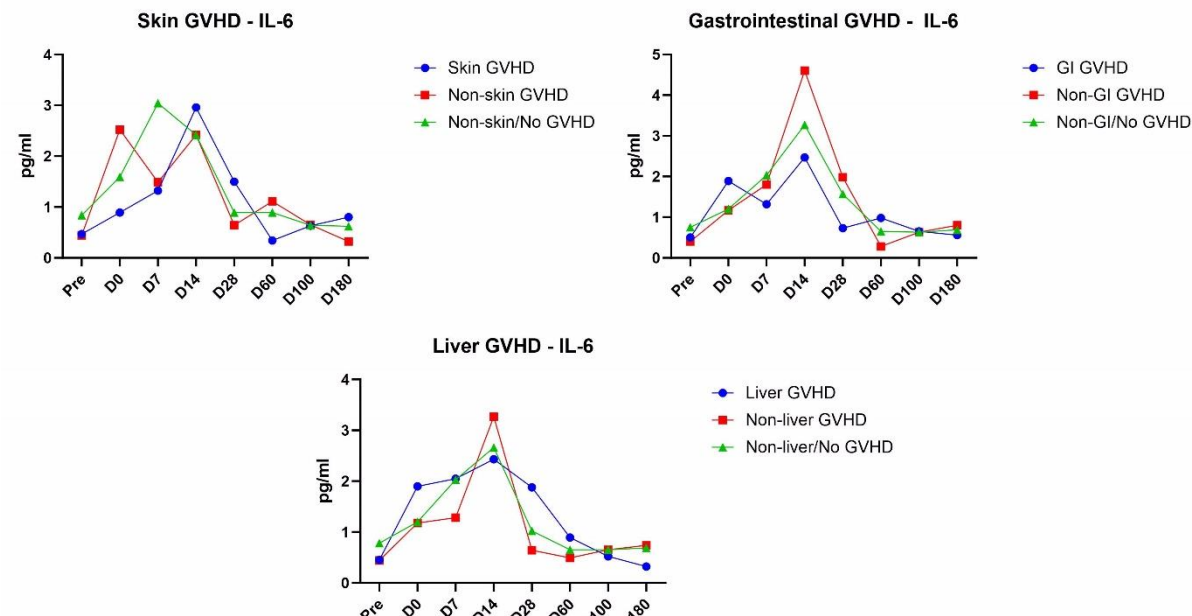


Figure 304: IL-6 secretion at serial time-points in acute GVHD subtype compared to non-subtype acute GVHD and non-GVHD cases: skin, gastrointestinal and liver.

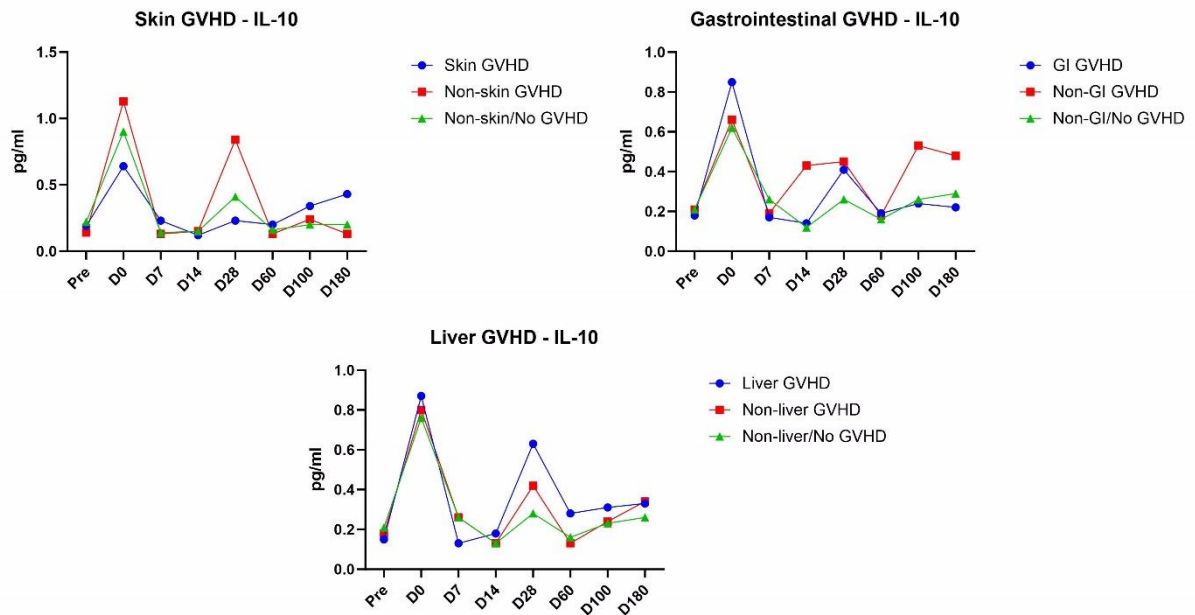


Figure 305: IL-10 secretion at serial time-points in acute GVHD subtype compared to non-subtype acute GVHD and non-GVHD cases: skin, gastrointestinal and liver.

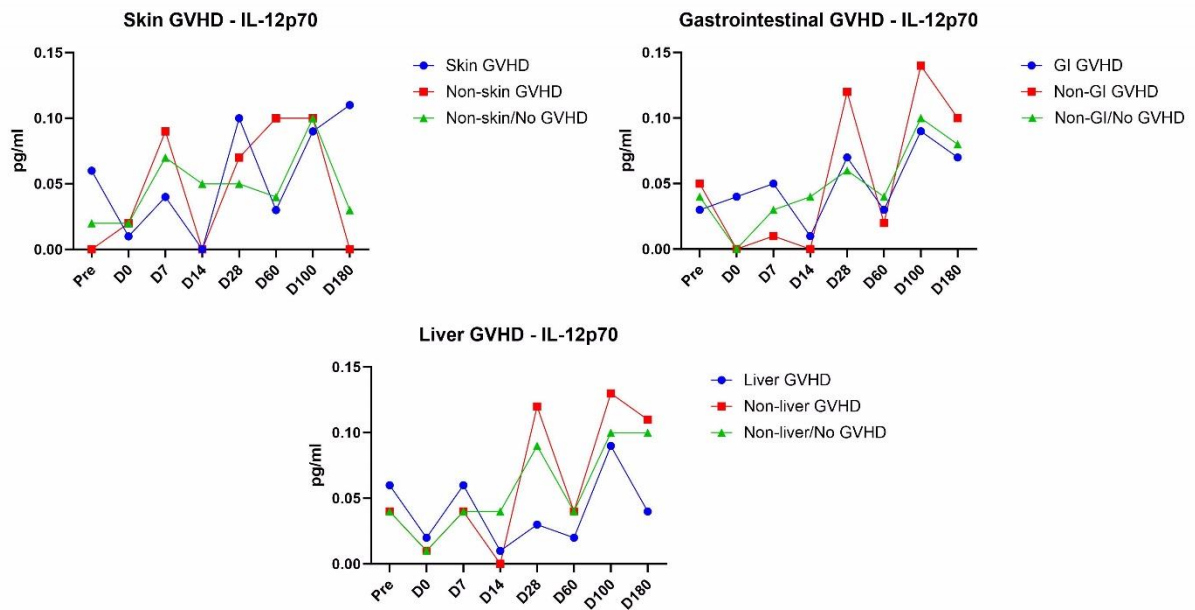


Figure 306: IL-12p70 secretion at serial time-points in acute GVHD subtype compared to non-subtype acute GVHD and non-GVHD cases: skin, gastrointestinal and liver.

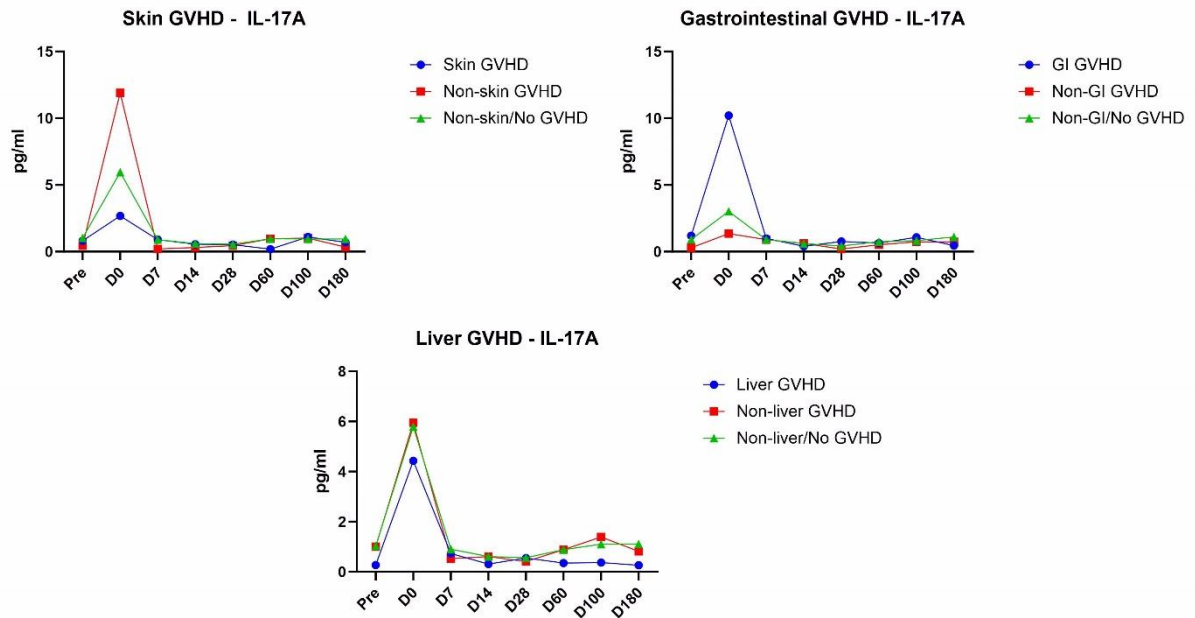


Figure 307: IL-17A secretion at serial time-points in acute GVHD subtype compared to non-subtype acute GVHD and non-GVHD cases: skin, gastrointestinal and liver. Increased IL-17A expression early in transplant in those who subsequently developed acute gastrointestinal GVHD is seen.

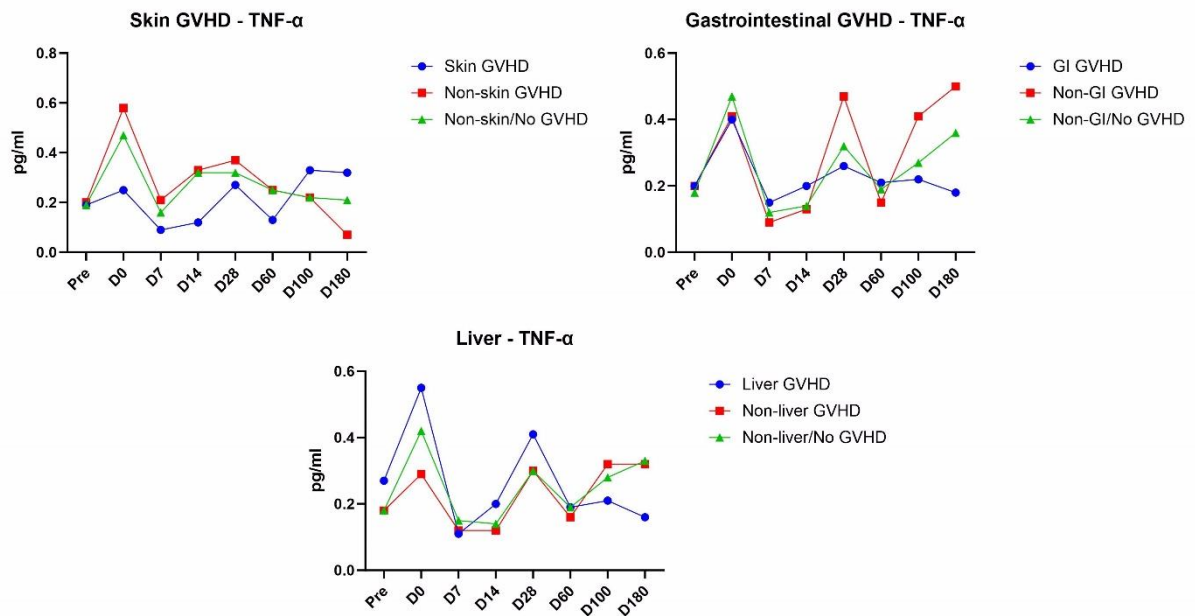


Figure 308: TNFα secretion at serial time-points in acute GVHD subtype compared to non-subtype acute GVHD and non-GVHD cases: skin, gastrointestinal and liver.

Table 35: Cytokine levels in acute GVHD subtypes

Time-point	IFN γ	IL-1 β	IL-2	IL-4	IL-6	IL-10	IL-12p70	IL-17A	TNF α
Pre	Skin ↓				Skin ↓		Skin ↑		
D0	GI ↑	GI ↑	Liver ↑					GI ↑ Skin ↓	
D7		GI ↑							
D14	GI ↓								
D28									
D60				Liver ↓	GI ↑ Skin ↓			Skin ↓	
D100									
D180	GI ↓			Liver ↓		Skin ↑ GI ↓	Skin ↑		GI ↓ Skin ↑

Outcomes in the over 70s

Collectively, 5 patients over the age of 70 were included in this analysis, including 4 in the group undergoing cytokine quantification. This represents the first cohort of patients availing of an allogeneic transplant over the age of 70 since the inception of the Irish allogeneic transplant programme in the mid- 1980s. These patients included several high-risk disease phenotypes; CMML with mutated *ASXL1*, *CSF3R*, *SRSF2* and *RUNX1* with a high CPSS-Molecular score, therapy-associated AML with a complex karyotype and mutations in *ASXL1*, *TET2* and *CBL*, MDS with excess blasts, mutated *ETV6* and a *KMT2A* PTD, AML with mutated *NPM1* and *KRAS* in CR2 and AML, myelodysplasia-related, with 11q+ and mutations in *FLT3*, *IDH2*, *DNMT3A* and *ASXL1*. No relapse or mortality events in this group had occurred at a median of 561 days follow-up, as was seen in all patients > 65 years old. Co-morbidity scores were lower in the over 70 age group including a higher KPS and lower age-adjusted HCT-CI. Graft and donor characteristics were similar to younger age groups and time to engraftment was quicker for both neutrophils and platelets. Acute GVHD outcomes were similar regardless of age group, although no chronic GVHD events were seen in the over 70s. Immune reconstitution appeared lower in older patients, with lower CD4 recovery at day 100, day 180 and 1 year in addition to consistently lower IgG recovery also. A summary of demographic particulars and transplant-related outcomes as compared to younger counterparts is provided in Table 36. In the 4 patients included in the cytokine analysis, no statistically significant difference in overall secretion of any cytokine was seen between those above or below the age of 70, with a small number of outliers noted (Fig. 309).

Table 36: Outcomes in the over 70s

Variable	70 + (n=5)	< 70 (n=175)
Relapse	0	53
Death	0	50
Age-adjusted HCT-CI	1 (1-5)	2 (1-8)
KPS	90 (80-100)	80 (50-100)
CIBMTR DRI	60%	30%
High	20%	63.5%
Intermediate	20%	6.5%
Low		
Weight	78.1kg	80.3kg
Days to transplant	209	306
Donor source	80% Unrelated	55.3% Unrelated
Cryopreservation	0	20
ABO mismatch	40%	46.4%
Donor age	37	39.8
Graft CD3 ⁺ T cells	23.28	21.27
Graft CD4 ⁺ T cells	14.3	13.44
Graft CD8 ⁺ T cells	8.86	7.72
Graft CD4 ⁺ /CD8 ⁺ T cells	0.28	0.36
Days to ANC engraftment	17	20
Days to PLT engraftment	13	18
Acute GVHD	60%	60.36%
Grade II-IV	60%	45.8%
Grade III-IV	20%	10.59%
Chronic GVHD	0%	16.67%
DLI	20%	37.1%
Day 100 CD4	46	104.4
Day 180 CD4	73.2	138.7
Day 365 CD4	90	192.5
Day 100 IgG	6.68	8.25
Day 180 IgG	6.22	8.77
Day 365 IgG	5.66	9.60
EASIX Pre-transplant	1.22	1.86
EASIX Day 0	16.27	11.49
EASIX at GVHD onset	1.27	2.03
EASIX 1 year	1.74	1.23

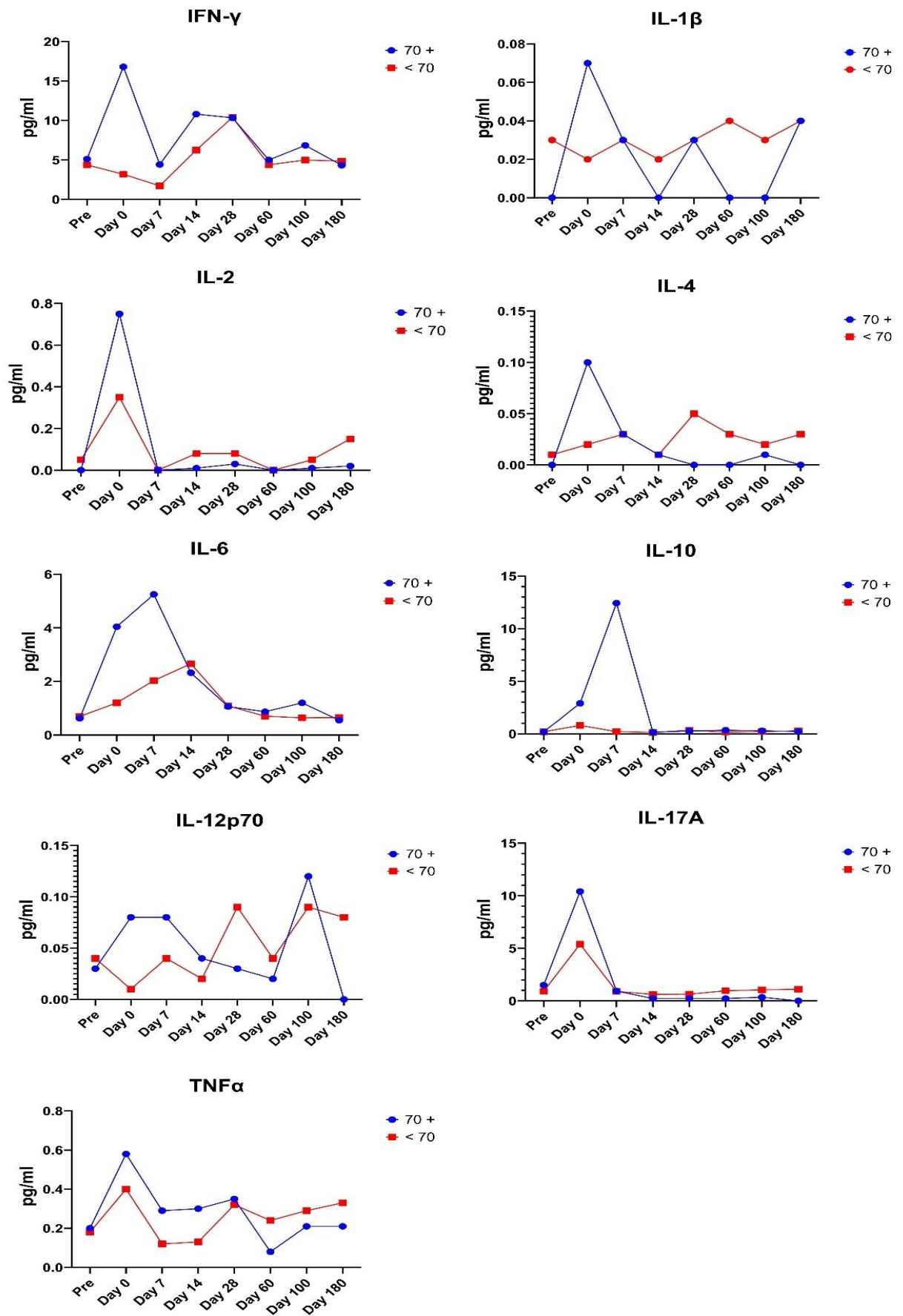


Figure 309: Median cytokine levels at serial time-points, ≥ 70 years old vs. < 70 years old.

Discussion

The latter group of 32 patients exhibited encouraging clinical outcomes across several domains, including survival, relapse, GVHD rates and infectious complications. Recipient and donor characteristics are broadly comparable to that of the retrospective group, and reflective of a consistency in transplant practice in recent years. Although median follow-up is shorter in the latter cohort of patients at 502 days, the number of adverse events for NRM and death at equivalent time-points are lower and may represent incremental improvements in this conditioning platform over time. Engraftment of both platelets and neutrophils is reassuringly high at 96.8% and 93.75% respectively, which tallies with the historical group of 143 patients. With 4 deaths, 2 due to relapse and 2 due to NRM at last follow up, the safety of this treatment approach is clear, although undoubtedly relapse-related deaths will increase with longer follow-up. Early relapse occurred in 5 of 32 patients, which is proportionately lower than the historical group, despite a similar number of high and intermediate risk disease types within the patient population. Rates of significant infection are also lower in this group, with no cases of invasive fungal disease and less viral re-activations, particular CMV due to the now routine use of letermovir prophylaxis. Liberal DLI use to prevent and treat relapse remains prominent in this group, with demonstrable clinical benefit in terms of relapse prevention in mixed donor chimerism, improved survival in relapse and limited post-DLI GVHD-related toxicity. CD4 recovery at early and intermediate time-points however remains a significant area of unmet need, with median recovery of 58, 95 and $128 \times 10^6/\text{L}$ at days 100, 180 and 1 year respectively.

In the pooled dataset of 175 patients, a 1- and 2-year overall survival rate of 80.34% and 72.35% respectively confirms FluBu3ATLG as a conditioning regimen capable of delivering a combination of modest treatment-related mortality and reasonable survival, including post-relapse. The most significant variables associated with reduced survival included high-risk disease by the CIBMTR DRI, high-risk myelofibrosis, ABO mismatch and graft CD8⁺ T cell content, while chronic GVHD and therapeutic or pre-emptive DLI use led to better overall survival. In the case of myelofibrosis, the excess mortality was driven by NRM, and alludes to additional toxicity experienced by patients with myelofibrosis, especially in those with significant splenomegaly at transplant (156). Interestingly, the negative impact of ABO mismatch on survival is clear in this dataset and adds to the mixed literature in this area, particularly in minor mismatch, which was the stronger association in this group (173, 174, 177, 569). The positive effect of chronic GVHD on survival replicates the findings of other groups, where a reduction in relapse corresponds with a survival advantage (321). Importantly, the benefit of judicious and liberal DLI use to prevent relapse pre-emptively in cases of mixed chimerism results in a survival advantage, and encouragingly, when DLI is used with additional salvage regimens, it prolongs median survival after post-transplant relapse. With over a third of patients receiving a DLI, the success of this RIC platform is significantly enhanced by this system of active surveillance and intervention.

The low 1- and 2-year NRM rates of 11.32% and 12.13% respectively are significantly below that expected by predictive scores and compare favourably with alternative reduced intensity platforms (44, 55). The predictors of NRM in this study included high-risk myelofibrosis, high grade acute GVHD, ABO mismatch and invasive fungal disease. Some of these variables are predictable, such donor and recipient ABO matching, and are modifiable *ab initio*, whereas predictive tools are needed for others. There is clear excess mortality associated with and due to invasive fungal disease, therefore the encouragingly low incidence of fungal disease,

particularly in the most recent cohort of patients, are supportive of an effective prophylactic antifungal regimen, high-quality environmental protection, and biomarker surveillance strategy. A median time to full neutrophil recovery of 20 days after this conditioning approach, particularly as no GCSF was used, is additionally helpful in abrogating some of the excess risk of fungal disease during prolonged cytopenia.

Relapse is the largest barrier to long-term positive outcomes however, with a 1- and 2-year cumulative incidence of relapse of 20.96% and 29.51% respectively, leading to a 1- and 2-year RFS of 69.9% and 61.7% respectively. Predictors of relapse include the CIBMTR DRI high category, adverse risk AML and MDS, high graft T cell content, graft cryopreservation and ABO mismatch. The association of disease risk groups and relapse risk is perhaps unsurprising; however, the contribution of graft-related variables is particularly interesting. The impact of graft cryopreservation has been highlighted by our research group previously, and particularly the risk of early relapse within a year of transplant. The recurrence of this finding in this group of homogeneously treated patients, even with modest numbers, supports this association and underpins the continued national preference for fresh PBSC grafts. T cell content in cryopreserved grafts was higher, despite a standardized mobilization, transportation, and sampling technique, yet in multivariable analysis, higher graft T cell content demonstrated a strong independent association with relapse risk. In both univariate and multivariate modelling, the impact of excess graft CD3⁺, CD4⁺, CD8⁺ and CD4⁺/CD8⁺ T cells was clear. Further characterisation of these T cells to identify the proportion of naïve alloreactive cells would help elucidate this finding. That ATLG exposure may play a role in relapse due to blunting of the GVL effect, and that persistence of ATLG may occur with reduced *in vivo* T cell levels during its peak plasma levels was a hypothesis tested in this study. If this assumption were true, low graft T cell content could similarly result in an increased relapse risk due to diminished ATLG adsorption and thus a longer plasma half-life. However, the inverse was identified and was consistent across both primary T cell subtypes and ATLG dose intensities. Donor regulatory T cells aid in successful engraftment, however the activity of new progeny in the recipient bone marrow niche and tumour microenvironment is likely to have complex time and context-specific relationships. The role for cytokine signalling and receptor SNPs during this transition period are likely of similar consequence. Variability in ATLG half-life may have implications in GVL strength, but a deterministic role is likely minor and influenced by additional variables including secondary immunosuppression and would best be aided by *in vivo* pharmacodynamic studies. The characterization of graft and *in vivo* T cell subpopulations by bespoke flow cytometric analysis is the subject of an ongoing tandem research project at our institution in this same patient group and may provide further insights into the phenomenon.

