Predictive Microbiome Post-Bone Marrow Transplantation and Acute Graft versus Host Disease

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Declaration

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To My Family

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Abbreviations

aGVHD – Acute Graft Versus Host Disease

ALL- Acute Lymphoblastic Leukaemia

AML -Acute Myeloid Leukaemia

ATG- Anti-thymocyte globulin

BM- Bone Marrow

BMI -Body Mass Index

BMLT- Bone Marrow Leukaemia Trust

BMLT- Bone Marrow Leukaemia Trust

BO- Bronchiolitis Obliterans

BOOP- Bronchiolitis Obliterans Organising Pneumonia

cGVHD- Chronic Graft Versus Host disease

CMV- Cytomegalo Virus

CNS- Central nervous system

DLCO- Diffusion capacity for Carbon monoxide

DLI- Donor Lymphocyte Infusion

DNA- Deoxy Ribonucleic acid

EBMT- European Blood and Marrow Transplant

EBV- Epstein Barr Virus

ECP- Extracorporeal Photopheresis

ES- Engraftment Syndrome

FEV1- Forced Expiratory volume in the first second

FLT3- Fms Related Receptor Tyrosine Kinase 3

FMT- Faecal Microbial Transplantation

GDPR- General Data Protection Regulations

GI- Gastro intestinal

GMS- Gut Microbiota Score

GVHD -Graft Versus Host Disease

HCC- Hepatocellular carcinoma

HCV- Hepatitis C Virus

HIV- Human Immunodeficiency Virus

HLA- Human Leukocyte Antigens

HMP- Human Microbiome Project

HODC- Haematology Oncology Day Care

HSCT- Haematopoietic Stem Cell Transplantation

IBD- Inflammatory bowel disease

IBMTR – International Blood and Marrow Transplant research

IBS- Irritable Bowel Syndrome

IBS-Irritable bowel syndrome

IF– Interferon-gamma

Ig- Immunoglobulin

IL - Interleukin

ILC - Innate Lymphoid Cells

IUBMR- Irish Unrelated Bone Marrow Registry

LPS- Lipopolysaccharide

MALT- Mucosa-associated Lymphoid Tissue

MAMP- Microbe-associated molecular pattern

MDS- Myelodysplastic Syndrome

MHC- Major Histo Compatibility

MHC- Major Histocompatibility complexes

mRNA- messenger RNA

MRSA- Methicillin-resistant Staphylococcus Aureus

NASH- Non-alcoholic Steatohepatitis

NO2- Nitrous oxide

NOD- Nucleotide oligomerization domain

NPM1- Nucleophosmin1

ODC- Ornithine Decarboxylase

OUT-Observed Taxonomic units

PBSC – Peripheral Blood Stem Cells

PCA- Principal Components Analysis

PCoA- Principal coordinates Analysis

RF- Random Forest

RNA – Ribonucleic acid

rRNA- Ribosomal RNA

SCFA-Short Chain fatty acids

SCT- Stem cell Transplant

SCT- Stem Cell Transplant

SDA- Spermine Dialdehyde

SMOX – Spermine Oxidase

TBI- Total Body Irradiation

TGF- Transforming growth factor

TLR- Toll-like Receptors

TNF- Tumour necrosis factor

Treg cells- T regulatory cells

VRE- Vancomycin resistant enterococcus

Summary

Haemopoietic stem-cell transplantation serves as one of the life-saving options for the management of refractory haematological malignancies and rarely for non-malignant conditions. The era of transplantation has significantly progressed due to the wider availability of suitable donors and better treatment options. However, there are several factors limiting the success of transplantation of which acute graft versus host disease continues to pose significant challenges. aGVHD is associated with reduced quality of life and contributes to one of the major causes of non-relapse-related mortality.

There is growing evidence to suggest the role of microbiota in various inflammatory and metabolic conditions of which intestinal microbes and their genomic components merit special attention. The microbiota exerts their effects through local mucosal actions and systemically through their metabolites and enzymatic pathways. Previous studies have demonstrated the changes in the microbial diversity post-stem cell transplantation and their significance on the outcomes, including the risk of GVHD. The impact of this microbial shift on the transplant outcomes is independent of the patient and donor-related factors.