The protective effect of chronic GVHD against relapse replicates that of many other series, but also underpins the importance of balancing prophylaxis with toxicity. While ATLG appears effective at preventing chronic GVHD, with a cumulative incidence of 17.3% at 2 years, this may ultimately be detrimental to relapse risk. Adopting an approach that balances chronic GVHD with relapse is complicated by the potential to induce excess mortality from extensive chronic GVHD itself and the treatment thereof, but also a counterbalancing a reduction in relapse-related mortality. The optimal modality and dosing of GVHD prophylaxis therefore is unclear and a one-size-fits-all approach to dosing is destined to result in suboptimal outcomes for some. We attempted to replicate the bespoke dosing schedules utilized in the paediatric setting for ATG, however we have been unable to confirm *in vivo* lymphocyte counts as a modulator of ATLG persistence in this adult population and a rational prospective clinical trial examining stratified ATLG dosing is required (287, 298).

Acute GVHD incidence was high at 60.34% and a 6-month and 1-year cumulative incidence of 37.71% and 47.72% respectively. Nevertheless, the burden of grade III to IV disease was modest at a peak cumulative incidence of 11.21%. Only a small number of patients developed steroid-refractory or poorly responsive disease and required additional immunosuppression, which is of particular benefit in an older patient group, where many such treatments can have significant off-target effects. Predictors of grade II and above disease included choice of calcineurin inhibitor, calcineurin therapeutic drug levels within the first month of transplant, non-AML disease subtype, use of an unrelated donor and younger donor age. The association of increased risk in those who were prescribed tacrolimus instead of ciclosporin is likely partially explained by the trend toward the use of tacrolimus with unrelated donors. Additionally, some patients with fragile renal function were treated with tacrolimus rather than ciclosporin and were therefore more likely to experience dose interruptions to preserve renal function. The association of risk with early erratic drug levels likely reflects dose interruptions for toxicity and the attendant rebound subtherapeutic trough levels thereafter. The association of high grade acute GVHD with high-risk myelofibrosis contribute to the excess non-relapse mortality seen in this patient group and endorses strategies to optimize disease burden pre-transplant. Younger donors were associated with unrelated donation, and the impact of this variable on increased acute GVHD risk is likely a manifestation of the known increased risk in alloSCT with unrelated donors. Chronic GVHD rates were surprisingly low at a peak cumulative incidence of 17.3%, although subtle limited chronic GVHD may have been underreported historically. The low incidence persists even in the most recent cohort of patients however, where documentation was robust. Predictors of chronic GVHD risk included two known risks: prior acute GVHD and alloSCT with female donors for male recipients (22).

The protective effect of pre-emptive DLI in mixed donor chimerism justifies our institutional policy and programme, particularly given the frequency of mixed donor chimerism beyond day 90, seen in 51.5% in this study. In addition to the positive effect on relapse risk, we noted a low burden of toxicity post-DLI, with no grade III or above acute GVHD, chronic GVHD or NRM events in this patient group. Interval chimerism analysis offers an objective measure of efficacy, which was confirmed in this study, as most patients experienced a rise in donor chimerism proportion following DLI. DLI appears well suited to the RIC setting, where doses can be safely collected during stem cell harvest and stored in an anticipatory manner. In most cases from this group the pre-harvested doses were sufficient and only a small number of donors were requested to undergo a further apheresis procedure. Administration is minimally time and resource intensive, being suitable for outpatient delivery and monitoring.

The ability of the EASIX score to predict a range of outcomes in this group of patients lends further strength to this increasingly used and simple prognostic system. Using rounded cut-offs at 4 distinct time-points we were able to identify a statistically significant correlation with survival, severe GVHD onset and relapse. The pre-transplant time-point was particularly informative, where a score above 3 was associated with future mortality, non-relapse mortality and grade III-IV acute GVHD. This score is easily incorporated into the pre-transplant assessment in tandem with the HCT-CI and KPS. At day 0, at a higher cut-off of 15, the score correlated with subsequent high-grade acute GVHD, which together with the pre-transplant score may help identify excess risk. At the onset of GVHD the score was predictive of mortality, again at a cut-off of 3 and at 1 year the score predicted lower long-term survival above a score of 1, in addition to an increased risk of relapse above this threshold.

Serial cytokine measurement was technically feasible with modification of the biobanking process in place at our institution. The collection, processing, storage, and analysis pathway was reproducible and robust, with study enrolment offered to all patients undergoing this form of reduced intensity transplant within the recruitment and observation period. The laboratory techniques are amenable to further streamlining within a clinical laboratory, and semi-automated and validated technologies are available for some of the analytes included. Although our samples were frozen and stored prior to analysis, it is conceivable that real-time analysis of a range of cytokines could be performed with meaningful turnaround times to guide transplant monitoring contemporaneously. Each of the pre-selected cytokines analysed offered an interesting insight into secretion patterns, the relationship between those different cytokines, and their effect on clinical outcomes. While the numbers of patients were limited by the duration of the recruitment period, we were able to recruit sufficient patients to analyse almost 250 distinct samples in 32 patients.

The results from IFN γ testing were robust, with quantifiable levels in most samples. The kinetics of this cytokine show an interesting peak and trough pattern, with elevated recipient levels pre-transplant, followed by early suppression after conditioning and ATLG, leading on to a peak level shortly after engraftment. The pre-transplant minor peak alludes to a degree of inflammation in those arriving at transplant, possibly provoked by cumulative treatment effect and its attendant complications. The major peak around day 28 is likely to reflect early donor inflammatory stimulation and sets the stage for induction of GVHD (445). While IFN γ is primarily pro-inflammatory, it does have pleiotropic effects, and this was most noted in that those with higher levels at the day 14 time-point were less likely to develop grade II-IV acute GVHD. No lung GVHD events were seen in this group, and therefore the protective effect of this cytokine in that organ were not assessable. The association between IFN γ and graft rejection was similarly not assessable as no complete rejection events occurred, however it is noteworthy that in the case of primary platelet engraftment failure, IFN γ levels were significantly higher than median.

A correlation between death and raised IFN γ levels pre-transplant and to a lesser degree at day 28, while likely multifactorial, points to an association of inflammatory processes and mortality, be it due to underlying disease or treatment complications. A recurrent pattern of IFN γ peaks shortly before onset of clinical acute GVHD was seen, which could translate into use as a predictive marker. GVHD site specific patterns were noted early in transplant, with reduced levels pre-transplant in those who developed skin acute GVHD and higher levels at day 0 in those who went on to develop gastrointestinal disease. At most time-points IFN γ had a negative correlation with chronic GVHD risk, but the association was weak and needs exploration in larger numbers.

IL-1 β showed less variation at serial time-points although trended towards a gradual increase over time. Secretion was low and resulted in non-quantifiable levels in some samples at each time-point. IL-1 β secretion was increased pre-transplant in those who went on to relapse, but also those who developed high grade acute GVHD. This finding mirrors the known association of IL-1 β with acute GVHD, but also appears to confirm its inability to stimulate a GVL effect. An increased IL-1 β level at day 0 and 7 was seen in those who subsequently developed gastrointestinal acute GVHD, which may have a predictive application. A weaker association with future chronic GVHD risk was then seen at day 100, although the median time to chronic GVHD onset was 280 days, with a range of other determinants potentially occurring between these two points.

IL-2 secretion patterns showed greater variation than IL-1 β , primarily driven by an early peak at day 0, but with fewer quantifiable levels at each time-point overall. An association with increased secretion and mortality at days 28 and 60 was evident. Higher levels did not appear to correlate with excess acute GVHD. In fact, those with lower levels of IL-2 around at these time-points were noted to trend towards an increased risk of grade II-IV acute GVHD. However, in those who developed liver GVHD, excess levels at day 0 were noted and may have predictive potential for this troublesome form of acute GVHD.

IL-4 secretion did not vary significantly through transplant and did not correlate strongly with clinical outcomes. However, in most instances, trends in association showed a negative correlation with GVHD, alluding to the pleiotropic nature of this cytokine. In those who developed liver acute GVHD, reduced IL-4 levels were noted at day 60, which further strengthens this pleiotropism, likely driven by Th1 suppression. IL-6 by contrast showed variation over time, with a rise from pre-transplant to day 28 followed by a reduction back to pre-transplant levels by day 100. In tandem with several other cytokines, increased IL-6 levels in the pre-transplant phase correlated with reduced survival. The proinflammatory nature of IL-6 is further demonstrated by the association of increased risk of high grade acute GVHD in those with raised levels between day 60 and 100. Pre-transplant IL-6 levels were low in those who developed acute GVHD of the skin and this was furthermore true at day 60 skin, but the inverse in gastrointestinal GVHD. This has both prognostic and therapeutic implications as a readily targetable cytokine with tocilizumab.

IL-10 levels were robustly quantifiable and displayed variability over time, with notable peaks at day 0 and day 28. Despite its anti-inflammatory nature, higher levels were found in those who developed high grade acute GVHD at the day 28 and 60 time-points. It is unlikely that IL-10 is a causative agent in these instances and the higher levels are likely to be a homeostatic counterbalance to the concurrent high levels of pro-inflammatory cytokines seen at these time-points. IL-12p70 levels showed a trend towards increase over time, and at the late time-point of day 100 were associated with an increased risk of death and subsequent relapse. IL-17A levels conversely peaked at the early time-point of day 0, but were again associated with death and relapse, in a bimodal distribution at the day 7 and 100 time-point respectively. The strong association between IL-17A levels and subsequent gastrointestinal GVHD at the day 0 time-point may represent early conditioning induced secretion from mucosal MAIT cells in the setting of inflammatory mucositis.

Secreted levels of IL-33 were the lowest of all cytokines tested, with quantifiable levels detected in only 40 of 244 samples and it is therefore difficult to draw meaningful conclusions from clinical correlation. Small variation in TNF α secretion was seen over time, with an early peak at day 0. A weak association between low levels early in transplant and later chronic GVHD development was seen, however the time differential between these two events was wide and subject to intervening confounding factors.

Discussion – over 70s

AlloSCT was offered on a case-by-case basis to those over the age of 70 for the first time during this study period. It is important to consider the inherent selection bias when assessing outcomes in this small group of patients. It is objectively clear that the fitness of patients referred for consideration of alloSCT in this age group tend to be higher than average, and

many of their age-comparators with similar diseases are not even referred due to pre-existing frailty. Additionally, of those referred and reviewed, those who then proceed to transplantation typically pass a range of pre-transplant screening and filtering. This is manifested in the higher KPS and lower age-adjusted HCT-CI scores. However, this is not a universal feature and the age-adjusted HCT-CI in this group included a range of up to 5. These patients required extra resources in the pre-transplant setting, with specialist geriatric assessment of frailty and fitness and frequently additional pre-transplant clinic reviews prior to admission for conditioning. While the burden of these interventions is shared by referring treatment centres, it does incur time costs within the alloSCT programme, particularly when treatment slots and transplant beds are in ever increasingly high demand.

Nevertheless, the objectively positive outcomes seen in these patients to date are encouraging of continuing this practice and provides incentive to expand access and attract resource investment for potentially curative and life lengthening treatment for older patients. It was notable that these patients did not require significant additional resources beyond commencement of conditioning as compared to other patients, with similar length of stay, post-transplant follow-up and interventions. With no mortality or relapse events seen in those over the age of 70 in this study at 561 days of follow-up, despite the inclusion of high-risk diseases, it appears that the FluBu3ATLG protocol can provide effective treatment without excess mortality risk for some older patients. Although limited by small numbers, the cumulative incidence of transplant-related toxicity in the form of high grade acute GVHD and chronic GVHD was no higher in this group and similarly there was no signal of increased opportunistic infections or organ dysfunction. Interestingly, time to engraftment of both neutrophils and platelets appeared significantly shorter in the oldest patients, which may help to limit some of the toxicity incurred by prolonged cytopenia. The primary detrimental finding identified for these patients was a notably lower CD4 cell reconstitution at all time-points post-transplant as compared to younger patients, which warrants further follow-up in a larger dataset. The patterns of serial cytokine secretion and the trends in EASIX levels were broadly age-agnostic and appear as valid in this older age group as in younger counterparts.

Conclusions

Strand 1: Aging and frailty, toxicity and the efficacy of reduced intensity conditioning

The expansion of the cohort presented in chapter 2 with an additional 32 subsequent patients confirms the continued growth in number and age profile of reduced intensity alloSCT for myeloid disease within the Irish system. Demand continues to be high, and with the newly included group of patients over the age of 70, the resources necessary to match that demand are growing. In the absence of alternative curative therapies, this trend is expected to align with the natural demographic shifts within the country. Age limit expansion has helped to formalize referral pathways, peri-transplant health optimization and builds upon important relationships with age-related health care specialists, who have proven increasingly important in ensuring access to alloSCT for appropriate patients. Objective assessment of frailty is now an important element of the pre-transplant review, and the validation of those assessments is an area of ongoing research interest. Similarly, risk scores and biomarkers of frailty have a

role in informing these frequently difficult decisions and allow the provision of more accurate and personalized appraisals to potential transplant candidates. Younger patients additionally stand to gain from this evolving process through enhanced awareness of toxicity and co-morbidity management, neither of which are confined to the over 70 age group.

It is clear from this expanded dataset that the FluBu3ATLG platform is a safe and effective conditioning regimen for older patients with myeloid disease, with a modest treatment induced mortality risk of 12.13% by 2 years and a manageable cumulative toxicity profile. Near universal primary engraftment rates and swift time to both neutrophil and platelet engraftment are reassuring. Overall survival of over 70% at 2 years offers a realistic chance for long-term positive outcomes, with especially low rates of severe acute GVHD and life altering extensive chronic GVHD. Importantly, in all domains assessed, outcomes were not significantly impacted by increasing age either in terms of clinical response, complications, or biological markers. There is, however, room for improvement, with scope to reduce the almost 30% relapse risk by 2 years post-transplant and increase timely immune reconstitution.

Strand 2: The donor and the graft

The right choice of donor in this reduced intensity setting is important, and frequently involves decisions between younger unrelated donors, with the attendant increased risk of GVHD and longer lead-time, or older siblings, where donor health and mobilization concerns are balanced by ease of access. Continued use of sibling donors in almost half of alloSCTs in this series ensures timely access to alloSCT for many patients and allows a degree of flexibility in the management of transplantation workload. It appeared in this dataset that donor age does not significantly determine relapse-free survival as both overall survival and relapse was donor age-agnostic. Use of a younger donor translated to an increased risk of acute GVHD, but this effect was likely biologically driven by the unrelated status of these donors rather than the age profile. The results of this study therefore do not strongly endorse the preferential use of younger donors using the FluBu3ATLG RIC conditioning regimen in older patients with myeloid disease.

Following correlation analysis of graft T cell content and clinical outcomes, an association between higher graft T cells and relapse was seen. The mechanism of this interaction is unclear although a further association with graft cryopreservation was evident. This corroborates previous findings at our institution highlighting an increased risk of relapse in those receiving cryopreserved stem cell grafts. The impact of high graft T cell content on relapse persists even amongst those who received fresh stem cell grafts and is an independent predictor for relapse. The preference for fresh stem cell grafts in our institution is supported by these data and further investigation into the kinetics of graft T cell interactions by flow-cytometric profiling is endorsed.

The recurrently poorer outcomes noted in those with ABO mismatch, in both minor, major or bidirectional form is a recurring finding throughout both phases of this study and supports the evidence of a similar effect in other series. The practicalities of adjusting for this variable are considerable, with almost half of patients receiving stem cell grafts from an ABO mismatched donor. Where multiple donors are available the weight of this finding should be considered. Conversely, the importance of CMV matching appears to have diminished in the era of effective CMV prophylaxis with letermovir, which appears to be well tolerated and its

continued use is supported by these data. The hierarchical matching of donor and recipient attributes is, as ever, an evolving conundrum that is subject to the many vagaries of retrospective and registry studies, being subject to temporal and contextual influences and should be interpreted in such a light.

Strand 3: Application of the EASIX score

We interrogated the EASIX score at several critical transplant time-points, building upon supporting published evidence. The score is an attractively simple adjunct to transplant monitoring and decision support and importantly is universally calculable and serially quantifiable. In this homogenous treatment population, we confirmed the robust predictive power of the score to anticipate non-relapse mortality and death at the pre-transplant time-point. We furthermore showed an expanded utility in identifying those at risk of severe acute GVHD. The day 0 time-point additionally offered an opportunity to identify those at risk of severe GVHD. We further confirmed the increased mortality risk in those with high scores at the time of acute GVHD onset, and again at 1-year post-transplant. The one-year post-transplant time-point showed a correlation between higher scores and subsequent relapse. As a marker of endothelial dysfunction, the score is particularly relevant in transplant medicine, where the endothelial interface is a key site of inflammatory interactions between graft, donor, and disease. For death and relapse, this score captures peripheral blood hallmarks of myeloid disease activity and threatened or impending relapse in the form of thrombocytopenia and rising LDH and is thus well placed in a myeloid-specific transplant programme. We endorse the recording of this score at these 4 time-points in standard practice, which in longitudinal analysis may strengthen its predictive capabilities.

Strand 4: The role of ATLG and its impact on relapse and immune reconstitution

Both the role and dose of ATG or ATLG in the reduced intensity conditioning setting is an area of ongoing debate and review. Comparisons between international series are compromised by a wide range of doses, schedules, and formulations. Due to differing immune stimulation source and manufacturing, the two most common ATG/ATLG formulations (Grafalon®/Thymoglobulin®) have variable lymphocyte specificity, pharmacodynamics, and pharmacokinetics. Although the formulations are known to share many attributes, evidence for bespoke dosing with Thymoglobulin® is mounting and there is therefore a need for confirmation with Grafalon®. There are challenges with ATG/ATLG delivery including acute toxicity and longer-term toxicity, driven by a long and variable half-life, which make alternatives such as PTCY an attractive option. Troublesome viral re-activation, primary opportunistic infection, and a potential increased risk of relapse due to prolonged T cell suppression and blunting of the GVL effect are areas in need of optimisation. Nevertheless, excess drug-related mortality is offset by a reduction in life-threatening high grade acute GVHD and extensive chronic GVHD. ATG/ATLG alternatives have not shown superiority in non-randomised trials in this setting, and randomised studies are ongoing. The risks of alternative modalities, in particular PTCY, must be carefully considered in older patients, as signals for opportunistic infection and cardiac toxicity may be prohibitive.

We report significant longitudinal experience using ATG-F/Grafalon® with standardized dosing over a long period. We did not identify a significant difference in relapse or CD4 recovery based on ATLG dose in this cohort of patients, although there was an increased risk of acute GVHD in those who received the higher dose in keeping with the associated use of unrelated donor stem cell grafts. We could not confirm lymphocyte volume as a determinant of variability in ATLG persistence, as neither lymphocyte count pre-conditioning or at the time of ATLG exposure correlated with clinical outcomes. A combination of lymphocyte volumes in both recipient and in the donor graft, which act as the substrate for serum unbound ATLG, in addition to recipient weight may together determine *in vivo* ATLG longevity but requires an exploratory therapeutic drug monitoring study to confirm this hypothesis in older adults exposed to ATG Grafalon®. Ongoing studies of the role of PCTY in this HLA-matched setting may challenge the role of ATG/ATLG in the future, but in the absence of demonstrable superiority, continuing endeavours to optimize the current ATLG approach by dose modification are justified.

Strand 5: DLI in reduced intensity transplantation

The success of this conditioning platform appears to depend in part on the rational application of DLI. Mixed donor chimerism beyond the third month of transplant is common, and without a meaningful means to modulate this, higher rates of relapse would likely occur. The strongest appeal for liberal DLI use seen in our dataset is seen in those with mixed chimerism, after weaning immunosuppression, who receive a pre-emptive DLI before relapse occurs. We have shown that relapse rates are significantly lower in this patient group as compared to those who did not receive a DLI. The additional benefit of DLI in extending survival in those who have relapsed is encouraging of a post-transplant combined treatment approach including hypomethylating agents and interval dose escalated DLI. Prophylactic DLI use in those with full donor chimerism, but with high-risk disease, is a potential additional area of utility with further prospective studies awaited. In this older and frailer patient group, the success of DLI is critically dependent on a low toxicity profile. With a third of the patient population receiving a DLI, it is encouraging that no acute GVHD events higher than grade II occurred post-DLI, nor were there any non-relapse mortality events post-DLI. By using a dose-escalation and chimerism-guided approach, the application of DLI, including in those over the age of 70 appears safe, effective and is operationally feasible.

Strand 6: Cytokine analysis in practice: potential and limitations

Serial cytokine analysis is feasible and conceivably adoptable in future routine clinical practice. The results of these analyses offer insights into the evolution of secretion over the transplant process and highlight the waxing and waning inflammatory and anti-inflammatory signalling driving clinical outcomes such as GVHD, relapse and survival. Patterns of cytokine secretion can potentially be used to predict several transplant outcomes, including GVHD, relapse and death. IFN γ levels peaked prior to GVHD onset in most cases and furthermore demonstrated specific association with gastrointestinal GVHD in excess at the day 0 time-point. Increased mortality in those with high IFN γ levels pre-transplant extend the potential predictive role for this cytokine. IL-1 β shows an association with death and relapse in excess at early transplant time-points, and furthermore has applicability in identifying patients at risk of gastrointestinal

GVHD at similarly early time-points. High IL-2 levels early in transplant are associated with liver acute GVHD while middle-transplant excess correlates with inferior survival. Reduced IL-4 levels at 2 months post-transplant are associated with liver acute GVHD and IL-6 at the pre-transplant and day 60 time-points associates with reduced survival and a lower risk of skin GVHD. Later IL-12p70 levels at day 100 associate with subsequent risk of death and relapse, as do IL-17A levels at this time-point. Increased IL-17A secretion early in transplant heralds subsequent gastrointestinal acute GVHD and has predictive potential.

Important limitations to consider in this research strand include the very nature of cytokines themselves. Many display pleiotropic effects, showing both positive and negative associations with given outcomes depending on the context and timing of analysis. Furthermore, interpreting the results of a singular cytokine must be considered in the context of other cytokines and cellular profiles in addition to extrinsic factors such as immunosuppression and disease activity. The numbers of patients in this study limits the strength of any associated statements, nevertheless, the trends and patterns herein described are encouraging of continued effort to develop a targeted, cytokine-inclusive biomarker panel. The validation of this approach will require further study in a larger data set with considerations of the optimisation of costs and resource utilisation.

Future directions

The results of this study support the continued use of the FluBu3ATLG platform as a safe and effective conditioning modality for older patients with myeloid diseases. The combination of a modest toxicity profile and robust long term survival rates remain attractive when compared to alternative approaches, and with long institutional experience, provides a local benchmark against which alternatives can be judged. The principle aims and hypotheses of this study have interrogated a range of domains including transplantation in older patients, the impact of graft characteristics on outcome, the role of ATLG in immune reconstitution, relapse and infection, the predictive power of the EASIX score in RIC alloSCT, the importance of a rational approach to DLI and the interplay and clinical correlation of cytokine secretion over time.

Further assessment of larger numbers of patients over the age of 70 is required to confirm the positive outcomes experiences so far with this subgroup of patients but early results are promising. A formalized pre-transplant assessment pathway with enhanced decision support from objective measures of frailty including serial grip strength manometry, timed up and go testing, scoring systems such as the Edmonton Frail Scale, and expert age-related physician input will help cater to the increasing demand for definitive therapy in this age group. Future validated transplant-specific frailty tools may help capture those patients who need additional inputs in an objective manner and ultimately allow for accurate longitudinal analysis of outcomes in this patient group.

The impact of various donor and recipient paired characteristics on outcome is an area of debate spanning the history of alloSCT. Although some variables are static such as ABO matching, others have become dynamic such as CMV matching due to access to therapies to modify risk. The hierarchical components of donor selection need support from an appropriate evidence base but also need to be pragmatic to ensure timely and appropriate access to donors. Recurrent findings at our institution of an increased risk of relapse in those who received cryopreserved stem cells is of concern and reinforces the default preference for

fresh cells with minor exceptions. Further correlation of outcome with the graft T cell content is required and this data is currently being collected for all transplant types within the programme, which will allow for future in-depth study of the impact of this variable. Although it appears ABO mismatch has a particularly deleterious effect in this dataset, this finding must be couched in the conflicting findings in this area in other case series, and the practical limitations of balancing competing pre-transplant variables must be considered. Further interrogation of large registry data to determine the weighted importance of these variables is required given the recent access to letermovir prophylaxis, PTCY-based GVHD prophylaxis in HLA mismatch and signals of a survival advantage in alloSCT performed with younger donors (183). No change to current practice can be recommended by these data but does offer a local context in the decision process.

These data support the design of a dose stratified prospective clinical trial for ATLG in combination with FluBu3. Although we were unable to identify specific determinants of ATLG exposure in this dataset, the retrospective and observational nature of this assessment may mask an underlying effect. At both the 60mg/kg and 30mg/kg dose level, there does not appear to be a significant difference in outcomes. Although acute GVHD incidence is high, moderate to severe disease and chronic GVHD have a comparatively low cumulative incidence and disease-related outcomes in those with chronic GVHD are superior. There is scope for investigation of lower dose ATLG within a formal randomized clinical trial, particularly in those with a matched sibling donor and those over the age of 65, where increased relapse is seen, although juxtaposed with lower high-grade acute GVHD and chronic GVHD. This may allow for more robust and timelier immune reconstitution and lower relapse risk by augmenting the GVL effect. An associated therapeutic pharmacokinetic and pharmacodynamic observation study would help identify variability in ATLG persistence and allow for more direct comparison with the paediatric studies in this area, with a goal of creating an adult dosing nomogram to guide individualized ATLG doses.

The EASIX score has shown predictive capabilities at multiple time-points and these data support its routine calculation pre-transplant, at day 0, at onset of GVHD and at 1-year post-transplant. Further correlation with larger numbers of patients and across additional disease types, age-groups and conditioning intensities will help solidify its role in transplant surveillance. Although not designed to directly guide treatment decisions, it offers objective time and patient-specific risk assessment. Over time, and with larger numbers, the score may correlate with additional outcomes or demonstrate predictive capabilities at additional transplant milestones and should be regularly reviewed.

Guided DLI use is encouraged by the findings of this dataset. For pre-emptive DLI, the timing of administration is variable as it must account for immunosuppression cessation, patient clinical status and chimerism testing turnaround time. Therefore, a degree of flexibility is necessary, however a formalized framework could support the decision-making process. The sequential dose escalating scheme utilized for these patients is safe, reduces the risk of relapse and these data support this current approach without modification. In relapsed patients, a clear survival benefit supports the current strategy of sequential dose escalated DLI, often in combination with disease-specific treatment. The duration of such treatment and number of DLI infusions however needs further review. Prophylactic DLI is an attractive approach for relapse-prevention and although the small numbers in receipt of such a DLI in this patient group had a positive outcome, international results are required before recommendation of a standardised approach.

Cytokine profiling offers an intriguing insight into the balance of inflammation and immune activation through the transplant process. The temporal correlation of cytokine secretion and clinical outcome needs further investigation, particularly using clinically available analytes and technologies. The levels of IFN γ , IL-1 β , IL6, IL-12p70 and IL-17A show the strongest associations with clinical outcomes and are thus candidates for narrower interrogation in future patient groups. The most significant time-points from this dataset appear to be the pre-transplant, day 0, day 28, day 60 and day 100 time-points, and in addition to a smaller panel of cytokine targets, reducing the sampling time-points to these key dates would yield the highest resource efficiency. With a focus on a smaller panel of cytokines there will be reagent cost savings and a reduction in analysis time. Further treatment groups should also be considered in the next phase of analysis, in the first instance including other non-FluBu3ATG based conditioning platforms in RIC alloSCT for myeloid disease, and potentially RIC alloSCT for lymphoid disorders. Additional targeted analysis of cytokines in DLI products, *in vivo* pre-infusion and post-infusion may elucidate the complexity of DLI and further interrogate heterogeneity of outcomes. If the initial findings here are reproduced, cytokine analysis at defined transplant time-points may offer an opportunity to prognosticate and ultimately support treatment decisions in clinical practice.

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Appendices

Appendix 1: Patient information leaflet and consent form



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Coláiste na Tríonóide, Baile Átha Cliath
The University of Dublin

PARTICIPANT INFORMATION LEAFLET

Biobanking of blood, bone marrow and lymph node samples for future research studies

Principal Investigators:

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Co-investigator:

Dr Tony McElligott, Assistant Professor, Trinity Translational Medicine Institute, St James's Hospital, Dublin 8. Email: tony.mcelligott@tcd.ie

You are being invited to take part in a research study to be carried out at St James's Hospital. Before you decide whether or not you wish to take part, you should read the information provided in this leaflet carefully. Take time to ask questions – don't feel rushed or under pressure to make a quick decision. You should understand the risks and benefits of taking part in this study so that you can make a decision that is right for you. You may wish to discuss it with your family, friends or GP.

PART 1 – THE STUDY

Why is this study being done?

Before a new treatment or test is used in hospitals, it is thoroughly tested to ensure that it works and is safe to use. Part of this testing involves studying tissue samples donated by patients. Such donated samples are also important for researchers who want to understand how and why diseases develop. A collection of tissue samples donated by patients is called a biobank. The St James's Hospital Haematology Biobank is a biobank that collects tissue samples donated by patients who may have a blood disorder.

Why am I being asked to take part?

We are inviting you to take part in our biobank because you have been diagnosed with a blood disorder.

Do I have to take part? What happens if I say no? Can I withdraw?

You don't have to take part in this study. If you decide not to take part, it won't affect your current or future medical care. You can change your mind about taking part in the study and opt out at any time even if the study has started. If you decide to opt out, it won't affect your current or future medical care. You don't have to give a reason for not taking part or for opting out. If you wish to opt out, please contact Dr Tony McElligott (01-8964864; email: tony.mcelligott@tcd.ie) who will be able to organise this for you.

How will the study be carried out?

Our biobank collects and stores **biological samples (blood, bone marrow and lymph node samples) and health information (data)** for use in future studies.

Our biobank aims to provide samples and health information (data) to researchers to help increase our understanding of blood disorders and to develop new tests and treatments. This is an ongoing study that will collect biological samples and data over many years.

What will happen to me if I agree to take part?

If you agree to take part, we will ask you to sign the **Biobank Consent Form**. We will give you a signed copy of your Consent Form and this Patient Information Leaflet to keep.

During your routine procedure we take extra samples of:

- **blood** - about two tablespoons (20mls)
- **bone marrow aspirate** - about two tablespoons (20mls) (where appropriate)
- **lymph node tissue** - additional lymph node biopsy (where appropriate)

You will not need extra procedures. We only take samples during your routine blood test, bone marrow biopsy or lymph node biopsy.

Are there any benefits to me or others if I take part in the study?

It is very unlikely that you will directly benefit from the research, because research takes a very long time. But an organised biobank should speed up this process. We hope your samples, along with many others, will be included in research that will help future patients. You will not get any money should research lead to a new test or treatment.

Are there any risks to me or others if I take part in the study?

No major physical risks are associated with donating samples to the biobank. If you decide to take part, you are agreeing to give an extra two tablespoons of blood and/or two tablespoons of bone marrow to the biobank. The extra lymph node biopsy sample is only

taken where appropriate. The blood, bone marrow and biopsy samples will only be collected during procedures that are part of your standard care. You will not require extra procedures. Taking part can carry a very small risk of accidental disclosure of data. This means that someone outside the research team could find out that you donated to the biobank. However, the research team will take steps to protect your privacy and minimise any risks.

Will I be told the outcome of the study? Will I be told the results of any tests or investigations performed as part of this study that relate to me?

It is very unlikely that the results of the research will have any impact on your current treatment, and you will not be informed about these results. However, if results become available that are unexpectedly relevant for you or your family, the Biobank will use reasonable efforts to inform your doctor so that s/he may discuss further options with you, as appropriate.

PART 2 – DATA PROTECTION

What information about me (personal data) will be used as part of this study? Will my medical records be accessed?

If you agree, we will collect your data from different databases in St. James's Hospital and store it in the biobank. This is because St. James's is the hospital where your blood, bone marrow and lymph node tissue will be tested and diagnosed. The data that will be collected will be limited to demographic data (age, gender, ethnicity) and disease specific data, such as type of blood disorder, what type of sample has been collected (i.e. diagnostic, monitoring, relapse), number and types of treatments you have received and the results of various diagnostic tests (e.g. histopathology, immunophenotyping and molecular).

We will store your information in two ways:

- **CODED DATA** (pseudonymised): anything that could identify you, such as your name, address or date of birth is removed, so researchers cannot link you to your samples. Your data will be coded by a dedicated Biobank scientist.
- **NON-CODED DATA**: researchers will be able to link your samples to you. This is because we link you to both your health information and samples.

What will happen my personal data?

Personal data will be processed **only as is necessary** to achieve the objective of Biobank and will not be processed in a way that damage or distress will be caused to you. This is an ongoing study that will collect and retain biological samples and data over many years.

Your CODED DATA will be stored on a Biobank Information Management System (database) and a Biobank Web portal. The Biobank Web Portal is an online catalogue where researchers search for samples.

Who will access and use my personal data as part of this study?

Only **hospital-approved staff**, within St. James's Hospital, may access and process your NONCODED DATA. Hospital-approved staff are approved researchers who work directly for clinicians at St. James's Hospital. Hospital approved staff may read and link your health information (data) from hospital charts, electronic patient records and databases, while doing their work on your samples. Researchers outside St. James's Hospital will only receive CODED DATA and will not know who you are. Your data may be processed to design studies or understand experimental results.

We will also give the National Cancer Registry Ireland (NCRI) your biobank code. The NCRI is a state appointed body which collects data on all cancer patients in Ireland.

The sharing of data allows the NCRI to identify you on their database and give the biobank information that is relevant to your samples. The NCRI will only pass on information about your blood disorder using this anonymous code. It will not pass on any information that would identify you.

Will my personal data be kept confidential? How will my data be kept safe?

We give your samples and data a unique code. This makes it difficult to identify who you are.

Only hospital-approved staff, within St. James's Hospital, may access your NON-CODED DATA. Hospital-approved staff are approved researchers, who work directly for clinicians at St. James's Hospital. Your NON-CODED DATA will be stored on secure databases in St James's Hospital.

We will not give any information that could identify you to researchers outside St. James's Hospital. Researchers from outside St. James's Hospital, will only receive CODED DATA. We will leave out anything that would identify you. In the course of processing your CODED DATA it may be transferred outside of the European Economic Area (EEA) in accordance with legally approved mechanisms to lawfully transfer data across borders.

The Biobank Web Portal will only show CODED DATA, so researchers will not know who you are. The NCRI will only pass on your CODED DATA. This will not identify you.

What is the lawful basis to use my personal data?

The lawful basis to use your personal data in this study is Article 6 and Article 9 of GDPR.

Article 6, 1 (e)– for the purpose of a task carried out in the public interest.

Article 9, 2 (j) – necessary for archiving purposes in the public interest, scientific or historical research purposes or statistical purposes.

What are my rights?

You have the following rights regarding your **data**.

- The right to access to your data and receive a copy of it.
- The right to restrict or object to processing of your data.
- The right to object to any further processing of the information we hold about you (except where it is de-identified).
- The right to have inaccurate information about you corrected or deleted.
- The right to receive your data in a portable format and to have it transferred to another data controller.
- The right to request deletion of your data.

By law you can exercise the following rights in relation to your personal data, unless the request would make it impossible or very difficult to conduct the research. You can exercise these rights by contacting your study Doctor or the St. James's Hospital Data Protection Officer, St. James's Hospital Dublin 8 at 01 410 3021 or dataprotection@stjames.ie

PART 3 – COSTS, FUNDING & APPROVAL

Will it cost me anything if I agree to take part?

No.

Who is funding this study? Will the results study be used for commercial purposes?

Science Foundation Ireland is funding this study.

We will allow researchers use your samples and data for ethically approved studies.

Researchers who can apply to use your samples and health information (data) may come from:

- hospitals;
- third-level institutions (colleges and universities);
- for profit pharmaceutical companies (companies that make drugs); and
- for profit genomic companies (companies who test DNA to identify disease related genes)

Each study must have ethical approval from a recognised hospital or university research ethics committee.

To help the biobank with costs, researchers may be charged a small administrative fee (not for profit).

Has this study been approved by a research ethics committee?

This study has been approved by the St James's Hospital / Tallaght University Hospital Research Ethics Committee.

PART 4 – FUTURE RESEARCH

Will my personal data and/or biological material be used in future studies?

We will allow researchers use your samples and data for ethically approved studies.

Researchers who can apply to use your samples and health information (data) may come from:

- hospitals;
- third-level institutions (colleges and universities);
- for profit pharmaceutical companies (companies that make drugs); and
- for profit genomic companies (companies who test DNA to identify disease related genes)

An Ethics Committee must approve each study before we will provide your samples or data. Your samples may be included in studies that investigate different aspects of blood disorders.

Future research may include research which looks to understand genetic influences related to:

- cancer growth;
- early detection;
- diagnosis;
- tests;
- treatment; and
- cancer in families

Your samples may also be used to develop new disease related tests and treatments. We cannot give the exact details of these studies, because the ideas for them have yet to be thought of.

The **data processing measures and safeguards** in existence for this study will be in place for any future studies (in addition to any future data processing regulations that may be introduced).

PART 5 – FURTHER INFORMATION

Where can I get further information?

Principal Investigator: Prof Elisabeth Vandenberghe

Consultant Haematologist

Department of Haematology

**St James' Hospital
Dublin 8**

E-mail: evandenberghe@stjames.ie

Co-investigator: Dr Tony McElligott

Assistant Professor

Trinity Translational Medicine Institute

**St James's Hospital
Dublin 8**

E-mail@ tony.mcelligott@tcd.ie

St James's Hospital is the data controller.

Research Team: Prof Vandenberghe, Dr McElligott, Hospital-approved staff (approved researchers who work directly for clinicians at St. James's Hospital).

Data Protection Officer: dataprotection@stjames.ie.

What happens if I wish to make a complaint?

Please contact Prof Vandenberghe.

Will I be contacted again?

The National Cancer Registry Ireland (NCRI) may also send you up to three short questionnaires about your quality of health and how you feel after your treatments (PROMS). We would ask you to fill in these short questionnaires and send them back to the NCRI. If you need help to fill out the questionnaires, just ask your nurse at a time that suits you both.



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Affix patient information
label here:

**Biobanking of blood, bone marrow and lymph node
samples for future research studies**

To be completed by the **PARTICIPANT**:

I have read and understood the information leaflet.	YES ☐	NO ☐
I have had the opportunity to discuss the study, ask questions about the study and I have received satisfactory answers to all my questions.	YES ☐	NO ☐
I have received enough information about this study.	YES ☐	NO ☐
I understand that I am free to withdraw from the study at any time without giving a reason and this will not affect my future medical care.	YES ☐	NO ☐
I agree to allow the researchers use my information (personal data) as part of this study as outlined in the information leaflet and that this information will be held indefinitely.	YES ☐	NO ☐
I agree to allow the researchers access my medical records as part of this study.	YES ☐	NO ☐
I give the biobank permission to share identifiable data with the National Cancer Registry of Ireland (NCRI) so that they may give additional nonidentifiable data to cancer researchers. I give permission to NCRI to send me questionnaires about patient related outcome measures (PROMS).	YES ☐	NO ☐
I agree to give a blood / bone marrow / lymph node sample (delete as appropriate) as part of this study.	YES ☐	NO ☐
I consent to take part in this research study having been fully informed of the risks, benefits and purpose of the study.	YES ☐	NO ☐
I give my explicit consent to have my data processed as part of this research study.	YES ☐	NO ☐
I give permission for my samples and coded data to be shared between hospitals, third-level institutions, for profit pharmaceutical companies and for-profit genetic companies. I agree that re-consent for the use of my samples and data in these future ethically approved studies is not required.	YES ☐	NO ☐

Participant's Name (Block Capitals):	
Participant's Signature:	
Date:	

To be completed by the **RESEARCHER**:

I have fully explained the purpose and nature (including benefits and risks) of this study to the participant in a way that he/she could understand. I have invited him/her to ask questions on any aspect of the study.	YES ☐	NO ☐
I confirm that I have given a copy of the information leaflet and consent form to the participant.	YES ☐	NO ☐

Researcher's Name (Block Capitals):	
Researcher's Title & Qualifications:	
Researcher's Signature:	
Date:	

Appendix 2: National Cancer Control Programme protocols

Fludarabine/Busulfan/ATG Grafalon® – RIC – MUD INDICATIONS FOR USE:

INDICATION	ICD10	Regimen Code	Reimbursement Status
Reduced intensity conditioning for matched unrelated donor allogeneic stem cell transplant in patients with myeloid disorders.	C92	00635a	Hospital

TREATMENT:

Conditioning chemotherapy is administered over **9 days**. Stem cells are infused on **day 0**.
Facilities to treat anaphylaxis **MUST** be present when conditioning therapy and stem cells are

Day (time)	Drug	Dose	Route	Diluent & Rate
-9,-8,-7,-6,-5,-4	Fludarabine ^a	30mg/m ²	IV infusion	100ml sodium chloride 0.9% over 30 minutes
-5,-4,-3 (10.30)*	Busulfan ^{b,c}	0.8mg/kg	IV infusion	(See note ^d) ml sodium chloride 0.9% over 2 hours
-5,-4,-3 (16.30)*	Busulfan ^{b,c}	0.8mg/kg	IV infusion	(See note ^d) ml sodium chloride 0.9% over 2 hours
-5,-4 (22.30)*	Busulfan ^{b,c}	0.8mg/kg	IV infusion	(See note ^d) ml sodium chloride 0.9% over 2 hours
-4,-3 (04.00)*	Busulfan ^{b,c}	0.8mg/kg	IV infusion	(See note ^d) ml sodium chloride 0.9% over 2 hours
NB: IV busulfan expires after 15 hours, infusion must begin at time specified				
- 3	e,f,g ATG Grafalon®	20mg/kg	IV infusion	(See note ^h) ml sodium chloride 0.9% over 12 hours
-2,-1	e,f,g ATG Grafalon®	20mg/kg	IV infusion	(See note ^h) ml of sodium chloride 0.9% over 10 hours
0	Stem cell infusion			
+1,+3,+6 (At least 24 hours post end of stem cell infusion)	Methotrexate ⁱ	10mg/m ²	IV infusion	50mls of sodium chloride 0.9% over 10 minutes
Dose rounding: Fludarabine doses ≤50mg to the nearest 2.5mg and doses >50mg to the nearest 5mg Busulfan to the nearest 1.2mg if <60mg, to nearest 6mg if >60mg. Oral busulfan available as 2mg and 25mg tablets. ATG Grafalon® to the nearest 20mg Methotrexate to the nearest 2.5mg				
^a All patients who have received fludarabine should receive irradiated blood products (lifetime recommendation).				
^b IV busulfan may be replaced with oral busulfan at the discretion of the haematology consultant. An oral dose of 1mg/kg is equivalent to the 0.8mg/kg IV dose. The dosing schedule for oral busulfan is 06:00, 12:00, 18:00, 23:59 . ^c If a problem with an infusion bag (i.e. leaking bag, short expiry) is discovered outside of 8.30am-5pm, an oral dose of busulfan 1mg/kg equivalent to the intravenous dose will be available from the MDA press on Denis Burkitt Ward. This can only be used after discussion with a haematology consultant and must be prescribed by haematology registrar or consultant on a chemotherapy prescription/NCIS. ^d Calculation of busulfan infusion solution: [(busulfan dose (mg) divided by 6) x 10] [to the nearest 10ml] NaCl 0.9% - concentration to be as close to 0.5mg/ml as possible.				
^e Patient monitoring is required during the ATG Grafalon® infusion: BP, pulse, respiration and temperature at 15, 30 and then 60 minute intervals for the duration of the infusion. ^f If an infusion reaction occurs during the administration of ATG Grafalon®, the infusion should be slowed. Chills and fever generally respond to antihistamines, antipyretics or corticosteroids. If the patient becomes hypotensive or experiences chest or back pain, indicating anaphylaxis, the infusion should be stopped and the medical team contacted immediately. ^g Platelets should be >50x10 ⁹ /L pre day 1 ATG Grafalon® treatment. If the patient has no reaction to ATG Grafalon®, platelets can be maintained at >30x10 ⁹ /L for the remaining days of ATG Grafalon® administration. Platelets should be maintained at >50x10 ⁹ /L in the setting of clinically symptomatic bleeding. ^h Each ml of ATG Grafalon® should be diluted with 6ml of sodium chloride 0.9% in accordance with SPC. Pharmacy to complete volume.				

administered.

Fludarabine/Busulfan/ATG Grafalon® – RIC – SIB

INDICATIONS FOR USE:

INDICATION	ICD10	Regimen Code	Reimbursement Status
Reduced intensity conditioning for sibling donor allogeneic stem cell transplant in patients with myeloid disorders.	C92	00636a	Hospital

TREATMENT:

Conditioning chemotherapy is administered over **9 days**. Stem cells are infused on **day 0**. Facilities to treat anaphylaxis **MUST** be present when conditioning therapy and stem cells are administered.

Day (time)	Drug	Dose	Route	Diluent & Rate
-9,-8,-7,-6,-5,-4	Fludarabine ^a	30mg/m ²	IV infusion	100ml sodium chloride 0.9% over 30 minutes
-5,-4,-3 (10.30)*	Busulfan ^{b,c}	0.8mg/kg	IV infusion	(See note ^d) ml sodium chloride 0.9% over 2 hours
-5,-4,-3 (16.30)*	Busulfan ^{b,c}	0.8mg/kg	IV infusion	(See note ^d) ml sodium chloride 0.9% over 2 hours
-5,-4 (22.30)*	Busulfan ^{b,c}	0.8mg/kg	IV infusion	(See note ^d) ml sodium chloride 0.9% over 2 hours
-4,-3 (04.00)*	Busulfan ^{b,c}	0.8mg/kg	IV infusion	(See note ^d) ml sodium chloride 0.9% over 2 hours
NB: IV busulfan expires after 15 hours, infusion must begin at time specified				
-3	e,f,g ATG Grafalon®	10mg/kg	IV infusion	(See note ^h) ml sodium chloride 0.9% over 12 hours
-2,-1	e,f,g ATG Grafalon®	10mg/kg	IV infusion	(See note ^h) ml of sodium chloride 0.9% over 10 hours
0	Stem cell infusion			
+1,+3,+6 (At least 24 hours post end of stem cell infusion)	Methotrexate ⁱ	10mg/m ²	IV infusion	50mls of sodium chloride 0.9% over 10 minutes
Dose rounding: Fludarabine doses ≤50mg to the nearest 2.5mg and doses >50mg to the nearest 5mg Busulfan to the nearest 1.2mg if <60mg, to nearest 6mg if >60mg. Oral busulfan available as 2mg and 25mg tablets. ATG Grafalon® to the nearest 20mg Methotrexate to the nearest 2.5mg				
^a All patients who have received fludarabine should receive irradiated blood products (lifetime recommendation).				
^b IV busulfan may be replaced with oral busulfan at the discretion of the haematology consultant. An oral dose of 1mg/kg is equivalent to the 0.8mg/kg IV dose The dosing schedule for oral busulfan is 06:00, 12:00, 18:00, 23:59 ^c If a problem with an infusion bag (i.e. leaking bag, short expiry) is discovered outside of 8.30am-5pm, an oral dose of busulfan 1mg/kg equivalent to the intravenous dose will be available from the MDA press on Denis Burkitt Ward. This can only be used after discussion with a haematology consultant and must be prescribed by haematology registrar or consultant on a chemotherapy prescription/NCIS ^d Calculation of busulfan infusion solution: [(busulfan dose (mg) divided by 6) x 10] [to the nearest 10ml] NaCl 0.9% - concentration to be as close to 0.5mg/ml as possible.				

^ePatient monitoring is required during the ATG Grafalon® infusion: BP, pulse, respiration and temperature at 15, 30 and then 60 minute intervals for the duration of the infusion.

^fIf an infusion reaction occurs during the administration of ATG Grafalon®, the infusion should be slowed. Chills and fever generally respond to antihistamines, antipyretics or corticosteroids. If the patient becomes hypotensive or experiences chest or back pain, indicating anaphylaxis, the infusion should be stopped and the medical team contacted immediately. ^gPlatelets should be $>50 \times 10^9/L$ pre day 1 ATG Grafalon® treatment. If the patient has no reaction to ATG Grafalon®, platelets can be maintained at $>30 \times 10^9/L$ for the remaining days of ATG Grafalon® administration. Platelets should be maintained at $>50 \times 10^9/L$ in the setting of clinically symptomatic bleeding. ^hEach ml of ATG Grafalon® should be diluted with 6ml of sodium chloride 0.9% in accordance with SPC. Pharmacy to complete volume.

Appendix 3: Laboratory techniques

Components:

- Diluent (3 and 57)
- Stop solution
- Read buffer
- 96-well plates with 10 assay spots – 6 x 9 spots and 6 x 1 spot (IL-33)
- Calibrators (1 and 6)

Additional materials:

- Tubes for reagent preparation and dilutions
- Multi-channel pipette for dispensing and washing
- Microtitre plate shaker
- Wash buffer
- Adhesive plate seals
- Deionized water
- Vortex mixer

Step 1: Overnight thaw of frozen serum aliquots in dummy 96-well plates.

Step 2: Designate MSD biotin binding linker (1-10), equilibrate diluents, wash buffer and read buffers to room temperature.

Step 3: Combine 200 μL biotinylated capture antibody to 300 μL of assigned linker, vortex and incubate at room temperature for 30 minutes.

Step 4: Add 200 μL stop solution, vortex and leave at room temperature for 30 minutes.

Step 5: Add 600 μL of each linker-coupled antibody to a single tube and vortex with additional stop solution for missing linker in plate 1 and 9 missing linkers in plate 2 (IL-33).

Step 6: Add 50 μL of multiplex coating solution to each well, seal plate with adhesive plate seal and incubate at room temperature while shaking for 1 hour.

Step 7: Wash each plate 3 times with 150 μ L/well of wash buffer.

Step 8: Reconstitute calibrator with 250 μ L of assay diluent. Dilute 50 μ L of reconstituted calibrator with 200 μ L of assay diluent. Prepare serial dilutions from 1-8 with 8 being zero calibrator.