Our study has evaluated the pre-transplant microbial changes in faecal samples of patients undergoing allogeneic stem cell transplantation and corresponding sibling transplant donors. Faecal microbial DNA analysis was carried out in the recipients and sibling donors from the samples collected prior to the pre-conditioning period. Furthermore, genomic sequencing of these microbial DNA was performed to investigate any shift in microbial composition and has been correlated with complications especially, the risk of aGVHD and survival following transplantation. With Random Forest Prediction models we were able to demonstrate the association between microbiome and the risk of aGVHD with an accuracy of more than 70%.

We hypothesise that there would be a specific microbial readout in the faecal microbiome obtained from the patients and donors prior to transplantation which would be predictive of post-transplant complications and in particular acute GVHD.

Our study has demonstrated specific changes in the pre-transplant microbial diversity which was associated with the risk of aGVHD and survival following transplantation. Further analysis has identified the activation of specific enzymatic pathways driving steroid responsiveness in aGVHD. Lower microbial diversity in the sibling donors was linked with severe GVHD in the corresponding recipients. From the study, it was evident that this microbial dysbiosis can occur weeks prior to stem cell infusions and affects the transplant outcomes which was independent of disease and treatment-related complications.

Microbial diversity and richness have been increasingly identified in the pathogenesis of various inflammatory and metabolic disorders including the risk of GVHD. Our study shed light on the fact that lower microbial diversity pre-transplantation is associated with complications such as aGVHD and increased mortality. Modulating or replacing this microbiome would serve as a potential therapeutic target in the management of aGVHD and improving survival following transplantation. The impact of the donor microbial diversity on the severity of aGVHD will be crucial when selecting suitable donors for stem cell transplantation with an improved outcome.

Chapter 1

Introduction

Microbiome in Bone Marrow Transplantation

Growing evidence suggests that the microbiome plays a role in various disease conditions, and a diverse microbiome is essential for maintaining a healthy state. A healthy microbiome is characterised by wider diversity and richness. Microbial richness is defined as the relative abundance of taxa among a microbial composition. Microbes and their metabolic products maintain a homeostatic state with the host immune system and regulate the inflammatory response. Stem cell transplantation has revolutionised the management of refractory haematological malignancies, serving as a rescue therapy and improving patient survival. Similar to many inflammatory and metabolic disorders, microbial dysbiosis can occur following stem cell transplantation and is implicated in the pathogenesis of graft-versus-host disease and the risk of mortality. Interestingly, microbial dysbiosis can occur weeks prior to transplantation and is affected by various host- and disease-related factors. Modifying this microbiota and its functional metabolic pathways could offer promising tools to alleviate the risk of complications and improve the overall survival related to the procedure.

1.1 Haematopoietic Stem Cell Transplantation

Haematopoietic stem cell transplantation (HSCT) remains a lifesaving treatment for refractory haematological malignancies and occasionally for non-malignant conditions. HSCT involves replacing dysfunctional and depleted haematopoietic cells and immune systems with healthy donor cells (Copelan 2006). The successful discovery of this procedure by Thomas and co-workers in the 1960s marked a new era in the field of managing haematological disorders (Thomas 1975). Although this had been initially developed as a rescue therapy for refractory

cancers, its beneficial role has been extended to treat certain autoimmune and non-malignant conditions. In 1968, a child with severe combined immunodeficiency syndrome underwent the first successful allogeneic stem cell transplantation in Minnesota. Over the last decade, the number of autologous and allogeneic stem transplantations has increased worldwide, providing a very optimistic view for managing certain refractory haematological malignancies.

Common sources of stem cells include the bone marrow (BM), peripheral blood (PBSC), and umbilical cord blood. In addition to availability and underlying disease conditions, several recipient factors affect the transplantation process, including age, comorbidities, and the patient's functional status. Human leukocyte antigen (HLA) matching is a key donor factor that determines the success of stem cell transplantation. Autologous stem cell transplantation involves transfusing the stem cells of a patient after undergoing purification processes. This is performed for different cancers, with multiple myeloma being one of the most common indications. Allogenic stem cell transplantation is performed mainly for refractory haematological malignancies, most commonly acute leukaemia. Indications for transplantation in chronic leukaemia and lymphomas are reserved for chemotherapy-resistant cases and for younger patients. Stem cell transplantation is occasionally considered for certain congenital or acquired non-malignant and immunological disorders.