Step 9: Dilute samples.

Step 10: Prepare detection antibody solution.

Step 11: Add 25 μ L assay diluent to each well and 25 μ L of sample or calibrator.

Step 12: Seal the plate and incubate on plate shaker for 1 hour.

Step 13: Wash plates 3 times with 150 μ L wash buffer.

Step 14: Add 50 μ L of detection antibody solution to each well, seal and incubate on plate shaker for 1 hour.

Step 15: Wash the plate 3 times with 150 μ L wash buffer.

Step 16: Add 150 μ L read buffer to each well and analyse on MSD read instrument.

Appendix 4: Cytokine expression: median, total and temporally correlated

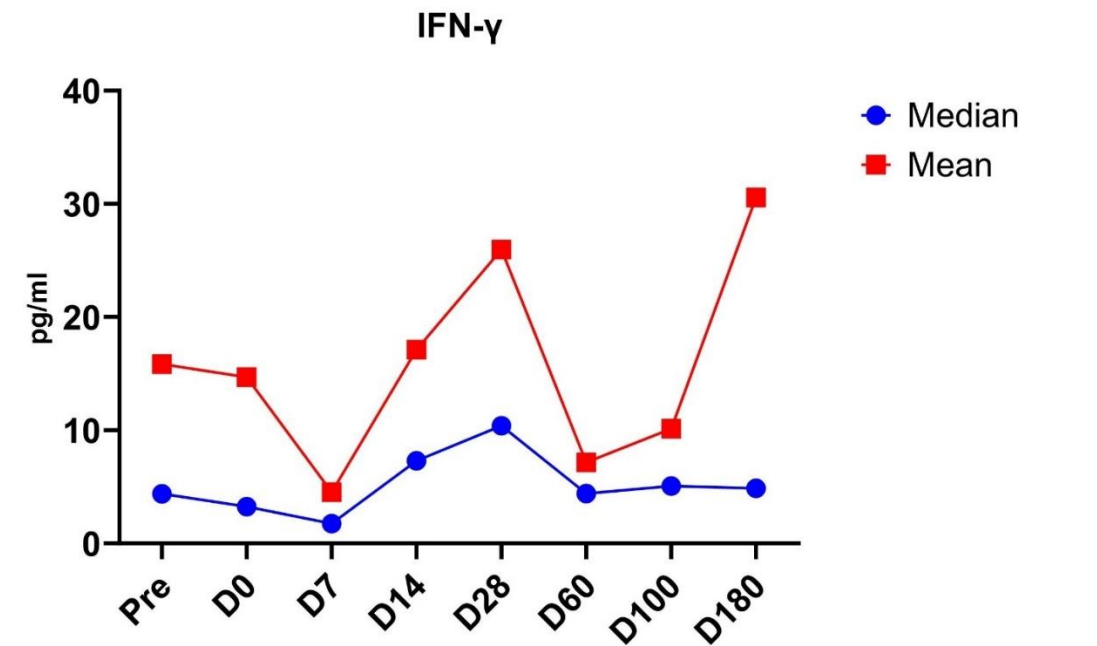


Figure 176a: IFN γ levels at serial time-points, median and mean.

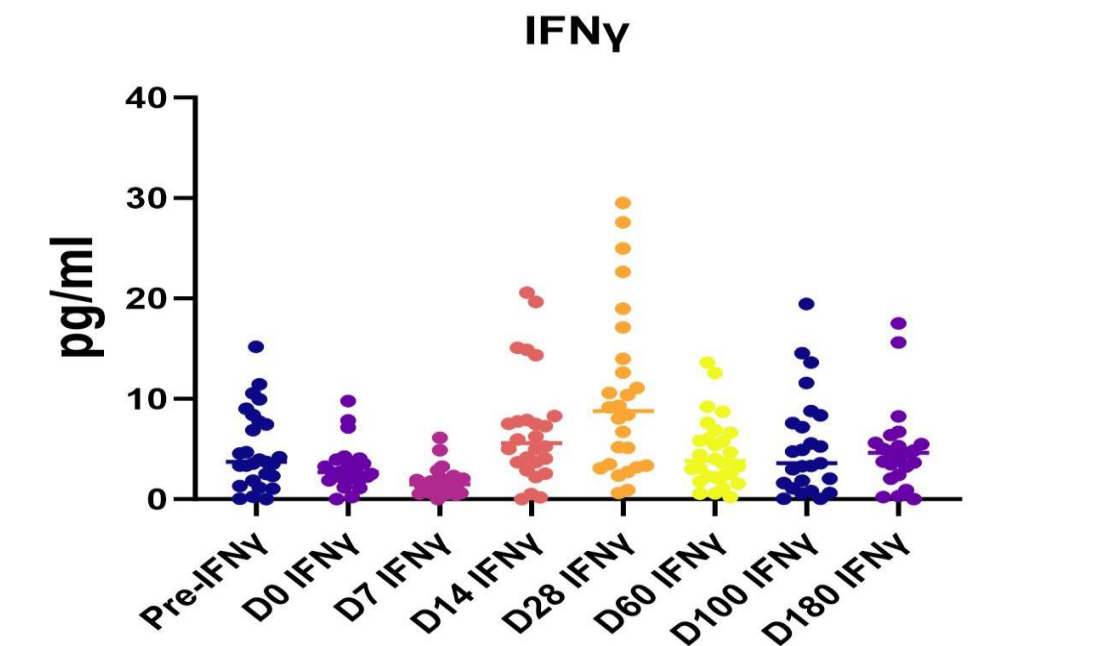


Figure 176b: Interferon-gamma levels at serial time-points, significant outliers not represented.

IFN γ



Figure 176c: Correlation matrix of IFN γ levels at serial time-points; red hues indicate negative correlation and blue indicate positive correlation. Asterisks denote statistical significance, * $p = 0.05$, ** $p = 0.01$, *** $p = 0.001$.

IL-1 β

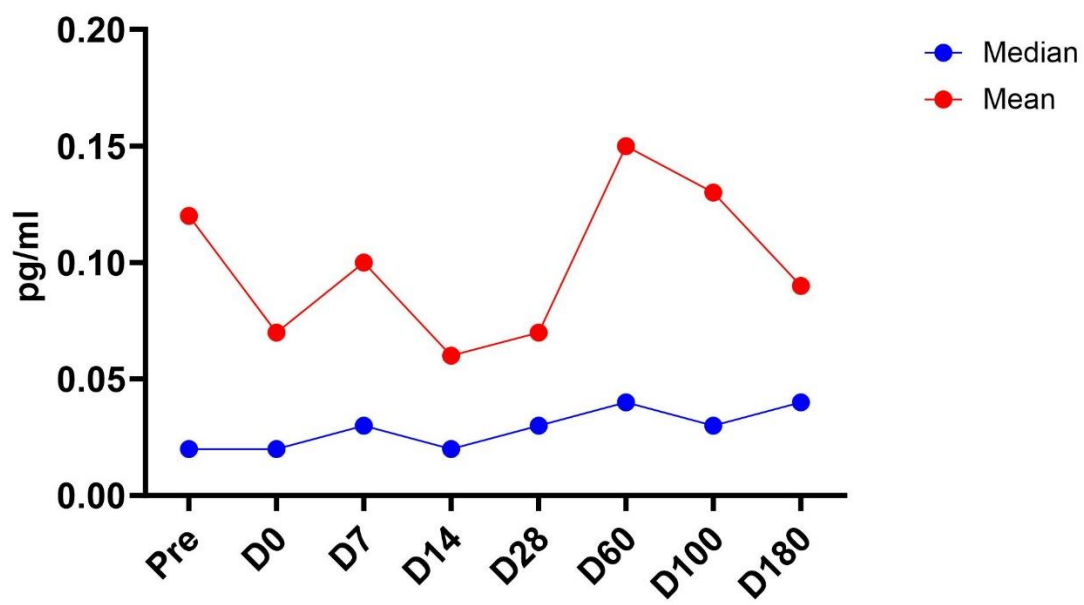


Figure 178a: IL-1 β levels at serial time-points, median and mean.

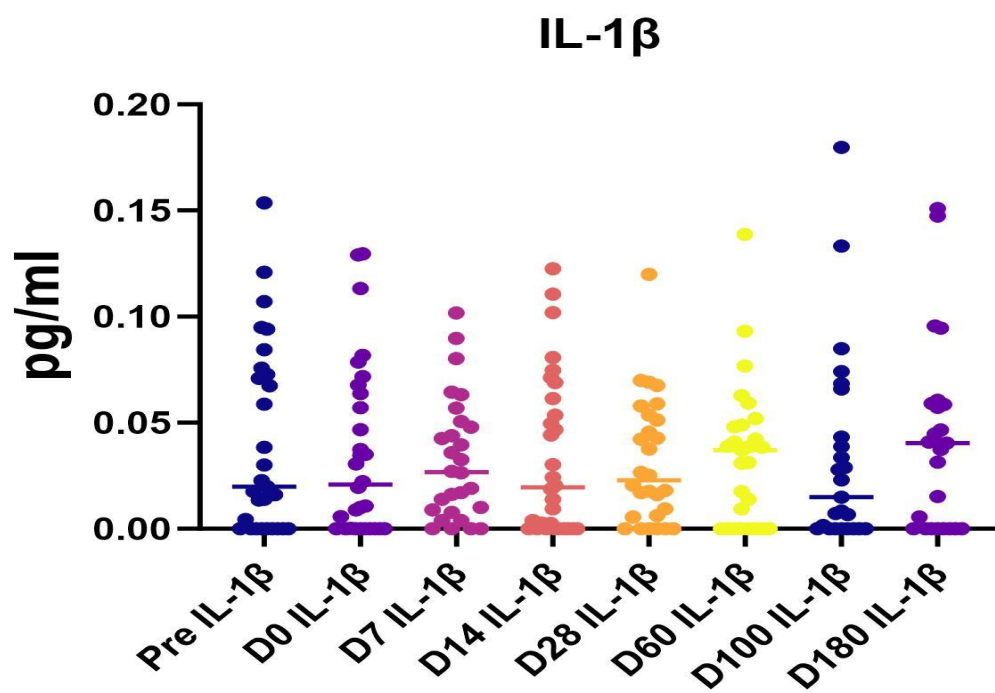


Figure 178b: Interleukin-1-beta levels at serial time-points, significant outliers not represented.

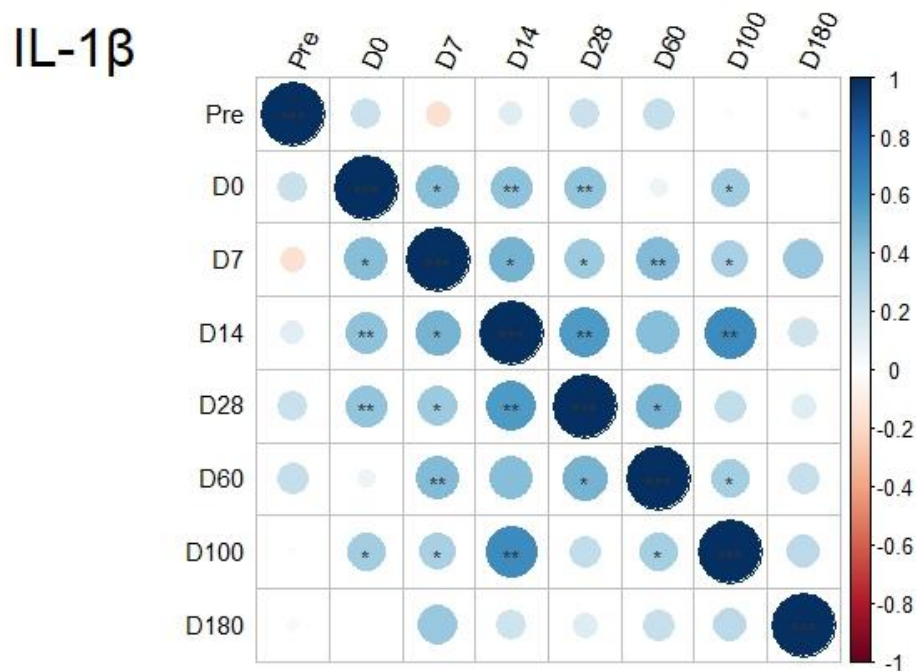


Figure 178c: Correlation matrix of IL-1β levels at serial time-points; red hues indicate negative correlation and blue indicate positive correlation. Asterisks denote statistical significance, * $p = 0.05$, ** $p = 0.01$, *** $p = 0.001$.

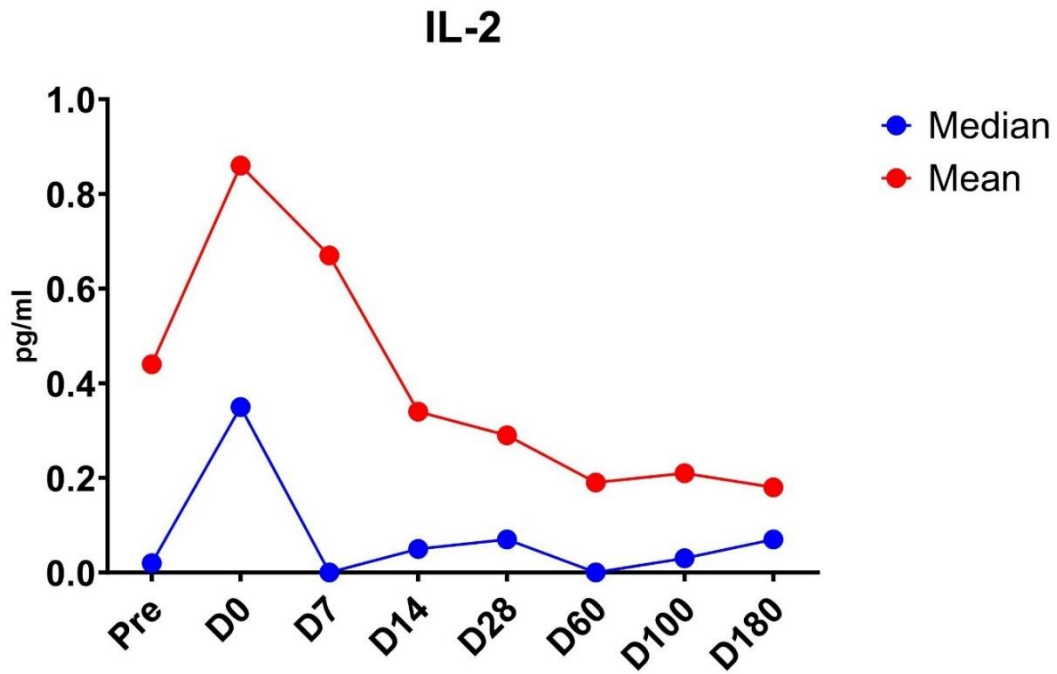


Figure 180a: IL-2 levels at serial time-points, median and mean.

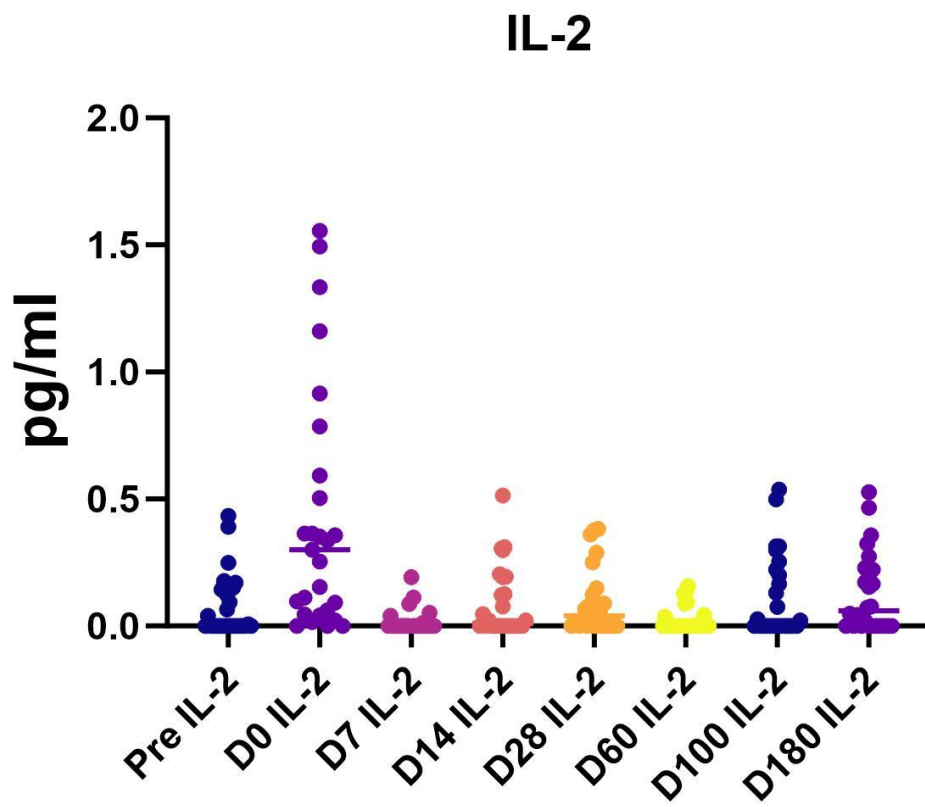


Figure 180b: Interleukin-2 levels at serial time-points, significant outliers not represented.

IL-2

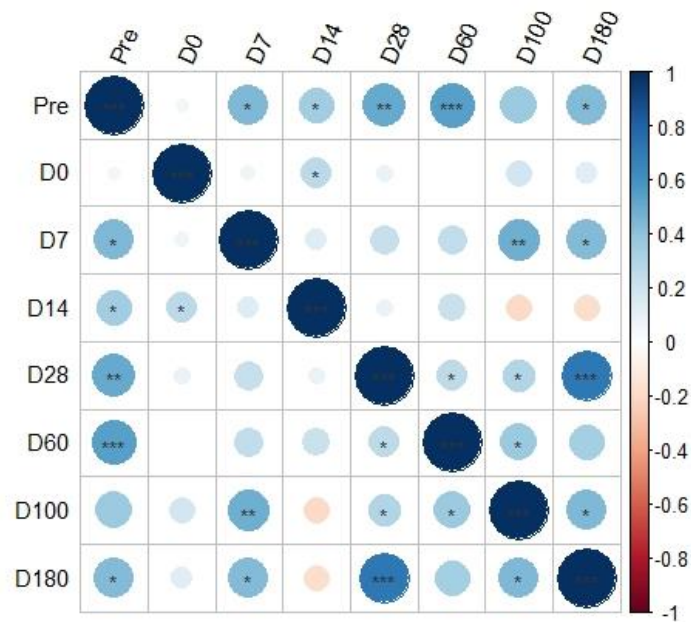


Figure 180c: Correlation matrix of IL-2 levels at serial time-points; red hues indicate negative correlation and blue indicate positive correlation. Asterisks denote statistical significance, * $p = 0.05$, ** $p = 0.01$, *** $p = 0.001$.

IL-4

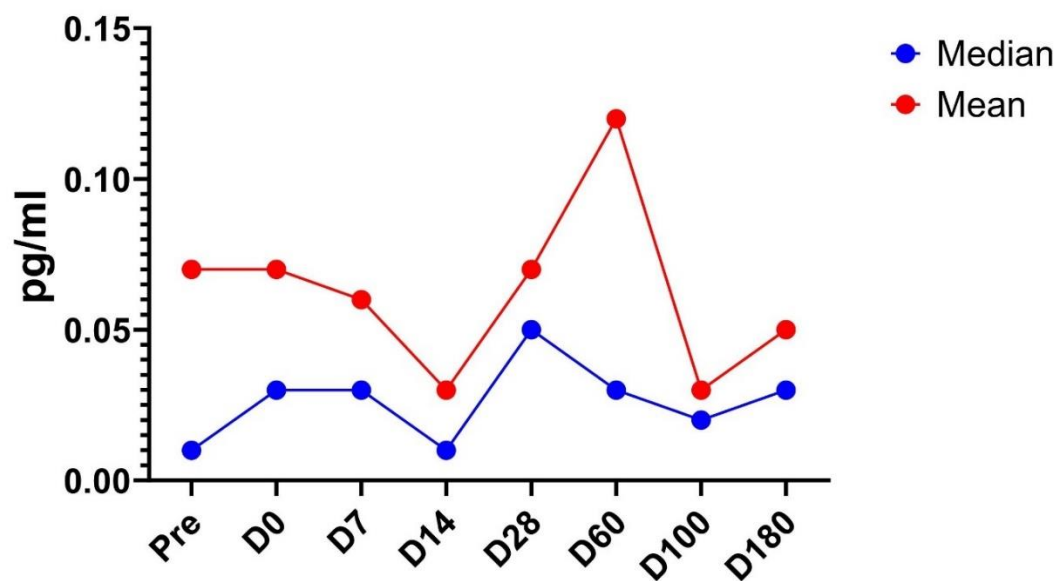


Figure 182a: IL-4 levels at serial time-points, median and mean.

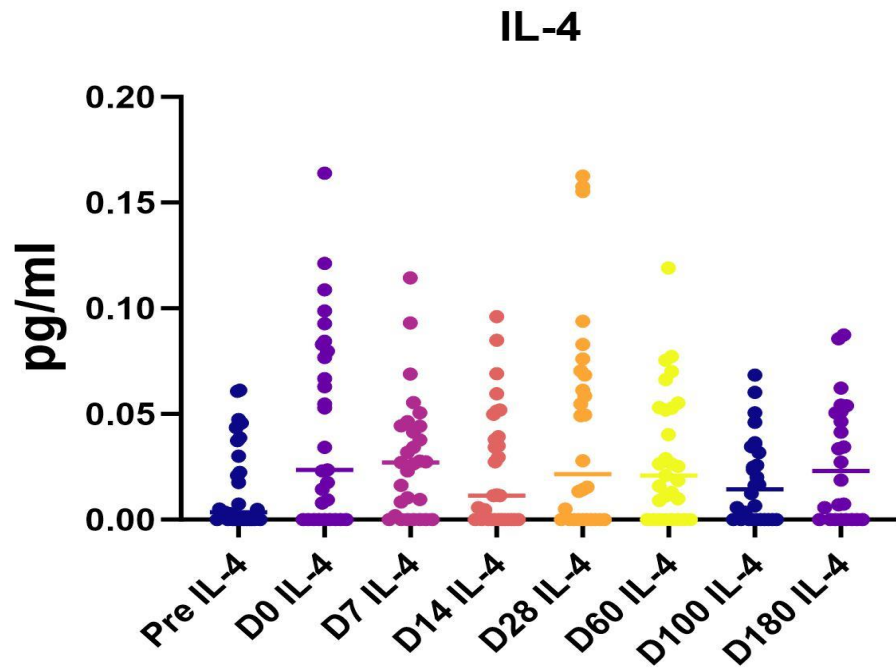


Figure 182b: Interleukin-4 levels at serial time-points, significant outliers not represented.

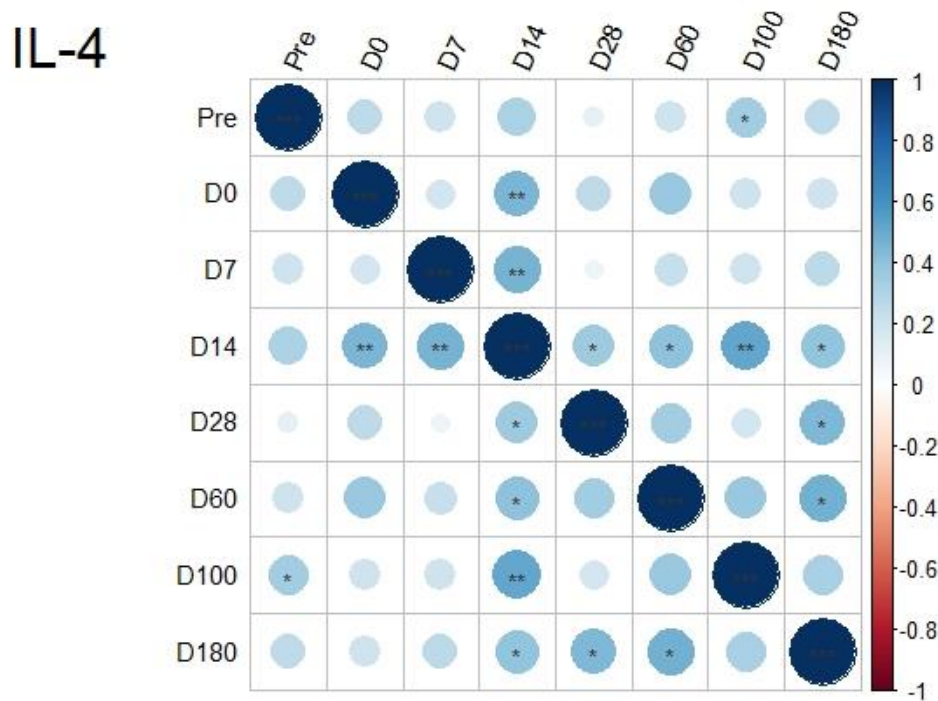


Figure 182c: Correlation matrix of IL-4 levels at serial time-points; red hues indicate negative correlation and blue indicate positive correlation. Asterisks denote statistical significance, * $p = 0.05$, ** $p = 0.01$, *** $p = 0.001$.

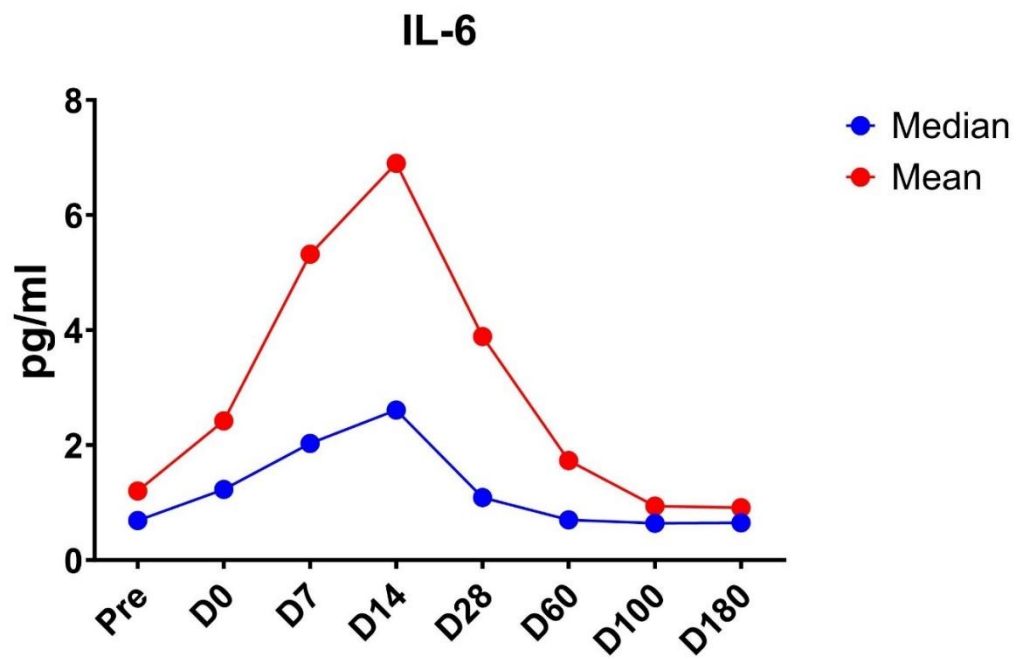


Figure 184a: IL-6 levels at serial time-points, median and mean.

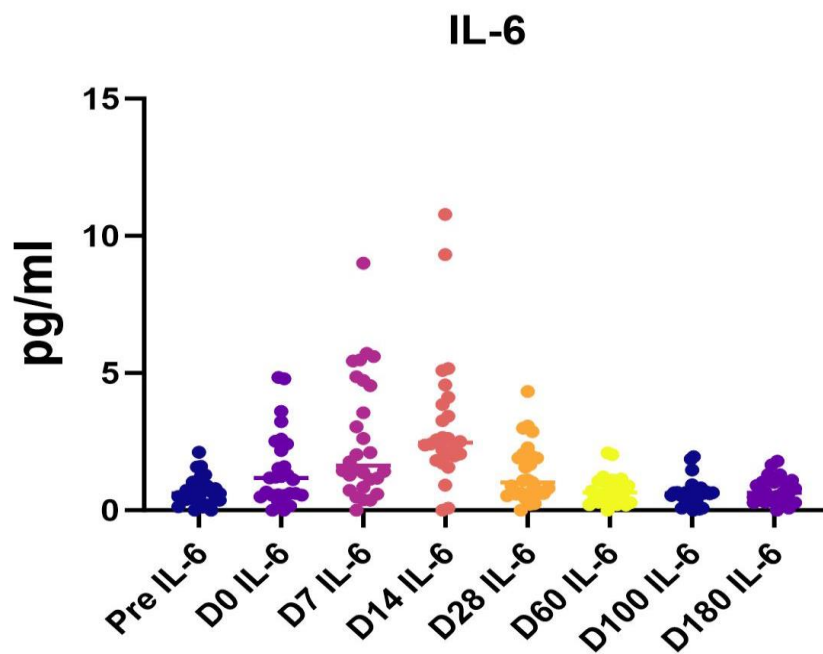


Figure 184b: Interleukin-6 levels at serial time-points, significant outliers not represented.

IL-6



Figure 184c: Correlation matrix of IL-6 levels at serial time-points; red hues indicate negative correlation and blue indicate positive correlation. Asterisks denote statistical significance, * $p = 0.05$, ** $p = 0.01$, *** $p = 0.001$.

IL-10

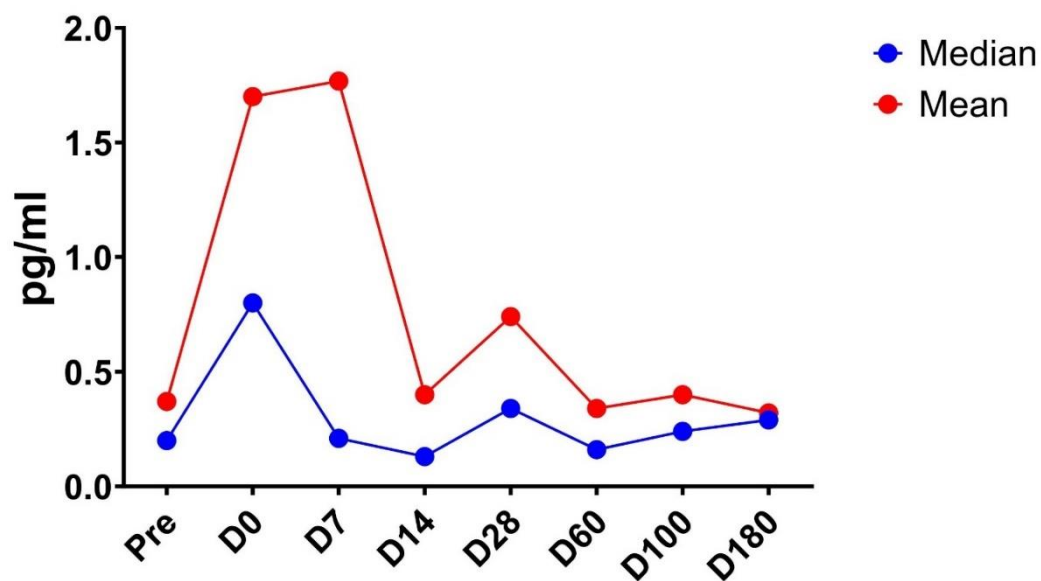


Figure 186a: IL-10 levels at serial time-points, median and mean.

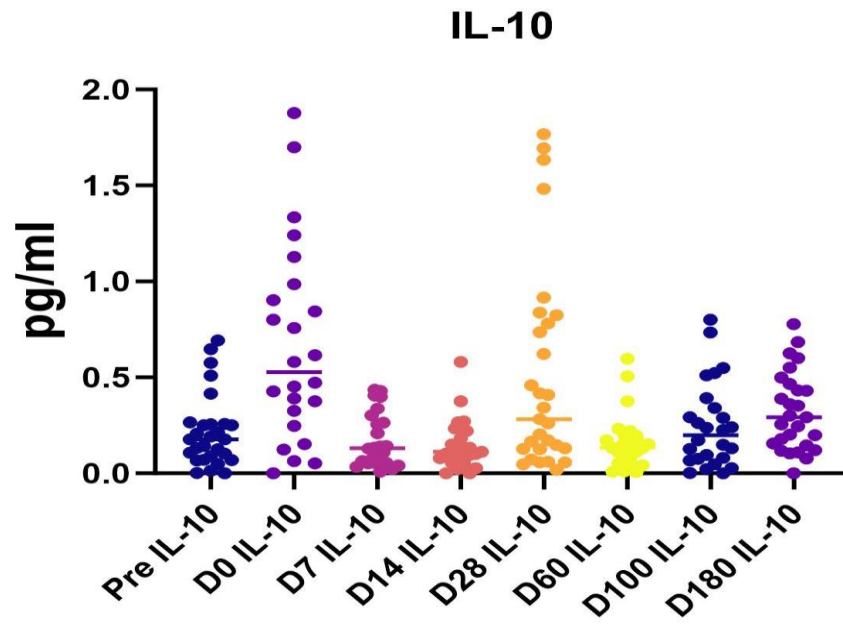


Figure 186b: Interleukin-10 levels at serial time-points, significant outliers not represented.

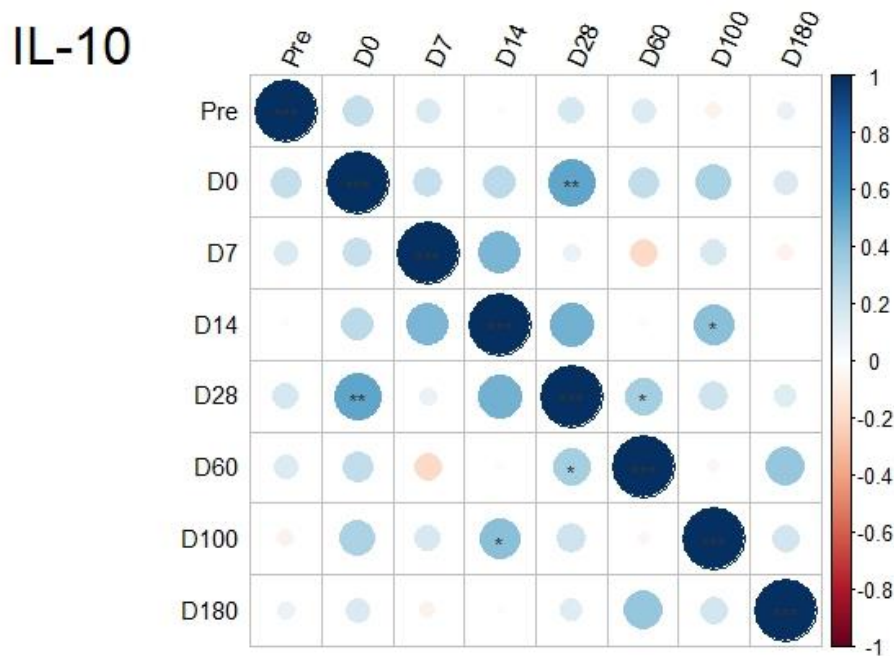


Figure 186c: Correlation matrix of IL-10 levels at serial time-points; red hues indicate negative correlation and blue indicate positive correlation. Asterisks denote statistical significance, * $p = 0.05$, ** $p = 0.01$, *** $p = 0.001$.

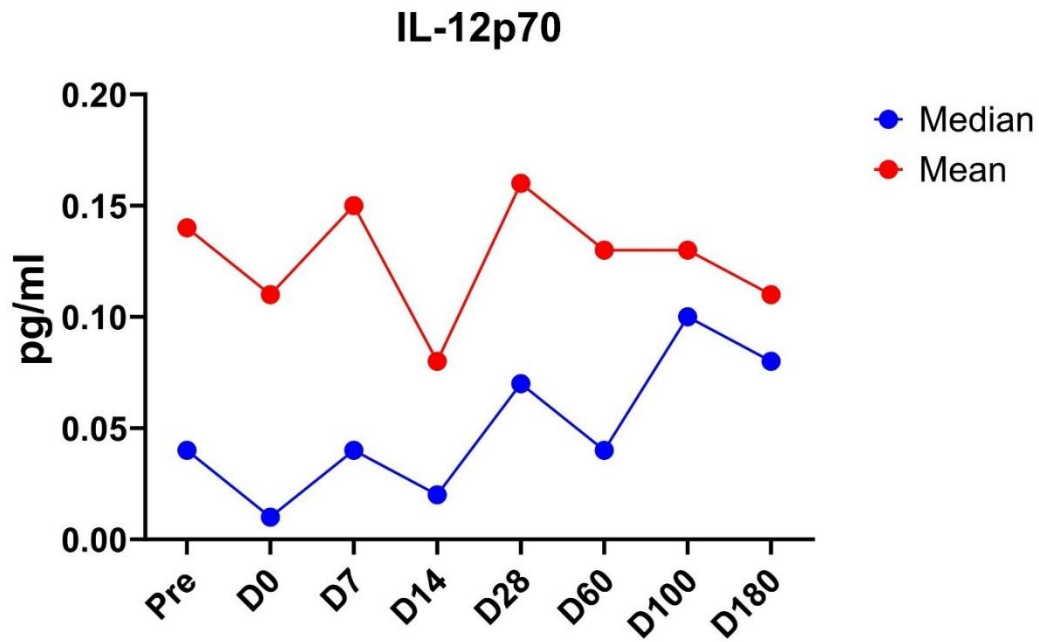


Figure 188a: IL-12p70 levels at serial time-points, median and mean.

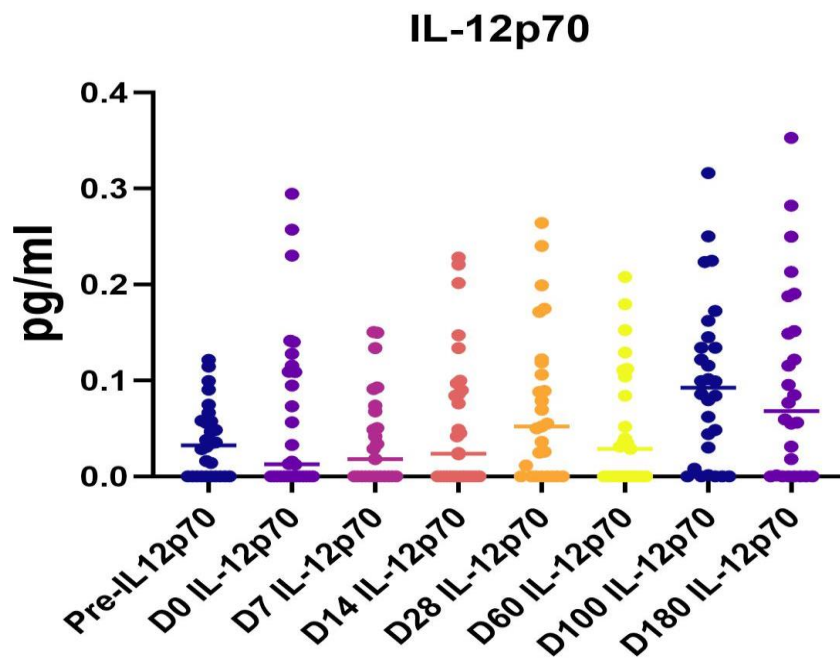


Figure 188b: Interleukin-12p70 levels at serial time-points, significant outliers not represented.

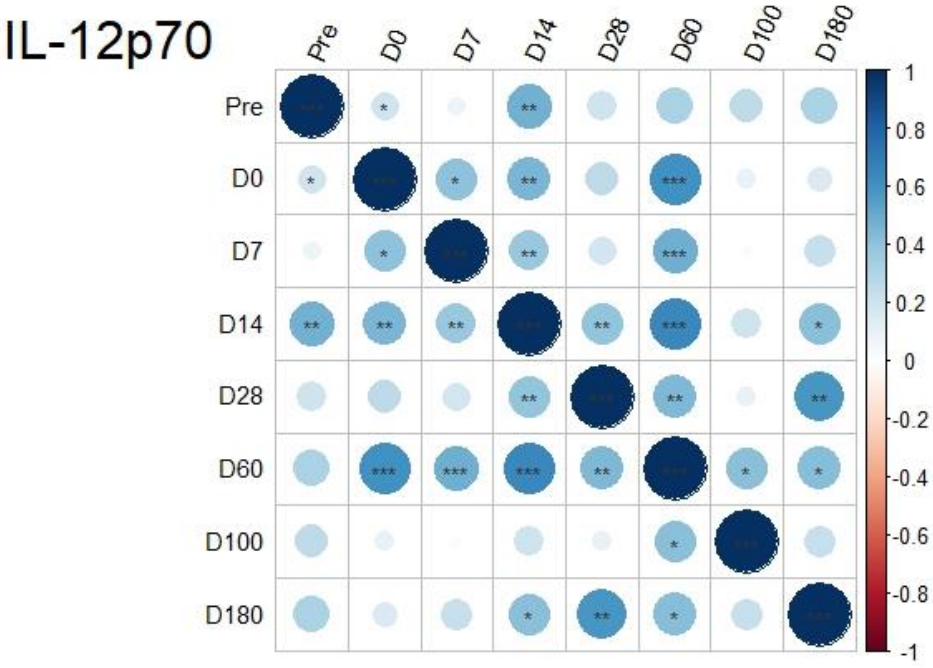


Figure 188c: Correlation matrix of IL-12p70 levels at serial time-points; red hues indicate negative correlation and blue indicate positive correlation. Asterisks denote statistical significance, * $p = 0.05$, ** $p = 0.01$, *** $p = 0.001$.

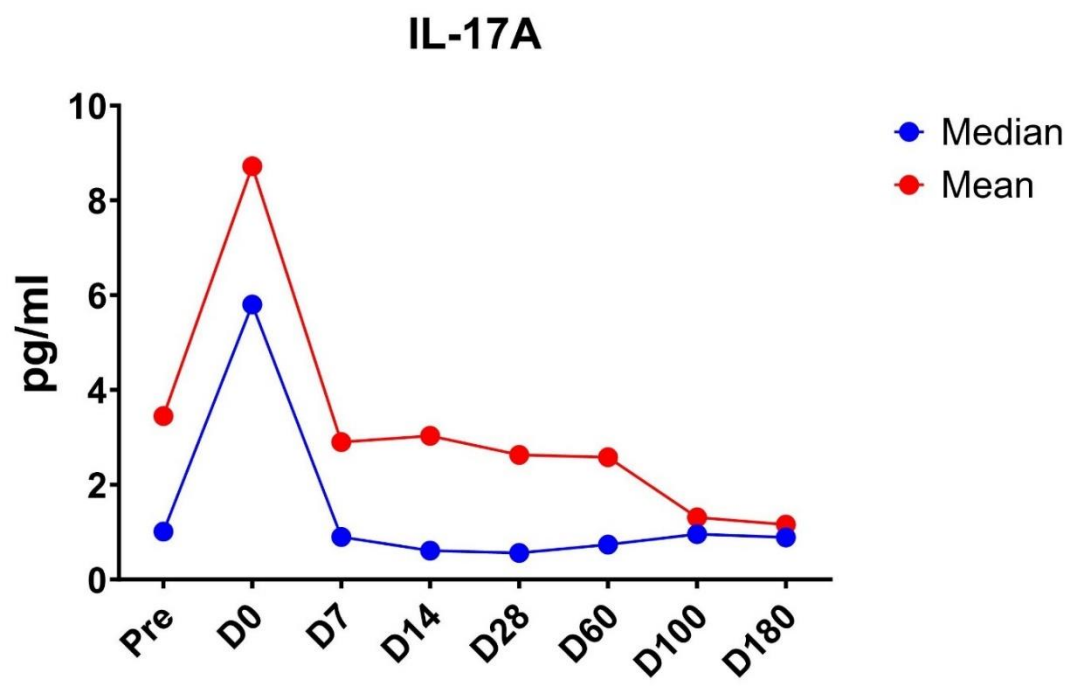


Figure 190a: IL-17A levels at serial time-points, median and mean.

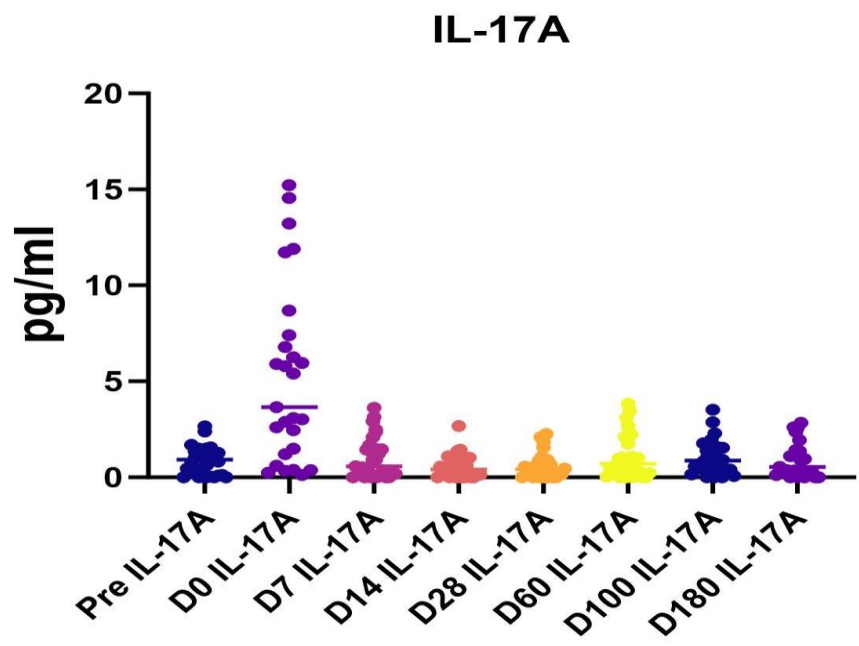


Figure 190b: Interleukin-17A levels at serial time-points, outliers suppressed.

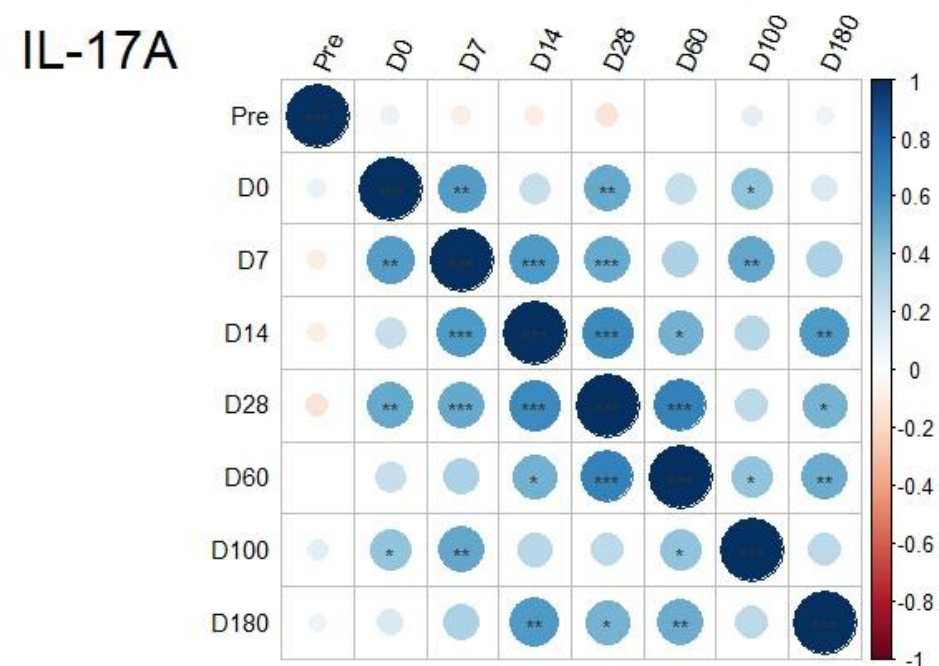


Figure 190c: Correlation matrix of IL-17A levels at serial time-points; red hues indicate negative correlation and blue indicate positive correlation. Asterisks denote statistical significance, * $p = 0.05$, ** $p = 0.01$, *** $p = 0.001$.

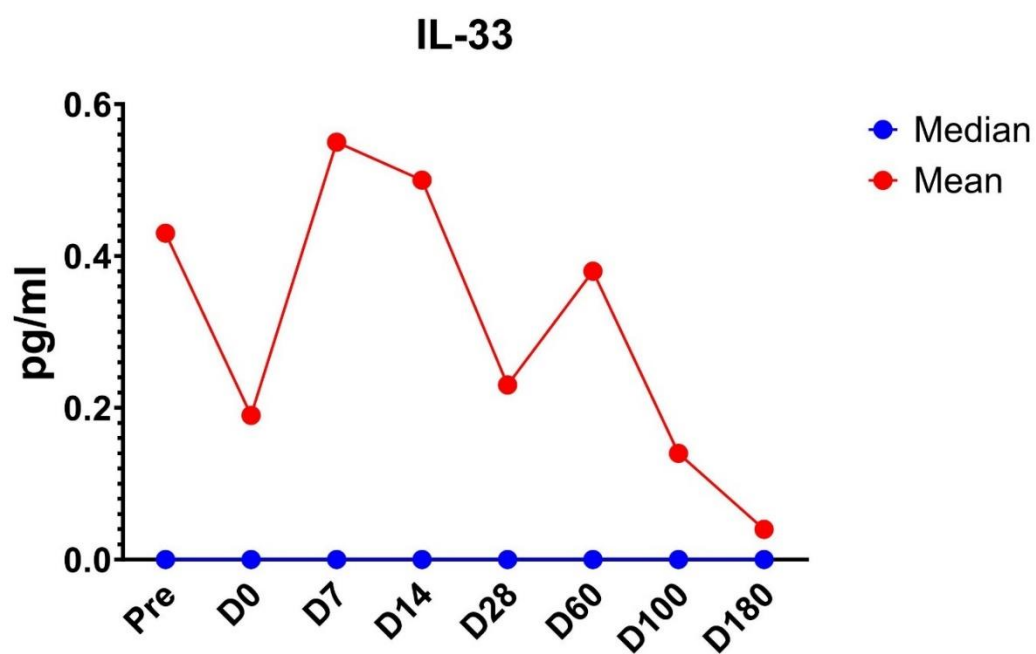


Figure 192a: IL-33 levels at serial time-points, median and mean.