After careful selection of donors and stem cell sources, patients undergo intense pre-conditioning therapy to facilitate the engraftment process. This includes chemotherapy or total body irradiation (TBI) which causes immunosuppression and has antitumour effects. Conditioning therapy can be myeloablative or non-myeloablative (low-intensity) depending on the underlying disease risk, age of the patient, and comorbidities (Barriga 2012).

After transplantation, stem cells travel to the bone marrow niches, proliferate into various mature haematopoietic cells, and appear in the peripheral blood. This process is referred to as

engraftment and is a key factor in determining sustainable effective haematopoiesis (Hutt 2018). For clinical purposes, engraftment is defined as a sustained peripheral neutrophil count $>500/10^6$ /L (Wolff 2002) and platelet count of $>20 \times 10^9$/L (Teltschik 2006). Two major factors affecting the engraftment process are the source of stem cells and pre-conditioning therapy (Hutt 2018). Patients who receive peripheral blood stem cells (PBSCs) have a faster recovery of neutrophils and platelets than recipients of bone marrow infusion. Engraftment syndrome (ES) is a clinical constellation of symptoms such as fever, pulmonary oedema, and multiorgan failure. This is characterised by the systemic vascular leak and pro-inflammatory response with the release of various cytokines and can occur at the time of neutrophil recovery post-transplantation (Chang 2014). With the advancement of effective pre-conditioning therapies and prophylactic measures against graft-versus-host disease, there are promising outcomes in the stem cell transplantation process.

1.2 Graft versus host disease

Graft-versus-host disease (GVHD) is caused by an immunological response between donor T cells and various antigens on the recipient's organs, resulting in inflammatory reactions and tissue damage. Along with infectious complications, GVHD is associated with significant morbidity and mortality after HCT. The risk of GVHD and outcomes of transplantation are affected by various recipient, disease, and graft factors, of which HLA compatibility appears to be one of the key determining factors for the risk and severity of GVHD. HLA proteins are present on cellular surfaces and play a major role in alloimmunity. They are encoded by highly polymorphic genes located on the short arm of chromosome 6, known as major histocompatibility complexes (MHC). Changes in polymorphisms can result in variability in HLA matching during transplantation and can affect the risk of GVHD (Shaw 2003). Minor histocompatibility complexes code for proteins outside HLA foci. Genetic differences in these

foci of minor complexes can also impact GVHD risk, even in HLA-identical grafts, especially chronic GVHD (Spierings 2013).

Acute graft versus host disease (aGVHD) develops within the first 100 days of transplantation and commonly affects organs such as the skin, gut, and liver, causing rash, nausea, diarrhoea, and impaired liver functions (Carreras 2019). The skin is the most common and first affected organ, usually coinciding with the engraftment of stem cells (Ferrara 2009). The severity of GVHD is determined by the extent of the involvement of these organs, and GVHD is classified as grade 1–4 (mild–very severe) (Glucksberg Classification 1974). Grades 3 and 4 have poor prognoses, with a high mortality rate of over 50% aGVHD in fully HLA-matched sibling donors ranges between 35% and 45%, whereas single HLA mismatch unrelated donors carry a GVHD risk of up to 80%.

Chronic GVHD (cGVHD) develops if there is ongoing inflammation and scarring in organs and is one of the main causes of late-onset non-relapse-related mortality. Chronic inflammation and fibrosis lead to permanent organ dysfunction, with significant physical, psychosocial, and functional impacts on a patient's quality of life. Older age, history of aGVHD, mismatched and unrelated donors, and stem cells from peripheral blood sources appear to be the likely risk factors for cGVHD (Carlens 1998). Most cGVHD cases develop within the first year after stem cell transplantation and are usually complicated by preceding aGVHD. However, in 30% of cases, cGVHD cases evolve de novo. (Arai 2015). Although the lungs, intestine, and other organs are rarely affected, the skin, nails, eyes, mouth, and liver are commonly affected by cGVHD.