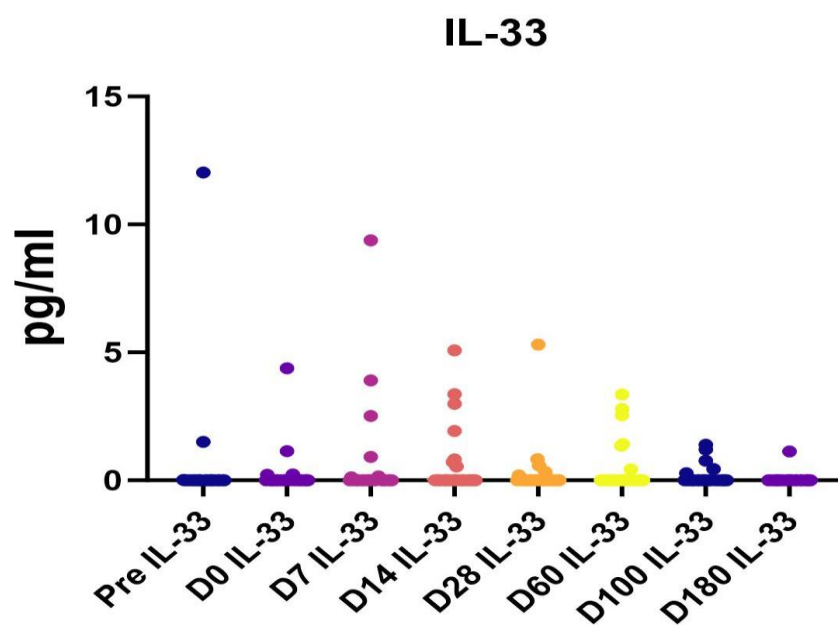


Figure 192b: Interleukin-33 levels at serial time-points.

IL-33

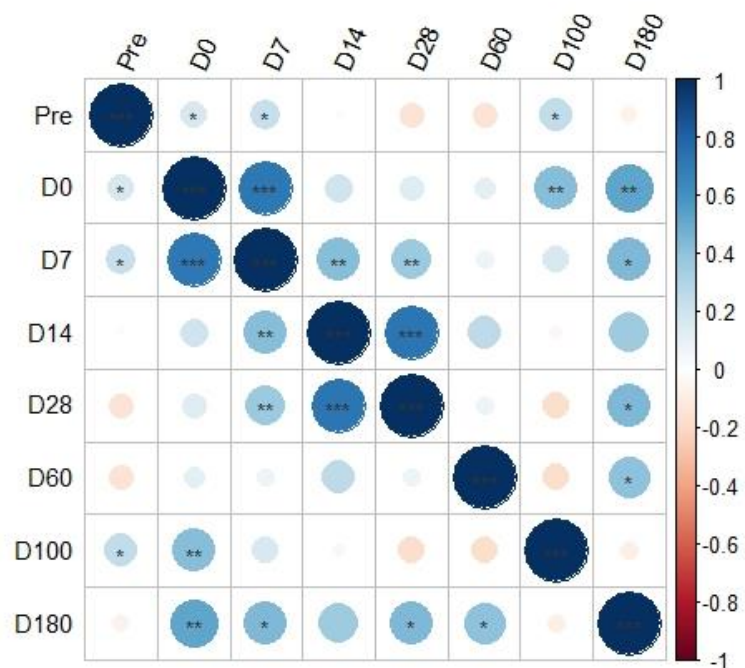


Figure 192c: Correlation matrix of IL-33 levels at serial time-points; red hues indicate negative correlation and blue indicate positive correlation. Asterisks denote statistical significance, * $p = 0.05$, ** $p = 0.01$, *** $p = 0.001$.

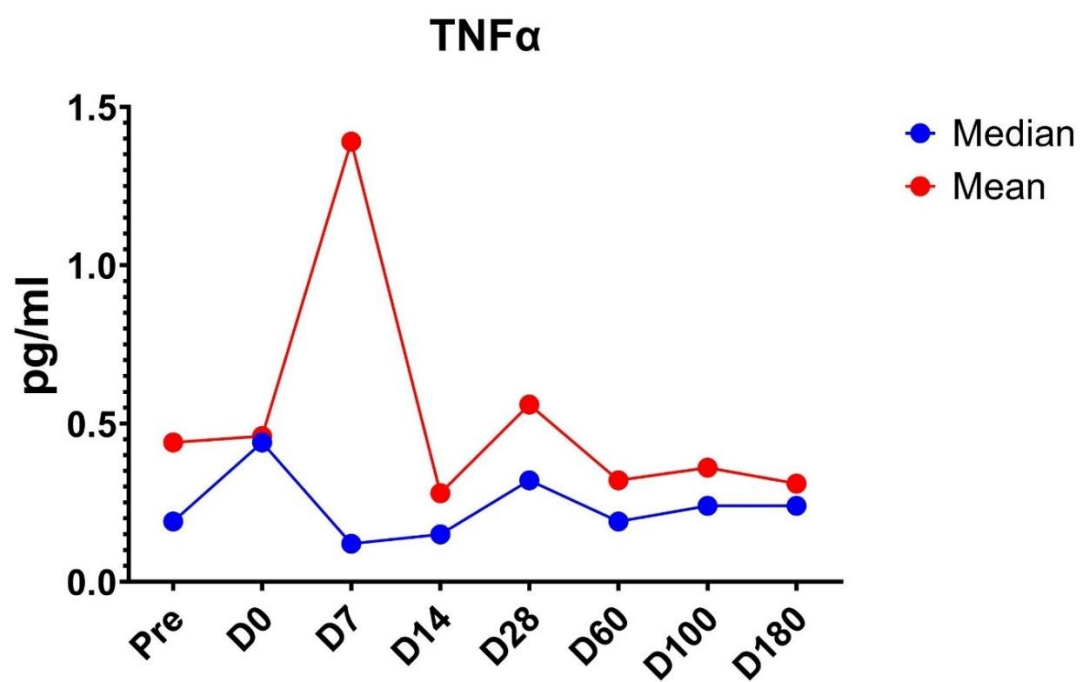


Figure 194a: TNFα levels at serial time-points, median and mean.

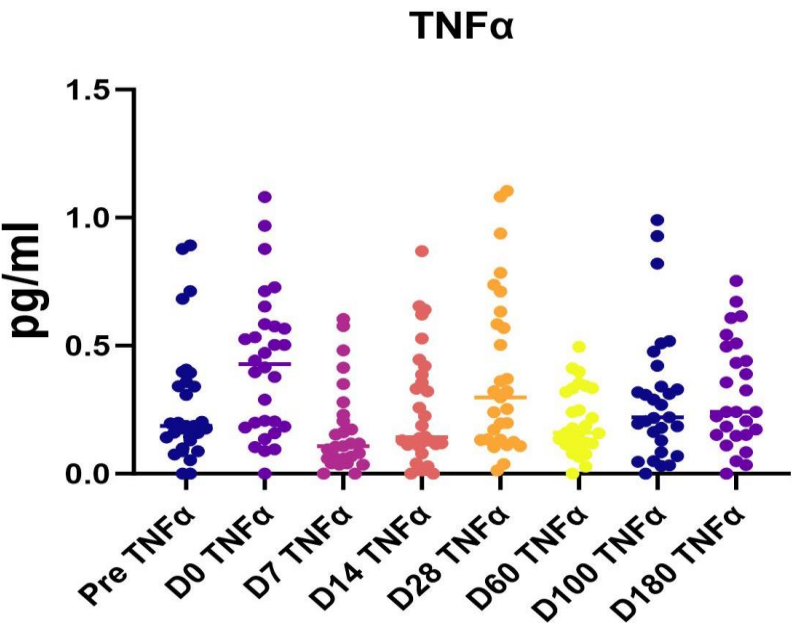


Figure 194b: TNFα factor alpha levels at serial time-points, significant outliers not represented.

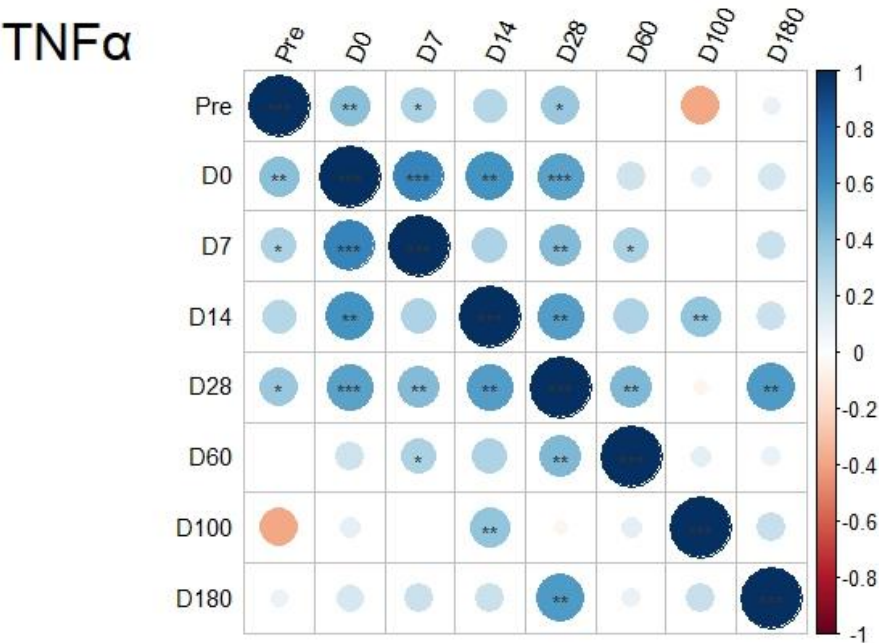


Figure 194c: Correlation matrix of TNFα levels at serial time-points; red hues indicate negative correlation and blue indicate positive correlation. Asterisks denote statistical significance, * $p = 0.05$, ** $p = 0.01$, *** $p = 0.001$.

Appendix 5: Chapter 2 Clinical Summary Paper

Allogeneic haematopoietic stem cell transplantation using reduced-intensity fludarabine, busulfan and anti-T lymphocyte globulin with strategic donor lymphocyte infusion in older patients with myeloid malignancy

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Competing Interests

The authors declare no competing financial interests.

Author contributions

CA was responsible for designing the data collection protocol, collecting data, conducting statistical analyses and writing the report. NO was responsible for designing the data collection protocol and providing report feedback. GL was responsible for collecting data. EC, CF, MNC, CAG, HFS and NG contributed to data collection and provided feedback on the report.

Funding

No funding was utilised to support this research.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files. Further anonymised composite data generated and analysed during the study are available from the corresponding author on reasonable request.

Ethics and Integrity

The study was conducted in accordance with regulatory and data protection requirements and was approved by the institutional ethics committee. All patients provided written consent for analysis of outcomes.

Novelty Statement

1. This study describes the modern context and competitiveness of an established reduced intensity conditioning protocol when combined with a methodical donor lymphocyte infusion programme in an era of evolving practice.
2. These data support the continued use of fludarabine, busulfan and anti-T-lymphocyte with fully matched donors for older patients with myeloid malignancy.
3. These results describe encouraging real-world outcomes in a large cohort of patients against a background of increasing use of alternative conditioning agents and graft-versus-host disease prevention strategies.

Word Counts and Metrics

Abstract: 200 words | Body of text: 3821 words

References: 50

Tables and figures: 6 | Supplementary tables: 1

Abstract

Fludarabine, busulfan and anti-T lymphocyte globulin (FLUBU3+ATLG) reduced-intensity conditioning is an established preparative regimen for allogeneic haematopoietic stem cell transplantation in older patients with myeloid malignancy. We examined its modern-day performance in 175 sequentially-treated patients on our national programme. Overall survival was 72.4% at 2 years (95% CI 64.6-78.6%) with a cumulative incidence of non-relapse mortality of 11%. The cumulative 2-year relapse incidence was 27% (95% CI 22.8-37.6%) and was partially ameliorated by chronic graft-versus-host disease (HR 0.35, 95% CI 0.12–0.98, $p=0.02$). Mixed donor chimerism was observed in 51.5% beyond day 90 but relapse was significantly reduced in these patients by adopting a pre-emptive donor lymphocyte infusion (DLI) strategy (HR 0.22, 95% CI 0.07 – 0.69, $p=0.005$). The use of DLI as part of post-relapse salvage was also effective, with an improved median survival duration of 6 months in recipients (HR 0.43, 95% CI 0.18–0.98, $p=0.01$). Outcomes in patients >65 years and a limited cohort >70 years are encouraging and compare favourably to published survival results using alternate reduced-intensity regimens. FLUBU3+ATLG, supported by modern supportive care and a pre-emptive DLI strategy, is well-tolerated by older patients across a spectrum of myeloid disease with modest toxicity and favourable long-term outcomes.

Introduction

Reduced-intensity conditioning (RIC) allogeneic haematopoietic stem cell transplant (HSCT) activity for older patients with myeloid malignancies has grown steadily over the last 2 decades worldwide [1]. Non-ablative doses of busulfan combined with fludarabine and *in vivo* T cell depletion using anti T-lymphocyte globulin (FLUBU3+ATLG) leads to high rates of donor engraftment, with limited acute and late toxicity for patients ineligible for myeloablative regimens [2, 3]. This combination is popular in Europe and has been the national standard RIC regimen for adults with myeloid malignancies in Ireland for almost 20 years. However, alternative alkylators such as melphalan and treosulfan, novel graft-versus-host disease (GVHD) prophylaxis strategies such as the use of post-transplant cyclophosphamide (PTCy), and developing post-transplant maintenance strategies have made choosing the optimal treatment approach ever more nuanced [4-7]. Furthermore, a paucity of direct comparator clinical trials limits appraisal of the various options.

Use of anti-thymocyte/T-lymphocyte globulin (ATG/ATLG) involves balancing risks; poor immune reconstitution contributes to excess infectious morbidity and weaker tumour surveillance but is countered by reduced toxicity from severe GVHD [8-10]. PTCy use has firmly established its role in the haploidentical and HLA-mismatched settings [11]. Predictable pharmacokinetic characteristics make PTCy an attractive alternative to ATG/ATLG, however extrapolation to matched unrelated and sibling donors is limited by a lack of direct comparisons in prospective trials and the largest study so far in the RIC setting did not utilise ATG/ATLG in the control arm [7, 12-14]. Alternative alkylators must additionally be considered, although comparative studies offer mixed results [15-19].

In the RIC setting, the balance of toxicity and graft-versus-leukaemia effect can be tenuous. Donor lymphocyte infusions (DLI) are a potent outcome modifier, particularly pre-emptively in those with mixed chimerism [20, 21] and with some promise incorporated into low-intensity multi-modal strategies [22-26]. However, DLI practice varies significantly between centres and there is a notable lack of prospective data or large-series retrospective data to guide usage.

Long-term disease-free survival for older patients with myeloid disease remains dismal without transplantation [27]. Over 60% of HSCT recipients in Europe are aged over 55 years, and trends in those above 65 show increasing growth year-on-year, as traditional chronologic age cut-offs are challenged by new insights into biological fitness and physiologic reserve [27, 28]. HSCT in older patients has demonstrable benefit [28-31]. However, outcomes are influenced by population variance, selection bias and treatment modality. Although survival benefit is reproducible, the impacts on morbidity and non-survival outcomes are controversial and there is a lack of prospective, randomised data [32, 33]. While there is little doubt of an unmet need for older patients, particularly in those with AML, for those patients who ultimately proceed to HSCT, the optimal RIC regimen must demonstrate low toxicity but also meaningful efficacy to justify expanding access to ever-older patients.

In this study we retrospectively examine our national outcomes using the FLUBU3-ATLG RIC platform, interrogating its performance in the modern era and exploring its suitability for increasingly older patients and furthermore examine the benefit of DLI in this large patient cohort.

Methods

Study cohort

Adult patients with AML, MDS and MPN at the National Adult Stem Cell Transplant Programme in Ireland between 2015-2022 were included (n=175). A RIC approach was considered the institutional usual conditioning intensity for patients over the age of 50 years and in those of younger age with significant co-morbidities. All patients in this study received fludarabine (180mg/m² over day -9 to -4), intravenous busulfan (8mg/kg over day -5 to -3), with rabbit ATLG (Grafalon® (Neovii, Switzerland): 30mg/kg (sibling donor) or 60mg/kg (unrelated donor) day -3 to -1), low-dose methotrexate (30mg/m² day +1/3/6) and a calcineurin inhibitor. We defined reduced intensity dosing of busulfan as a total dose of <9mg/kg, as per Giralt et al and the CIBMTR [34]. Pharmacokinetic monitoring of busulfan was not employed. All received unmanipulated peripheral blood stem cell grafts. Eleven single-locus HLA mismatched grafts were included in the earlier phase of observation, all of whom received ATLG-based GVHD prophylaxis (PTCy use in this setting was later adopted but such cases are not included in this study).

DLI Strategy

Chimerism analysis of total/CD3⁺ fraction in peripheral blood was performed using STR-PCR (sensitivity 3%) at day 45 and day 90 post-transplant and individualized thereafter, but typically 3 monthly. While the international consensus definition for mixed chimerism is <95%, we defined <97% donor chimerism as mixed based on in-house PCR sensitivity, preferentially in the CD3⁺ compartment. DLI use was considered pre-emptive in those with persistent mixed donor chimerism following withdrawal of immunosuppression, therapeutic when post-relapse or prophylactic if in remission with full donor chimerism. DLI was employed at a minimum of 100 days post-transplant, after withdrawal of immunosuppression and with a starting dose of 0.1x10⁷/kg CD3⁺ cells in pre-emptive/prophylactic approaches, and typically 0.3x10⁷/kg CD3⁺ cells in relapse. Dose escalation followed a pattern of 0.1, 0.3, 0.5, 1, 3 and 5x10⁷ CD3⁺ cells/kg on an 8-12 week interval with clinical monitoring for GVHD at weeks 3, 6

and prior to subsequent doses. DLI administration was terminated at re-attainment of full donor chimerism, GVHD onset or dose exhaustion. CD4 and IgG levels were assessed at 3, 6 and 12 months and viral re-activation was defined by whole-blood PCR.

Outcomes

Study endpoints were overall survival (OS) and relapse-free survival (RFS), non-relapse mortality (NRM) and relapse incidence, engraftment rate and time, acute and chronic GVHD, immune reconstitution and viral reactivation. Engraftment was defined as neutrophils $\geq 0.5 \times 10^9/L$ for 3 consecutive days and platelets $\geq 20 \times 10^9/L$ unsupported for 7 days. Acute and chronic GVHD were graded by modified Glucksberg and NIH criteria.

Statistical analysis

Categorical characteristics were described using medians and ranges. Comparison of categorical variables was conducted using Fisher's exact test and comparison of medians with the Kruskal-Wallis H test for non-parametric data. Survival estimates were calculated using Kaplan-Meier method. Cumulative incidence of NRM and relapse (as competing risks) were calculated using the Fine and Gray method. Univariable analyses used a Cox model for OS and RFS, and Gray's test for relapse, NRM and GVHD. A Cox proportional-hazards model using a backwards step-wise approach was used for multivariable regression. Hazard ratios and 95% confidence limits are provided as a summary measure. *P* values are two sided with a significance of 0.05. Analysis was conducted using *STATA* (Version 18.0, StataCorp, Texas, USA).

The study was conducted in accordance with regulatory and data protection requirements and was approved by the institutional ethics committee. All patients provided written consent for analysis of outcomes. All co-authors had access to the data and vouch for its completeness and accuracy.

Results

Patient and disease characteristics

Patient characteristics are summarised in Table 1. The median age was 60 years (range 27-74), increasing from 56.9 in 2015 to 60.8 in 2022. Baseline characteristics were similar across age groups, although those >60 years had a slightly lower median age-adjusted haematopoietic cell transplant comorbidity index score (aaHCT-CI) (2 vs. 3). 93.1% of patients had intermediate or adverse risk disease, and the remainder (6.9%) were transplanted in $\geq$ CR2. In the AML group, all patients were in haematological CR or CR with incomplete haematological recovery at the time of transplant except for one patient who was in a morphological leukaemia free/aplastic state. In those with MDS only 1 patient was transplanted with progressive disease at the time of transplant and 5 in the MPN subgroup. 42.8% of donors were siblings, with a median age of 54 (unrelated donors: 26 years).

Table 1: Demographics	
Number of patients	175
Sex, n (%)	
Male	106 (60.5)
Female	69 (39.5)
Age, n (%)	Median: 60, range 27-74
<50 yr	12 (6.8)
50-54 yr	30 (17.1)
55-59 yr	44 (25.1)
60-64 yr	62 (35.5)
65-74 yr	27 (15.5)
Age-adjusted HCT-CI, median (range)	2 (1-8)
Disease type, n (%)	
AML	89 (50.8)
MDS	46 (26.4)
MPN	40 (22.8)
CIBMTR Disease risk index, n (%)	
Low	12 (6.9)
Intermediate	109 (62.3)
High	54 (30.8)
Donor type, n (%)	
HLA-matched sibling	75 (42.8)
HLA-matched unrelated	89 (50.9)
HLA mismatched related/unrelated: 1 locus	11 (6.3)

Donor age (yr)	Median: 38, range 18 – 67 Sibling: Median 54, range 24 – 67 Unrelated: Median 26, range 18 – 64
Graft cryopreservation, n (%)	20 (11.4)
ABO matching, n (%)	
Minor mismatch	31 (17.7)
Major/bidirectional mismatch	50 (28.6)
CMV matching, n (%)	
Recipient Neg / Donor Neg	97 (55.5)
Recipient Pos / Donor Pos	49 (28)
Recipient Pos / Donor Neg	15 (8.5)
Recipient Neg / Donor Pos	14 (8)

Neutrophil engraftment without granulocyte colony-stimulating factor occurred in 99.4% at a median of 20 days (range 11 - 42) and platelet engraftment occurred in 96.5% at a median of 18 days (range 11 – 116). Due to the logistical constraints during the COVID pandemic, several transplants were conducted using cryopreserved stem cell grafts (11.4%). Although no significant difference in rate or time to engraftment was seen compared to fresh, a signal for an increased risk of relapse with this approach led to reverting preference for fresh grafts following logistical easing. Time from diagnosis to transplant was 52 days longer in those who received a cryopreserved graft. No complete primary graft failure events occurred, although 4 patients had failure of primary platelet engraftment. No sinusoidal obstructive syndrome events requiring defibrotide were seen in this cohort.

OS and RFS

The median OS was not reached by the median follow-up of 652 days. OS at 1 and 2 years respectively was 80.3% (95% CI 73.5% - 85.5%) and 72.4% (95% CI 64.6% - 78.6%) (*Fig. 1a*). RFS was 70% (95% CI 62.5% - 76.1%) at 1 year and 61.7% (95% CI 53.7% - 68.7%) at 2 years (*Fig. 1b*). OS did not differ by age, sex, disease type or donor source on univariable analysis. Older donor age was not associated with inferior survival (donor age ≤ 40 : HR 0.64, 95%CI 0.37–1.14, $p=0.13$). Univariable predictors of mortality included CIBMTR-DRI high-risk category (HR 1.84, 95% CI 1.04 – 3.29, $p=0.03$) (*Fig. 2a*), high-risk myelofibrosis (HR 4.36, 95% CI 1.34 – 14.16, $p=0.01$) and ABO mismatch (HR 2.32, 95% CI 1.30 – 4.14, $p=0.003$). Superior

survival was seen in those who developed chronic GVHD (HR 0.35, 95% CI 0.12 – 0.98, $p=0.02$) (Fig. 2b). A multivariable model for relapse and survival is presented in Table 2 (see supplementary data table for unadjusted hazard ratios for univariable predictors).

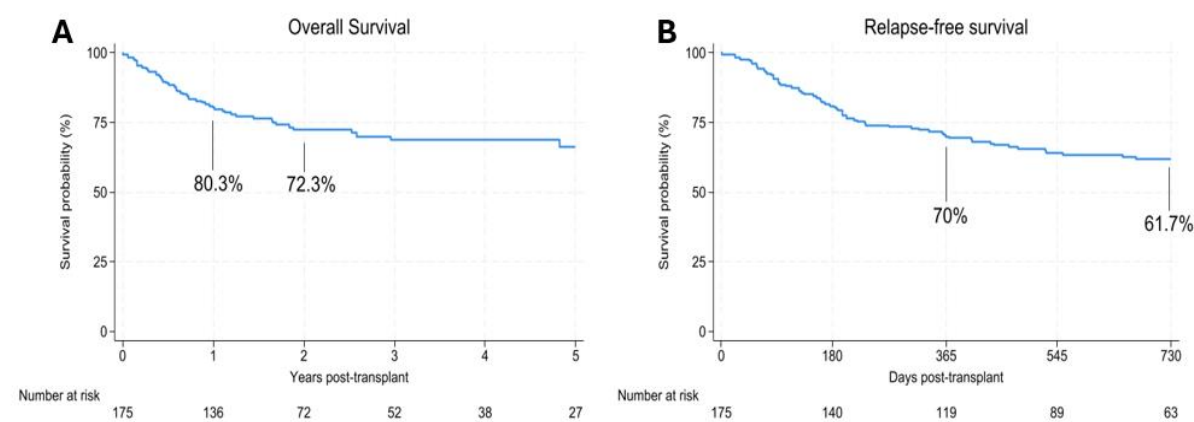


Figure 1. A: Overall survival, year one to five. B: Relapse-free survival, year one and two.

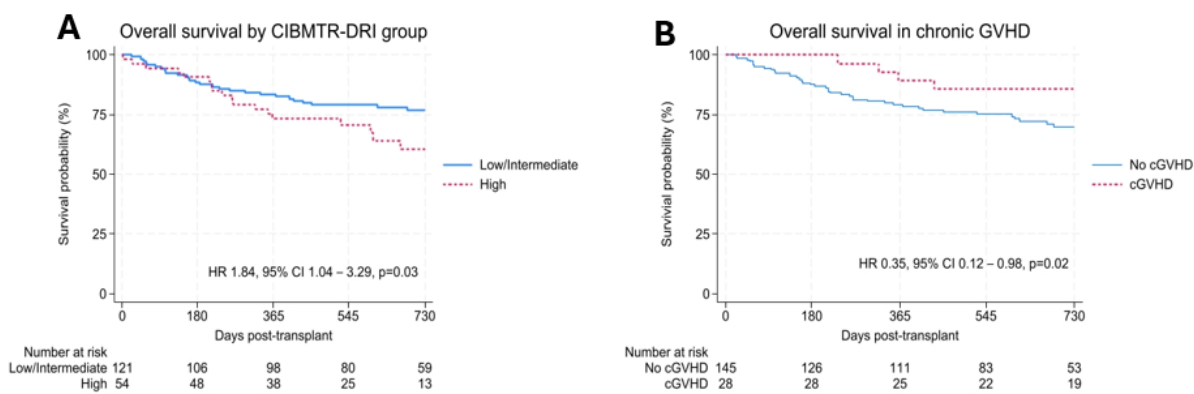


Figure 2: A: Overall survival stratified by CIBMTR-DRI, intermediate and low risk vs. high risk. B: Overall survival by chronic GVHD status. *CIBMTR-DRI* = Centre for International Blood and Marrow Transplant Research – Disease Risk Index. *GVHD* = graft-versus-host disease.

Table 2: Multivariable models for relapse and overall survival			
Outcome	Number of events	Hazard ratio (95% CI)	P Value
Relapse	53		
CIBMTR-DRI: High	54/175	2.16 (1.22 – 3.81)	0.008*
Graft cryopreservation	20/175	2.79 (1.39 – 5.60)	0.004*
Age >60	89/175	0.83 (0.48 – 1.42)	0.5
Chronic GVHD	28/105	0.35 (0.14 – 0.86)	0.02*
Death	50		
Age-adjusted HCT-CI 3+	78/175	1.28 (0.71 – 2.30)	0.4
CIBMTR-DRI: High	54/175	1.72 (0.95 – 3.12)	0.07
Age >60	89/175	1.16 (0.65 – 2.08)	0.6
Chronic GVHD	28/105	0.33 (0.12 – 0.95)	0.04*

Relapse and NRM

The cumulative incidence of relapse at 1 and 2 years was 19.5% (95% CI 15.5 – 28.1) and 27% (95% CI 22.8 – 37.6) respectively, occurring at a median of 204 days (range 30–2186) (*Fig. 3a*). Relapse was highest in the MPN subgroup; 30.5% at 2 years vs. 25% in AML and 27.5% in MDS. Risk was similar across ages, sexes, donor types and donor ages. Higher rates of relapse were seen in the high-risk CIBMTR-DRI group (HR 1.82, 95% CI 1.05 – 3.15, $p=0.03$) and after receipt

of a cryopreserved graft (HR 2.38, 95% CI 1.11 – 5.1, $p=0.02$) (Fig. 3c). Relapse was unaffected by acute GVHD incidence, however there was reduced risk for those who developed chronic GVHD (HR 0.40, 95% CI 0.17 – 0.94, $p=0.03$) (Fig. 3d).

The cumulative incidence of NRM at 1 and 2 years was 10.4% (95% CI 7.3 - 17.5%) and 11% (95% CI 7.9% - 18.5%) and occurred at a median of 134 days (Fig. 3b). NRM was lowest in the oldest age group, with no events occurring in those aged >65 years. Higher aaHCT-CI scores were not predictive of NRM risk in this cohort at any cut-off level. Patients with MPN had the highest risk, with a 2 year cumulative incidence of 16.8%, compared to 11.5% in MDS and 7.8% in AML. Within the MPN group, those with myelofibrosis had a 17.5% 2 year cumulative incidence. Univariable predictors of NRM included high-risk myelofibrosis (HR 8.61, 95% CI 1.91 – 38.69, $p=0.005$), grade III-IV acute GVHD (HR 5.29, 95% CI 2.15 – 13.03, $p<0.001$) and the development of invasive fungal disease (HR 8, 95% CI 3.11 – 25.6, $p<0.001$).

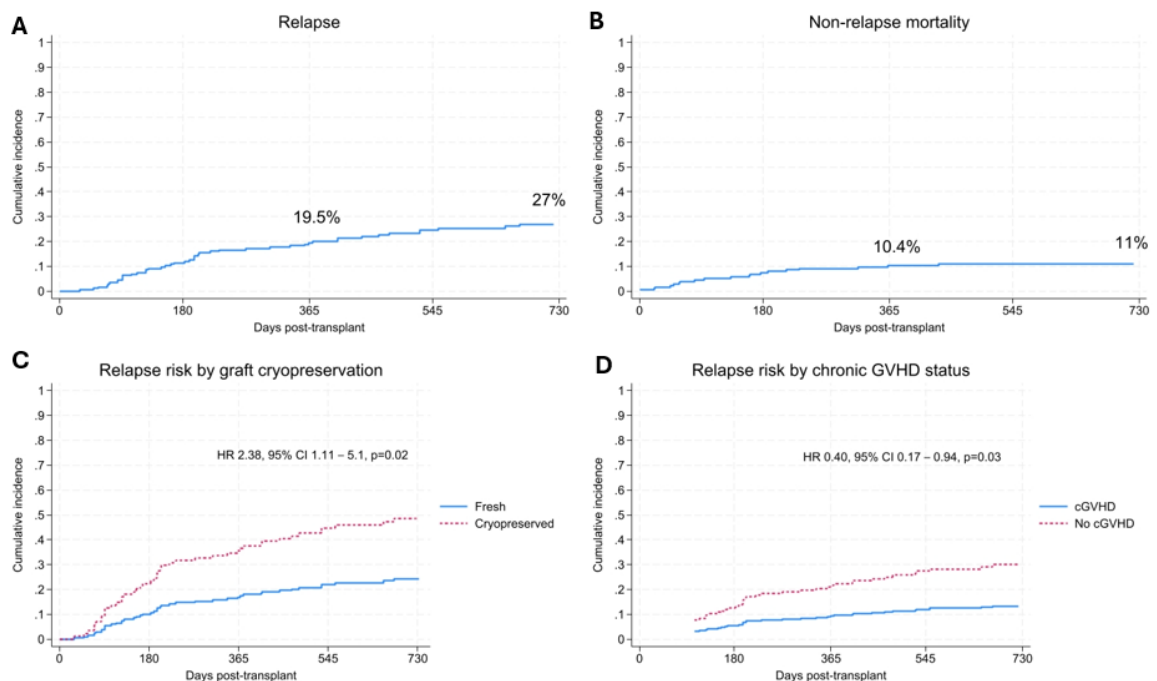


Figure 3. A: Relapse cumulative incidence, competing risk NRM. B: Non-relapse mortality cumulative incidence, competing risk relapse. C: Relapse cumulative incidence based on graft format, fresh vs. cryopreserved product. D: Relapse cumulative incidence following chronic GVHD vs. no chronic GVHD. *GVHD = graft-versus-host disease*.

GVHD

All-grade acute GVHD occurred in 60.3% and the cumulative incidence of grade II-IV and grade III/IV disease at 2 years was 47.7% and 11.2% respectively. Of those who developed GVHD, 72.8% required steroids and 21.6% of those patients required second-line or more treatment. Univariable predictors of grade II-IV acute GVHD included labile calcineurin drug levels within the first month of transplant as defined by supra-therapeutic plasma drug levels on more than one occasion requiring dose interruption (HR 4.38, 95% CI 1.34 – 14.23, $p=0.01$). For grade III-IV disease, associations included non-AML disease types (HR 2.18, 95% CI 1.31 – 3.51, $p=0.001$), the use of an unrelated donor of any age (HR 3.18, 95% CI 1.07 – 9.48, $p=0.03$), and donor age <40 years, 96% of whom were unrelated (HR 3.85, 95% CI 1.30 – 12.50, $p=0.01$). The cumulative incidence of chronic GVHD at 1 and 2 years was 12.9% and 17.3% respectively. Univariable predictors of chronic GVHD were prior acute grade II-IV GVHD (HR 4.69, 95% CI 1.93 – 11.36, $p<0.001$) and female donor to male recipient (HR 2.61, 95% CI 1.01 – 6.87, $p=0.05$).

Chimerism and Donor lymphocyte infusions

64 of 175 patients received at least one DLI dose, with 113 doses administered in the observation period. The median time to first DLI was 186 days post-transplant. 48% (n=84) of patients had a mixed donor chimerism at day 90 or beyond and 50% (n=42) of these received a pre-emptive DLI. Reasons for non-receipt of a pre-emptive DLI included ongoing immunosuppression, active or prior severe GVHD, lack of available donor cells or the

development of significant morbidity due to aggressive disease relapse. A further 3 received a prophylactic DLI. Of the 53 patients who relapsed, 19 received a DLI as part of salvage therapy, most commonly alongside azacitidine.

OS was significantly better in those with mixed chimerism if DLI was administered (HR 0.22, 95% CI 0.07 – 0.69, $p=0.01$) (Fig. 4a). A similarly lower risk of subsequent relapse was seen in this group (HR 0.33, 95% CI 0.14 – 0.80, $p=0.01$) (Fig. 4b). The median survival duration post-relapse in those who received salvage therapy incorporating DLI was 365 days, compared to 174 days in those who did not ($p=0.007$). Pre-emptive DLI appeared particularly effective at preventing relapse in the AML subgroup (HR 0.06, 95% CI 0.01 – 0.31, $p=0.001$).

Acute GVHD of any grade or chronic GVHD occurred in 46.8% of DLI recipients. 92.3% of post-DLI acute GVHD was grade II and required steroid treatment, 16.6% of whom required second-line or more treatment. No grade III-IV acute GVHD or extensive chronic GVHD events occurred post-DLI for any indication. The median number of DLI doses received by those who did not develop GVHD was 2 (range 1-5) and 1 for those who developed GVHD (range 1-5).

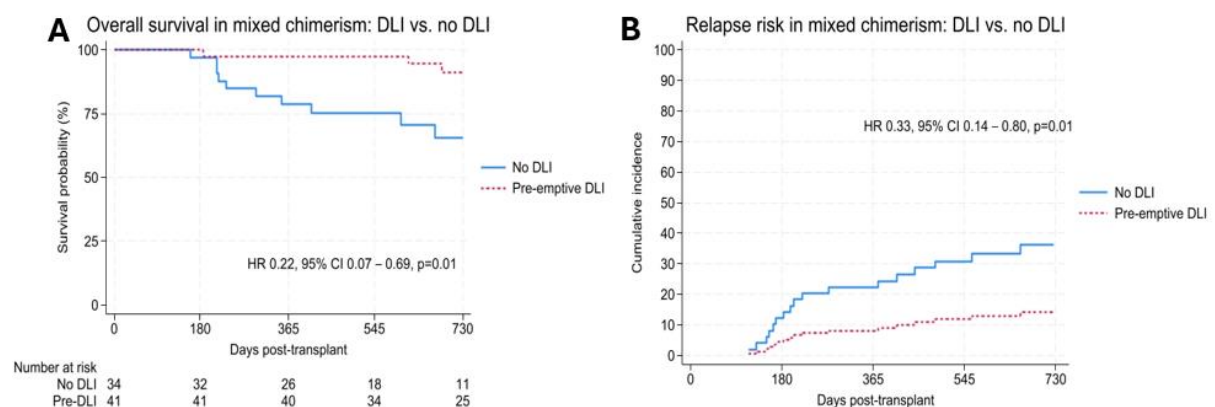


Figure 4. A: Overall survival in persistent mixed chimerism, DLI receipt vs. no DLI. B: Relapse cumulative incidence in persistent mixed chimerism, DLI receipt vs. no DLI. *DLI* = donor lymphocyte infusion.

Infections and immune reconstitution

Low-level viral re-activation was frequent but associated with a low case-fatality rate. CMV was detected by whole blood PCR pre-emptive monitoring in 27.4% of patients and one patient died from CMV-related disease. The adoption of letermovir prophylaxis in the latter phase of the observation period led to a 45.2% relative risk-reduction of CMV detection. Low-level EBV re-activation was detected in 41.7% of patients and there were six cases of post-transplant lymphoproliferative disorder, all of whom were successfully treated with a course of rituximab. Adenoviral DNA was detected in blood in 10.2% (n=18) of patients, of whom 11.1% (n=2) developed fatal organ damage. There was one case of toxoplasmosis, which was fatal, and four of *pneumocystis jirovecii*, with one fatality. Confirmed or probable invasive fungal disease occurred in 5% of patients during neutropenia, steroid-use, or immunosuppression and was associated with subsequent death in one third of cases.

The median CD4 recovery at 3-, 6- and 12-months, was 68.5, 112.5 and 158 x 10⁶/L respectively and recovery in those over 70 years of age (n=5) was comparatively weaker at those points, with medians of 46, 73.2 and 90 x 10⁶/L respectively. A trend toward reduced CD4 recovery at 6 months was seen in those who experienced prior grade III-IV acute GVHD ($p=0.06$) but no difference in recovery was seen based on ATLG dose. The recipient lymphocyte count at the time of ATLG exposure was not predictive of future CD4 count recovery as the median total lymphocyte count at that time-point was $\leq 0.1 \times 10^9$ /L following five days of fludarabine. Immunoglobulin recovery was comparatively robust, with median IgG levels at 3-, 6- and 12-months within the normal range.

Discussion

In this retrospective study, we report on a large series of patients with myeloid disease, transplanted using the FLUBU3ATLG RIC platform at our national adult allogeneic stem cell transplant unit. This study demonstrates an excellent rate of primary neutrophil engraftment of 99.4% which was achieved without the use of post-transplant GCSF. The use of GCSF in this setting is variable internationally, and the encouraging engraftment rates may reduce costs and limit some side-effects. We observe favourable survival outcomes and limited toxicity, while importantly showing a survival benefit of a pre-emptive DLI strategy.

Transplant practice varies across the world despite efforts for harmonisation and co-operation between international programmes. The historical lack of large-scale, multi-centre randomised control trials to address clinical challenges, such as identifying optimal conditioning strategies for the elderly, has to date underpinned a reliance on experiential practice. Developments in transplant medicine, including donor choice, conditioning design, GVHD prophylaxis, supportive care, monitoring and relapse management have expanded significantly in recent years and pose a challenge to adopt practices that improve patient outcomes while maintaining the benefits of institutional familiarity. For example, we did not corroborate recent evidence suggesting a survival benefit when using younger donors, including in the AML subgroup; a finding which emphasizes the need for continued contextualising and auditing of international developments in local practice [5]. As RIC HSCT activity matures and expands, this dataset provides an opportunity to benchmark and inform the future of RIC practice, and indeed poses the question of whether, with fine-tuning, the FLUBU3+ATLG platform should maintain a place in the modern landscape.

Balancing the toxicity of conditioning and NRM risk is an important consideration for older patients. The safety profile of busulfan-based treatment is long-established and is adaptably dosed to produce a range of conditioning intensities, suitable for a wide age range [35-37]. Alternative alkylators, however, are popular and need to be considered. Direct comparator

studies are limited, and a trade-off between relapse and NRM attenuates differences, particularly in melphalan-based approaches, which typically demonstrate a higher NRM incidence compared to busulfan [4, 16-19, 38-41]. Treosulfan shows promise in balancing the risks of toxicity and efficacy as reported by Beelen et al., however the busulfan dose in that study was lower than is used in our protocol (6.4 vs 8mg/kg) [15]. The competitively low 2 year NRM rate of 11% demonstrated in our study cohort is a strength of this busulfan-based approach, particularly as post-transplant relapse prevention and management strategies improve. While the NRM incidence in our myelofibrosis subgroup was higher than in other disease subgroups, recent focus on splenic debulking and the use of JAK inhibitors throughout transplant may in the future confirm a reduction in risk [42, 43].

The low NRM risk in the oldest age group in this study highlights the importance of careful candidate selection. At our institution, transplant candidates are typically reviewed by a transplant physician on at least two occasions prior to proceeding, providing an opportunity to optimize co-morbidities and co-ordinate care with primary treating teams. In the latter phase of the study older patients received a peri-transplant review by a specialist physiotherapist and a geriatrician with special interest in haemato-oncology to further enhance fitness and identify potential vulnerabilities. A recent large observational study by Sorrow et al. including 315 patients who received a transplant for AML or high risk MDS confirmed the survival benefit of transplant in older patients, including a 35% relative-risk reduction of death in those >65 years [33]. However, in multivariate modelling this benefit was masked in all but those with high-risk disease and those who never achieved CR1, exposing the pitfalls of selection bias and the lack of prospective and randomized clinical trials in this area.

The phase II results reported by Kroger et al. on behalf of the EBMT, combining FLUBU3 with ATG/ATLG in myelofibrosis, was an important milestone in encouraging RIC activity and the

protocol proved effective across a range of myeloid malignancies [3]. While a survival disadvantage above a recipient age of 55 years was reported in that paper, in our study this effect was not replicated. This may reflect patterns in candidate selection in older patients, incremental refinements in supportive care over the intervening years and modern strategies for monitoring, preventing and treating relapse.

The strongest competitor to ATLG-based GVHD prophylaxis is the rapid adoption of PTCy. In the landmark BMT CTN 1703 study, Bolanos-Meade et al. reported 1 year OS and DFS estimates of 77% and 67% respectively, with 1 year incidence of NRM and relapse reported at 12.3% and 20.8% respectively, using a RIC-PTCy platform. The incidence of neutrophil recovery by day 28 and platelet recovery by day 100 was 90.3% [44]. Patients on the study arm had a median age of 64 years, 87% were recorded as white and at least 79% of patients had myeloid indications for transplant. 96.7% had fully HLA-matched donors, siblings in 28%. This population is quite similar to ours and facilitates, with limitations, comparison of our results – which show comparable survival outcomes with no neutrophil engraftment failures. Sibling donors have historically been abundant in Ireland and the impetus to expand haploidentical use has been modest, leading to substantial experience with ATLG-based prophylaxis. Direct comparison of ATG/ATLG in the sibling and matched unrelated setting alongside study of the appropriate cyclophosphamide dose and timing in older patients are areas of ongoing study worldwide (NCT06001385, NCT03983850) [45, 46]. The attractive properties of PTCy, with predictable pharmacokinetics and low rates of GVHD are counterbalanced by potential toxicity, especially for the elderly.

Immune constitution, counterbalanced by severe GVHD and infectious complications is a concern in RIC HSCT. The perceived capacity for the patient to endure and overcome these complications is frequently a subjective measure of transplant candidacy. It is therefore encouraging to note the modest severe acute GVHD cumulative incidence of 11.2% and a risk

of chronic GVHD of 17.3% at 2 years indicates the efficacy of ATLG. Importantly, despite modest CD4 recovery in this cohort, infectious complications were manageable, with limited CMV activity in the letermovir era.

A liberal but methodical approach to DLI use is an important modifier of outcome in this patient group. The clear reduction in relapse risk and significantly improved survival of patients who received a pre-emptive DLI for mixed chimerism highlights the dynamism of RIC HSCT, where persistent mixed donor chimerism is common. The use of a dose-escalated treatment schema was demonstrably well tolerated in all age groups and no severe GVHD or NRM events occurred post-DLI, which are events of concern in frailer patients. Importantly, when appraising the risks of DLI, the availability of novel effective therapies for GVHD including ruxolitinib, belumosudil and axatilimab, helps to temper the severity of that risk [47-49].

A small number of cases with prophylactic DLIs are included in this dataset with positive outcomes and support the growing evidence of benefit, although randomized studies are awaited (NCT03597321) [21, 50]. While outcomes for those who relapse post-allograft remain unsatisfactory, many are now offered salvage therapy, including hypomethylating agents, targeted therapy, and complementary DLI. The doubling of survival in those who received a DLI in this study is encouraging, although subject to the retrospective bias of candidate selection.

Conclusion

FLUBU3+ATLG is an effective RIC regimen for selected older patients with myeloid malignancies with a tolerable safety profile. Institutional and international familiarity, coupled with incremental refinements in supportive care have helped benchmark its position in modern-day clinical practice and sets the thresholds that the many exciting developments on the horizon for RIC HSCT practice must exceed. The results here described support the need

for randomised studies of the role of HSCT in older and frailer patients and encourages the incorporation of rational DLI use in study designs.

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Supplementary Table 1: Unadjusted hazard ratios for acute GVHD, chronic GVHD and NRM			
Outcome	Number of events	Hazard ratio (95% CI)	P Value
Acute GVHD II-IV	81		
Recipient age > 65 years	27/175	1.03 (0.57 – 1.88)	0.9
Donor age < 40	90/174	1.13 (0.74 - 1.75)	0.56

Unrelated donor	98/175	1.14 (0.75 – 1.76)	0.53
Tacrolimus use (vs. CSA)	64/175	1.59 (1.02 – 2.50)	0.04*
CNI levels outside target in first 30 days > 1 occasion	114/175	4.38 (1.34 – 14.23)	0.01*
Acute GVHD III-IV	19		
Recipient age > 65 years	27/175	1.50 (0.49 – 4.56)	0.46
Non-AML disease-type	86/175	2.18 (1.31 – 3.51)	0.001*
Donor age < 40	90/174	3.84 (1.30 – 12.50)	0.01*
Unrelated donor	98/175	3.18 (1.07 – 9.48)	0.03*
Tacrolimus use (vs. CSA)	64/175	2.54 (1.03 – 6.28)	0.04*
Chronic GVHD	28		
Recipient age > 65 years	27/175	0.66 (0.20 – 2.17)	0.49
Donor age < 40	90/174	1.32 (0.61 – 2.88)	0.48
Unrelated donor	98/175	0.66 (0.31 – 1.41)	0.28
Female donor to male recipient	23/175	2.61 (1.01 – 6.87)	0.05*
Prior aGVHD grade II-IV	81/174	4.69 (1.93 – 11.36)	<0.001*
Prior aGVHD grade III-IV	19/174	4.62 (1.86 – 11.48)	0.001*
NRM	19		
Recipient age > 60 years	89/175	1.35 (0.54 – 3.35)	0.51
High-risk myelofibrosis	May-28	8.61 (1.91 – 38.69)	0.005*
Acute GVHD grade III-IV	19/174	5.29 (2.15 – 13.04)	0.001*
Invasive fungal disease	9/175	8 (3.11 – 20.60)	<0.001*
Relapse	53		
Recipient age > 65 years	27/175	1.43 (0.73 – 2.79)	0.29

CIBMTR DRI High	54/175	1.86 (1.18 – 2.94)	0.007*
Graft cryopreservation	20/175	2.38 (1.11 – 5.10)	0.02*
ABO Mismatch	81/175	1.79 (1.04 – 3.08)	0.03*
High graft T cell content	85/175	2.30 (1.29 – 4.11)	0.004*
Chronic GVHD	28/173	0.40 (0.17 – 0.94)	0.03*
Pre-emptive DLI	43/82	0.33 (0.14 – 0.80)	0.01*
Mortality	50		
Recipient age > 65 years	27/175	0.66 (0.67 – 1.46)	0.26
aaHCT-CI > 2	78/175	1.29 (0.75 – 2.26)	0.35
CIBMTR DRI High	54/175	1.74 (1.04 – 2.90)	0.03*
High-risk MF	May-28	4.36 (1.34 – 14.17)	0.01*
ABO Mismatch	81/175	2.34 (1.30 – 4.15)	0.003*
High graft CD8 ⁺ content	85/175	1.92 (1.07 – 3.45)	0.02*
Chronic GVHD	28/173	0.36 (0.12 – 0.98)	0.04*
Pre-emptive DLI	43/82	0.22 (0.07 – 0.69)	0.005*
Therapeutic DLI	19/51	0.43 (0.18 – 0.98)	0.03*

Appendix 6: Chapter 2 cytokines summary paper

Serial cytokine expression in reduced intensity conditioning haematopoietic stem cell transplant for myeloid malignancies: predicting GVHD and mortality

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Abstract

Inflammatory signalling underpins many outcomes in allogeneic haematopoietic stem cell transplantation. Serum cytokine levels may have utility as dynamic biomarkers to describe and predict transplant complications including GVHD, NRM, relapse and overall mortality. We performed an observational study of 32 sequential transplants for myeloid malignancies, conditioned with reduced intensity fludarabine, busulfan and anti-T lymphocyte globulin in Ireland. A panel of 10 cytokines including IFN γ , IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A, IL-33 and TNF α was analysed by electrochemiluminescence at sequential time-points from pre-transplant to day 180. Correlation with clinical outcomes identified recurrent associations, including pre-GVHD peaks in IFN γ and an increased risk of GVHD with higher IL-1 β pre-transplant and IL-6 at day 60. Liver GVHD associated with excess IL-2 at day 0 and reduced IL-4 at day 60, whereas gastrointestinal GVHD occurred with excess IFN γ , IL-17A and IL-1 β at day 0. In those who relapsed, higher IL-1 β pre-transplant and IL-12p70/IL-17A at day 100 was seen. In those who died, higher IFN γ and IL-6 pre-transplant, IL-17A at day 7, IL-2 at day 28 and IL-12p70 at day 100 were seen. Exploratory serial cytokine measurement using modern techniques is informative and could help guide transplant management alongside clinical predictive tools.