1.3 Infectious complications post system-cell transplantation

Infectious complications are common and cause significant mortality and morbidity after stem cell transplantation (Sahin 2016). Infections from viral, bacterial, and fungal agents can occur in the post-transplantation period, mostly from the immunosuppressive state and shift of

beneficial commensal organisms in various mucosal surfaces. Neutropenia and mucosal damage are the leading risk factors for infections in the pre-engraftment period. Although bacterial infections predominate, opportunistic infections from viral and fungal agents can also occur during this time with immunosuppressive treatments for GVHD, adding to the complexity of managing these patients in the critical window of the engraftment phase.

Bacterial infections by gram-negative bacilli are common and still account for a high mortality rate, whereas gram-positive organisms can cause infections related to indwelling access catheters. Infections from encapsulated bacteria, such as Haemophilus, Streptococci, and Neisseria, can occur in the later phase of transplantation due to hypogammaglobulinemia and functional asplenia. Fungal agents such as Candida are commonly associated with infections in the early post-transplant period; however, invasive aspergillosis can occur at a later stage and is associated with high mortality and morbidity. Although there is a significant reduction in mortality with better antibiotic and antifungal prophylactic regimens, infections in the posttransplant period continue to be a challenging cause of graft failure and death.

1.4 Relapse following stem cell transplantation

With the increased availability of suitable donors, advanced treatment regimes, and supportive care, there has been significant improvement in the outcomes of stem cell transplantation in terms of GVHD, post-transplant infections, and overall survival. Unfortunately, relapse-related mortality and morbidity remain challenges in the management of haematologic malignancies. According to recent ICBMTR (Centre for International Blood and Marrow Transplant Research) data, relapse of the primary disease accounts for approximately 27–56% of mortality following stem cell transplantation, depending on the donor source (Horowitz 2018). Several factors affect the risk of relapse, including underlying diagnosis, disease risk, genomic mutations, and the intensity of the conditioning regimens (Ossenkoppele 2016). Nowadays, with the use of reduced-intensity pre-conditioning, more patients undergo HSCT

with fewer side effects but with a compromised risk of relapse. Most disease relapses occur in the first year following HCST, with a worse prognosis if it occurs within the first few months (Barrett 2010). Myeloid disease seems to have a higher risk of relapse than other diseases in HCT. Apart from the disease- and treatment-related factors, the host microenvironment also plays a key role in the risk of relapse (Horowitz 2018). The treatment of relapses depends on the disease and patient factors, which include salvage therapy, disease-specific agents followed by immunotherapy, donor lymphocyte infusion (DLI), and consideration of the second transplant in a very selected case (Barrett 2010).

1.5 Microbiota and microbiome:

For decades, human microbes have been used for their symbiotic role in blocking the invasion of pathogenic microorganisms through mucosal surfaces. Research into human-associated microbiota dates back to when Antonie van Leeuwenhoek first began to study microorganisms in the 17th century. Advances in science and molecular techniques in the 20th century have brought us a long way in understanding the role of microbes in human health and disease.

Microorganisms living in close association with different parts of the human body are collectively called microbiota (Manzo 2015). The human body inhabits trillions of microbes on different epithelial and mucosal surfaces, including the skin and respiratory, gastrointestinal, and genitourinary tracts, forming an ecological system critical to human health. The gastrointestinal tract contributes to 95% of microbial colonies, primarily the lower intestine, where the numbers exceed the total number of cells in the human body. Microbial colonies on various epithelial and mucosal surfaces are established from an early stage of life (Palmer 2007) and co-evolve to adapt to the functions of their cellular counterparts. Some of the key cellular functions include energy harvesting, storage, and metabolic regulation (Gill 2006). The indispensable role of microbes in host cell immune function, maintenance of mucosal integrity, and homeostasis account for protection against various diseases.