Introduction

Cytokines are secreted by a range of cell types and bind specific target receptors and instigate signal transduction[1]. Target receptors, once engaged, lead to downstream changes in transcription, protein expression and ultimately cell behaviour. Cytokines have direct and indirect effects, either by direct stimulation of a metabolic pathway or by stimulation of an effector class of cells such as T cells, where they can encourage differentiation and proliferation. Cytokines are detectable in serum albeit at different concentrations depending on the cytokine and timing of sampling. Secretion patterns may be temporally correlated with clinical outcomes in allogeneic haematopoietic stem cell transplant (alloHSCT), such as disease activity, inflammation, and graft-versus-host disease (GVHD)[2-11]. In addition to temporal and situational secretion variance, differences in secretion can be seen on a patient basis due to polymorphisms in the genes encoding both cytokine and receptor[1, 12-14]. In alloSCT, the inflammatory milieu is delicately balanced by opposing effects, which are frequently influenced by extrinsic manipulation and host/donor interactions and affected by underlying disease and conditioning therapy[15].

Understanding the pro- and anti-inflammatory cytokines responsible for these processes and their evolution over time may delineate the biology and variability of alloHSCT. Many cytokines demonstrate site-specific and dose-dependent effects, where a given concentration may have either inflammatory or anti-inflammatory consequences or varying clinical manifestations depending on the organ site[16-19]. Several inflammatory and pleiotropic cytokines such as interleukin (IL) -1, 2, 4, 6, 12, 17, 21, 22 & 33, tumour necrosis factor alpha (TNF α) and interferon-gamma (IFN γ) in addition to anti-inflammatory cytokines including IL-10 and transforming growth factor beta (TGF β) have been studied in the alloHSCT context[20-41]. T cell biology is intrinsically associated with cytokine expression as both secretors and principal targets for cytokine mediated effects. T cell differentiation, proliferation and exhaustion are driven by cytokines produced by other T cells, endothelial, mucosal, epithelial, and stromal cells and other haematological cells including myeloid cells, antigen presenting cells, NK cells, B/plasma cells and tissue macrophages[42-45]. This complex network of up- and down-regulation of activity is heavily dependent on cytokine patterns, and in alloHSCT involves the combining of two non-identical immune systems.

The archetypal clinical correlate for cytokine activity is GVHD, which is a consequence of unbalanced inflammatory signalling. GVHD is a stereotypical clinical syndrome and affects around half of all RIC alloHSCT patients, although with varying degrees of severity and organ specificity[46]. Several cytokines have shown temporal- and dose-related associations with patterns of clinical GVHD. A relationship between IFN- γ , TNF α , IL-1 β , IL12p70 and IL-17A and GVHD is well established, and organ-specific associations are known, such as TNF α , IFN γ and MAIT cell-derived IL-17A in gastrointestinal GVHD. The associated graft-versus-leukaemia (GVL) effect has also been demonstrated with IL-2, IL-12p70 and IFN γ . Time-dependent effects are seen with IL-2, IL-12p70 and IL-33, amongst others. Additional correlation between cytokines and the risk of non-relapse mortality (NRM) and disease relapse are less well defined, but inflammation, mediated by cytokine signalling, is known to play a key role in

malignant disease pathophysiology and adverse transplant outcomes [47, 48]. Emerging clinical scores are proxy measures of this inflammatory activity, as seen with the the Endothelial Activation and Stress Index (EASIX) [49-51].

Commercially available multiplex immunoassays using chemiluminescence techniques allow for high through-put, and the techniques are well established in clinical medicine[52]. Compared to cellular analysis with flow cytometry, where expertise in gating is required, cytokine analysis requires comparatively less result analysis. Cytokines and their relationship to transplant milestones are of interest as they may help predict transplant complications, allowing early intervention, or represent therapeutic targets in and of themselves[53-61]. The impact of timing of measurement is important, as cytokine expression in the early transplant phase is reflective of both residual host and newly engrafting donor profiles. Similarly, in later phases of transplant where mixed donor chimerism occurs, cytokine expression can be the product of both recipient and donor immune activity. T cell depletion and steroids reduce global cytokine secretion by suppressing T cell proliferation, and calcineurin inhibitors reduce IL-2 mediated cell signalling. Serial measurement may help to account for these myriad mediators of secretion by mapping trends and accounting for outliers.

Methods

Study cohort

32 adults with AML, MDS and MPN at the National Adult Stem Cell Transplant Programme in Ireland in 2021/2022 were included. All received fludarabine (180mg/m² over day -9 to -4), intravenous busulfan (8mg/kg over day -5 to -3), rabbit ATLG (Grafalon®: 30mg/kg sibling, 60mg/kg unrelated donor over day -3 to -1), low-dose methotrexate (30mg/m² over day +1/3/6) and a calcineurin inhibitor. Graft source was unmanipulated peripheral blood stem cells.

Analysis

We measured 10 cytokines using multiplex electrochemiluminescence (U-PLEX: Mesoscale Discovery®, USA) at 8 time-points (pre-transplant, D0, D7, D14, D28, D60, D100 & D180). The panel included IFN- γ , IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A, IL-33 and TNF- α . Results were correlated with clinical endpoints including relapse-free survival, acute and chronic GVHD incidence and severity, immune reconstitution of CD4⁺ T cells, relapse, NRM and overall survival. GVHD was graded by Glucksberg and NIH criteria [62, 63]. Immune reconstitution was assessed at 3, 6 and 12 months by CD4 and IgG levels. Samples were processed in duplicate and run as per manufacturer's instructions, with an overnight supernatant incubation protocol and reference calibrators. Analyte concentrations were calculated using Discovery Workbench software (version 4.0). Chimerism analysis utilised STR-PCR with a sensitivity of 3%.

Statistical analysis

Categorical characteristics were described using medians, ranges, and proportions. Comparison of categorical variables was conducted using Fisher's exact test and comparison of medians with the Kruskal-Wallis H test for non-parametric data. Survival was calculated using Kaplan-Meier estimates. The cumulative incidence of NRM, relapse, acute and chronic

GVHD and immune reconstitution was calculated in a competing risks model (Fine and Gray's). Univariable analyses used a Cox model for OS and Gray's test for relapse, NRM and GVHD. A Cox proportional-hazards model using a backwards step-wise approach was used for multivariable regression. Hazard ratios and 95% confidence limits are provided as a summary measure. *P* values are two sided with a significance of 0.05. Analysis was conducted using STATA (Version 18.0, StataCorp, Texas, USA).

The study was conducted in accordance with regulatory and data protection requirements and was approved by the institutional ethics committee and all patients provided written consent. All co-authors had access to the data and vouch for its completeness and accuracy.

Results

Patient characteristics and outcomes

Patient characteristics are summarised in Table 1. The median age was 60 years (range 47-74) and the median follow up was 502 days. Transplant indications were AML (n=17), MPN (n=9) and MDS (n=6). 30 patients had intermediate or high risk (CIBMTR-DRI) disease and 2 had relapsed favourable risk disease. Half of donors were unrelated, and all were 10/10 HLA-matched. Median neutrophil engraftment, without GCSF support, occurred at 18 days and platelet engraftment at 19 days. There were no episodes of primary graft failure or rejection.

The estimated overall survival at 1 year was 87.25% (range) and the relapse-free survival was 76.67% (range). There were 2 non-relapse mortality events and X relapse events by last follow up. Acute GVHD grade II or higher occurred in almost half (46.8%), with grade III or higher in 9.3%. There were 12 episodes of skin GVHD, 12 gastrointestinal and 6 liver. Chronic GVHD occurred in 12.9% at last follow-up, half of which was extensive. Donor T cell chimerism was mixed in 15/26 patients at 3 months and pre-emptive DLI was used in 8 patients, with a further 3 receiving a therapeutic post-relapse DLI. The median CD4 recovery at 3 and 6 months was $58 \times 10^6/\text{kg}$ and $95 \times 10^6/\text{kg}$ respectively.

Table 1	
Patient characteristics	
Patient number	32
Age, n (%)	Median: 60 (47 – 74)
Sex, n (%)	
Male	20 (62.5%)
Female	12 (37.5%)
Disease subtype, n (%)	
AML	17 (53.1%)
MDS	6 (18.7%)
MPN	9 (28.1%)
CIBMTR- DRI, n (%)	
Low	2 (6.25%)
Intermediate	19 (59.3%)
High	11 (34.3%)
Age-adjusted HCT-CI, median (range)	Median: 2 (1 - 6)
Donor source, n (%)	
MUD	16 (50%)

	Sibling	16 (50%
Donor age, median (range)		Median: 38 (20 – 67)

Cytokine secretion

Cytokine secretion varied throughout transplant and between individual cytokines (Fig.1). Median secretion levels were consistently above the lower limit of detection (LLOD) for IFN- γ , IL-6, IL-10, IL-17A and TNF- α , and there was variability for IL-1 β , IL-2, IL-4, IL-12p70 at each time-point (Table 2). A nadir at day 7 in IFN- γ secretion was followed by a peak level at day 28. Early peaks at day 0 were seen with IL-2, IL-10, IL-17A and TNF α secretion. Peri-engraftment peaks occurred with IL-6 and IL-4 at days 14 and 28. Both IL-1 β and IL-12p70 showed less variability between timepoints and increased marginally over time. Median IL-33 levels were consistently below the LLOD using this assay. Examining the relationship between cytokines, positively correlated secretion patterns were seen between each cytokine at most timepoints, with occasional individual negative correlations between IFN γ and other cytokines in the early timepoints.

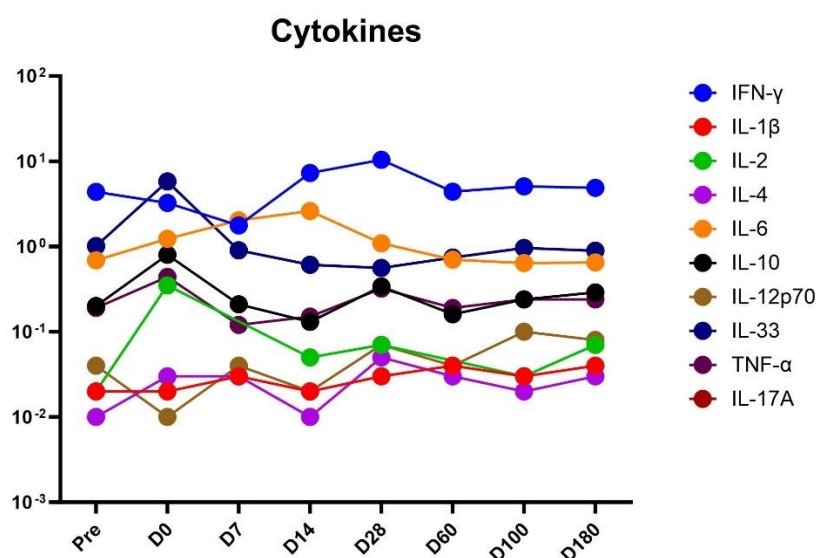


Figure 1: Logarithmic representation of median measurable cytokine levels at serial time-points. Levels of <0.01 pg/ml not represented.

Table 2: Median cytokine levels (pg/ml)								
	Pre	D0	D7	D14	D28	D60	D100	D180
IFN γ	4.38	3.24	1.76	7.30	10.39	4.40	5.08	4.86
IL-1 β	0.02	0.02	0.03	0.02	0.03	0.04	0.03	0.04
IL-2	0.02	0.35	0.00	0.05	0.07	0.00	0.03	0.07
IL-4	0.01	0.03	0.03	0.01	0.05	0.03	0.02	0.03
IL-6	0.69	1.23	2.03	2.61	1.09	0.70	0.64	0.65
IL-10	0.20	0.80	0.21	0.13	0.34	0.16	0.24	0.29
IL-12p70	0.04	0.01	0.04	0.02	0.07	0.04	0.10	0.08
IL-17A	1.01	5.80	0.90	0.61	0.56	0.74	0.96	0.89
IL-33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TNF α	0.19	0.44	0.12	0.15	0.32	0.19	0.24	0.24

Clinical correlation

There were no significant differences in median cytokine level based on patient age, gender, disease subtype or donor type at any timepoint. IFN- γ secretion was lower in those who subsequently developed grade II-IV acute GVHD at the day 14 time-point ($p=0.004$) but not significantly different between those who developed acute GVHD and those who did not at other time-points. Early higher IFN- γ levels at day 0 were seen in those who subsequently developed gastrointestinal GVHD as compared to skin or liver disease ($p=0.03$). A recurrent trend of peak IFN γ secretion occurring at 1 or 2 time-points in advance of the onset of clinical acute GVHD was noted. Higher levels were also noted at the pre-transplant time-point in those who subsequently died ($p=0.01$).

Higher IL-1 β secretion was seen at the pre-transplant time-point in those who subsequently developed grade II-IV acute GVHD ($p=0.04$) and at the day 100 time-point in those who developed subsequent chronic GVHD ($p=0.05$). IL-1 β secretion at day 0 was higher in those who developed gastrointestinal acute GVHD ($p=0.05$) and similarly at day 7 ($p=0.04$). Higher secretion was seen pre-transplant in those who relapsed post-transplant ($p=0.03$) but no difference in survival was seen. IL-2 secretion was similar between those who developed acute and chronic GVHD and those who did not. However, in acute GVHD subgroups, higher levels were seen at day 0 in those who later developed liver GVHD ($p=0.01$).

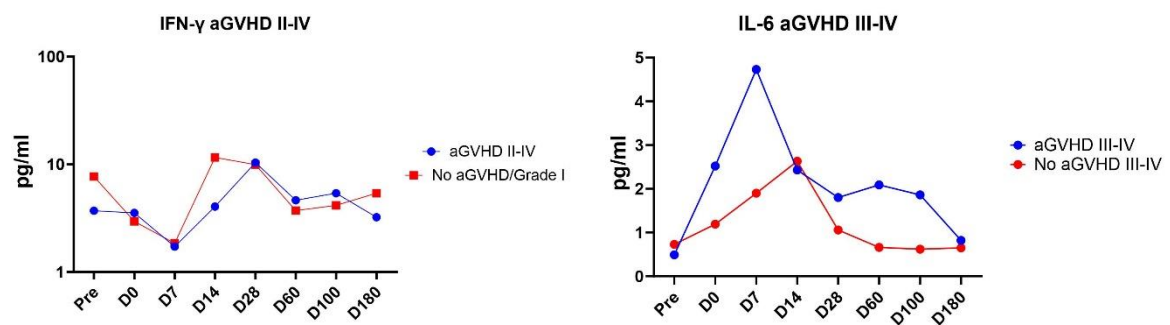


Figure 2. A: Median secretion of IFN- γ comparing those who developed grade II-IV acute GVHD vs. those who developed grade I or no GVHD. B: Median secretion of IL-6 comparing those who developed grade III-IV acute GVHD vs. those who developed grade I-II or no GVHD.

IL-4 levels did not show variance between those who did or did not develop acute or chronic GVHD but there was a suggested protective effect against liver GVHD at the day 60 time-point, where lower levels were found in those who developed this form of GVHD. IL-6 levels at day 60 were associated with grade III-IV acute GVHD ($p=0.04$). Those who had raised levels at the pre-transplant time-point experienced more mortality events ($p=0.002$). Higher IL-10 levels were seen at the day 28 time-point in those who subsequently developed grade III-IV acute GVHD, and furthermore at day 60 ($p=0.007$). Higher IL-12p70 levels at the day 100 timepoint were seen in those who later died ($p=0.04$) and a similar association with subsequent relapse was seen at this time-point ($p=0.03$).

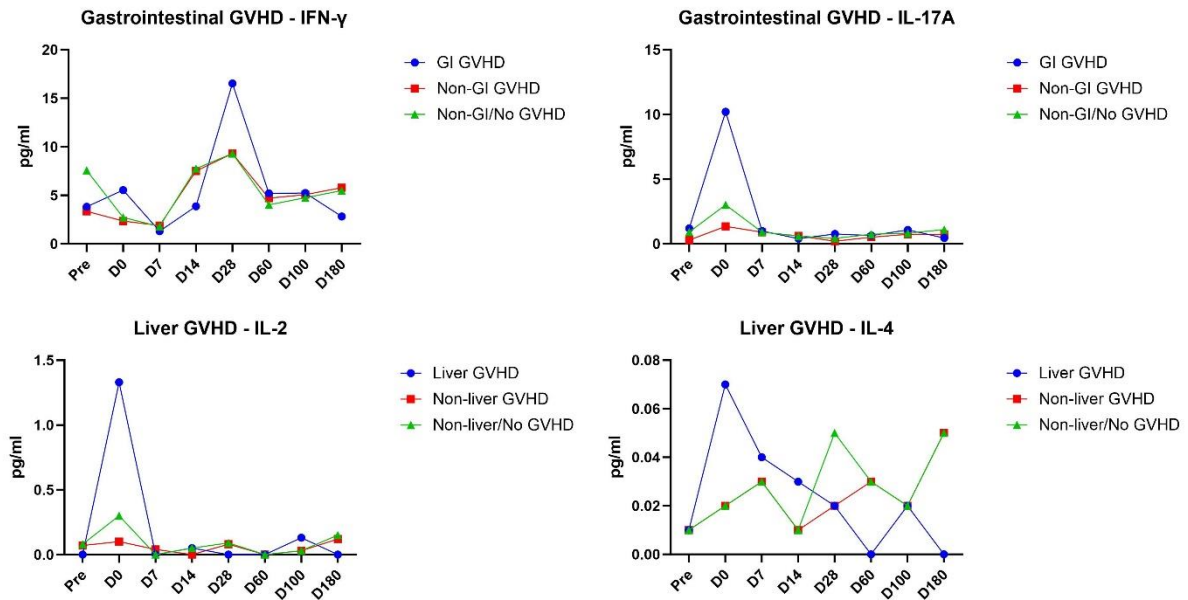


Figure 3. A: Median secretion of IFN-γ comparing those who developed gastrointestinal GVHD vs. those who developed non-gastrointestinal GVHD or no GVHD. B: Median secretion of IL-17A comparing those who developed gastrointestinal GVHD vs. those who developed non-gastrointestinal GVHD or no GVHD. C: Median secretion of IL-2 comparing those who developed liver GVHD vs. those who developed non-liver GVHD or no GVHD. D: Median secretion of IL-4 comparing those who developed liver GVHD vs. those who developed non-liver GVHD or no GVHD.

An association with IL-17A and gastrointestinal acute GVHD at day 0 was seen ($p=0.005$) and early higher levels at day 7 were observed in those who subsequently died ($p=0.04$). Day 100 levels were also higher in those who relapsed subsequently ($p=0.03$). No meaningful clinical observations could be made with IL-33 due to globally low secretion and there was no evident differential TNFα secretion between clinical outcomes.

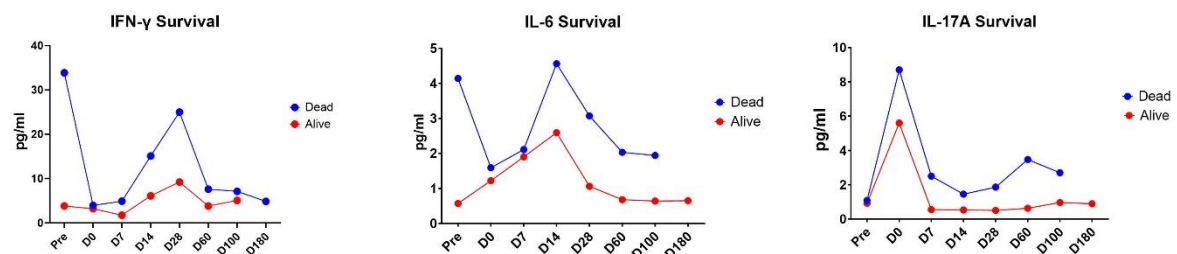


Figure 5. A: Median secretion of IFN-γ comparing those who died vs. survivors. B: Median secretion of IL-6 comparing those who died vs. survivors. C: Median secretion of IL-17A comparing those who died vs. survivors.

Discussion

RIC alloHSCt involves a careful balance of GVL and GVHD modulated primarily by T cells and their signalling. The key role of cytokines in immunogenicity and tolerance is of pertinence in the RIC setting, where successful outcomes are not predicated on the upfront conditioning intensity. The methods by which adverse transplant outcomes are abrogated include tailoring immunosuppression, DLI and prophylactic maintenance or consolidation anti-cancer therapy. Currently these interventions are guided by clinical features, disease risk, chimerism analysis and MRD monitoring. However, predicting outcomes on an individual basis remains challenging and therefore patient-specific tools such as cytokine biomarkers are attractive adjuncts that may help predict an outcome before its manifestation. To fulfil this role, testing must demonstrate reproducibility, cost effectiveness, and meaningful turn-around times.

Within the format of this exploratory study, serial cytokine measurement was readily achieved alongside standard of care practice at our institution. The laboratory technique is not labour-intensive and although our samples were frozen prior to batch analysis, real-time testing of some of the cytokines could conceivably be performed with actionable turnaround times. Each of the pre-selected cytokines here studied offered interesting insights into secretion patterns, inter-cytokine relationships, and their temporal association with clinical outcomes. While the numbers of patients were limited by the study duration, sufficient patients were recruited to accrue almost 250 distinct samples across the 32 patients.

IFN γ kinetics showed a pre-transplant minor peak, perhaps indicating a degree of baseline inflammation prior to conditioning, possibly provoked by cumulative pre-transplant treatment effect and its attendant complications. The major peak at day 28 likely reflects early donor inflammatory stimulation and sets the stage for induction of GVHD in some. While IFN γ is primarily pro-inflammatory, it also has pleiotropic effects, and this was most noted in that those with higher levels at the day 14 time-point were less likely to develop grade II-IV acute GVHD. The association between IFN γ and graft rejection was not identified, as no complete rejection events occurred, however it is noteworthy that in the case of primary platelet engraftment failure, IFN γ levels were significantly higher than median [35]. A correlation between death and raised IFN γ levels pre-transplant, and to a lesser degree at day 28, while likely multifactorial, suggests an association between inflammation and mortality, be it due to underlying disease or treatment complications. A recurrent pattern of IFN γ peaks shortly before onset of clinical acute GVHD, is encouraging for its use as a predictive biomarker. A site-specific relationship was additionally noted in gastrointestinal GVHD, which could prompt additional organ monitoring or guide early investigation in those not yet displaying stereotypical clinical signs.

IL-6 levels at the pre-transplant time-point correlated with reduced survival, much like IFN γ . The proinflammatory nature of IL-6 is further demonstrated by the association of increased risk of high grade acute GVHD in those with raised levels between day 60 and 100. IL-6 is an attractive study target for GVHD management given the now increasing familiarity with blockade in many transplant centres due to tocilizumab use in CAR-T therapy, although the optimal timing is unclear and required prospective study [56, 57, 64-69].

Higher IL-10 levels were found in those who developed high-grade acute GVHD at the day 28 and 60 time-points, which likely reflects oppositional secretion to the proinflammatory cytokines also in excess at these time-points such as IFN γ and IL-6. IL-17A shows promise as a

robustly quantifiable cytokine with the potential clinical correlates of relapse, death and gastrointestinal GVHD, particularly with higher levels early during transplant. This may represent early conditioning induced recipient secretion from mucosal MAIT cells in the setting of inflammatory mucositis and has a potential for synergistic predictive capability alongside IFN γ . TNF α measurement was robust but in this series no significant clinical correlations could be identified, however the published data suggests that in a larger series, differential associations may become more apparent [28, 70].

IL-1 β , IL-2, IL-4 and IL-12p70 were less consistently measurable throughout the series, but nevertheless a number of potential clinical correlations with secretion levels were identified, and could be explored in a larger series or with a more sensitive assay. Increased IL-1 β levels pre-transplant in those who went on to relapse, but also those who developed high grade acute GVHD, alludes to the known association between IL-1 β with acute GVHD, without an attendant GVL effect [26]. While early IL-1 β excess was seen in those who subsequently developed gastrointestinal acute GVHD it was more robustly measured by IFN γ and IL-17A.

An association with increased IL-2 secretion and mortality at days 28 and 60 was noteworthy, and furthermore, in those who developed liver GVHD, early excess at day 0 were noted in advance of clinical detection. IL-4 in most instances showed a negative correlation with GVHD, and therefore in those who developed liver acute GVHD, reduced IL-4 levels were noted at day 60, which suggests there may be a protective role for this cytokine in this disease process. Late excess of IL-12p70 was seen in those who went on to relapse or die, which if replicated in larger numbers could offer dynamic longitudinal risk assessment. IL-33 measurement was ill-suited to this monitoring modality, being immeasurable at many timepoints, and therefore measurement of ST2 levels may have greater yield [71, 72].

The natural limitations of this observation study must be considered when interpreting results. The number of patients and the incidence of events of interest during the study period limit the power to support the observed associations, and although several clinical correlations were statistically significant, they are exploratory in nature. This patient cohort was homogeneously treated with an established RIC regimen, however the wider context of evolving practice in T cell depletion, particularly the increasing use of post-transplant cyclophosphamide, should be considered, as alternative GVHD prophylactic measures are likely to impact cytokine expression and potency.

The results of these analyses offer insights into the patterns of secretion throughout transplant and highlight the waxing and waning inflammatory and anti-inflammatory signalling driving clinical outcomes such as GVHD, relapse and survival. These data support further study of this approach in a predictive model, alongside clinical predictive tools, to identify transplant-related adverse risks and allow timely intervention. The findings of this study support extending application to a larger number of patients with extended follow up. To streamline resource utilisation, a smaller panel comprising the most robust cytokines, IFN γ , IL-6, IL-10 and IL-17A would help solidify their predictive capabilities. Additional exploration in alternative conditioning regimens, conditioning intensities, disease indications and in donor samples may then further expand the generalisability of these findings.

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