The microbiome is a frequently used term in association with various health and disease conditions, including genomic components encoding various metabolic and inflammatory pathways. The effects of microbiota on host cell function are influenced by various pathways at the neuronal, immunological, and hormonal levels (Dupont 2020). Until recently with new molecular gene sequencing techniques, little has been known about the significance of these microorganisms on various host cell functions. Large-scale studies, including the Human Microbiome Project (HMP), have provided a better understanding of the different compositions and functions of microbiota (Integrative HMP Research 2014).

The genomic content of microorganisms differs considerably, fitting their structural and functional roles in various environmental niches. Host genetics and environmental factors play important roles in modulating microbial configuration. Although there is a wide range of variability in microbial composition between people, a healthy microbiome is characterised by its diversity and richness. Microbial diversity is measured in various terms mainly alpha and beta diversity, the terms coined by R.H. Whittaker in the 1970s. Alpha diversity was measured by the number (richness) and relative proportions (evenness), whereas beta diversity denotes the proportion of specific taxa in the microbial population. (Whittaker 1972, Dupont 2020)

Contrary to previous acceptance, emerging data have shown the impact of the microbiome on various disease states and as promising treatment targets. Advances in metagenomics and high-throughput sequencing in the early 2000s have greatly accelerated our understanding of microbiome diversity and identified potential links to health and diseases.

In the context of HSCT, a healthy microbiota is essential for shaping local and systemic immune responses and maintaining normal haematopoiesis (Kosravi 2014). Earlier murine studies have shown that germ-free mice have aberrant haematopoiesis and (Khosravi 2014, Balmer 2014)impaired immune function, highlighting the role of healthy microbiota in the

outcome of stem cell transplantation. Several factors, such as diet, environmental influence, and use of antibiotics, which affect the composition of microorganisms and maintain a homeostatic state between these microbes and body cells are essential for a healthy state. Microbial metabolites play an important role in interacting with the host immune system and in regulating inflammatory responses. Microbial dysbiosis has been linked to many metabolic disorders and inflammatory conditions, highlighting its possible pathogenic roles. Microbial dysbiosis is characterised by reduced alpha diversity and changes in taxon composition, with a relative predominance of pathogenic microbiota (Khosravi 2014, Balmer 2014). The link between microbial dysbiosis and GVHD and the various outcomes of stem cell transplantation has been widely studied with cause-association effects. Although several mechanisms, such as the alteration of microbial composition from mucosal damage and dysregulation of the immune response from altered microbiota-derived metabolites, are implicated in the risk of GVHD, more exciting genomic and metabolic pathways are yet to be revealed.

1.6 Microbiome and haematopoiesis:

There is a cross-link between microbiota and several haematological disorders. The microbiome plays an important role in shaping local and systemic immune responses and regulating normal haematopoiesis (Khosravi 2014). Aplastic anaemia which is characterised by pancytopenia and marrow failure is linked to several viral infections, such as hepatitis virus, parvovirus B19, EBV, and CMV infections. (Rauff 2011) Iron is an important co-factor for haematopoiesis and a key regulator of bacterial infections. Iron metabolism disorders are associated with certain bacterial infections (Manzo 2015). Chronic inflammation-related anaemia can alter cytokine production and iron metabolism. Numerous lymphomas such as gastric MALT (Mucosa-associated lymphoid tissue) are associated with chronic *EBV*, *HIV*, *HCV*, and *H pylori* infections (Manzo 2015). Human T-cell lymphotropic virus I is implicated in adult T-cell leukaemia and lymphoma (Matutes 2007).

Several systemic inflammatory and metabolic disorders, infections, and prolonged antibiotic usage are associated with haematological abnormalities, such as anaemia and neutropenia, reflecting the role of microbial communities in bone marrow function. Murine models have shown that intestinal inflammation causes significant stress on haematopoietic progenitor stem cells (Griseri 2012, Kishikawa 2003, Sharma 1996), explaining the possible association between aplastic anaemia and IBD. Nutritional disorders such as malnutrition and obesity are associated with changes in intestinal microbial communities and abnormalities in haematopoietic function (Yan 2018).

Earlier murine studies have shown that germ-free mice exhibit aberrant haematopoiesis and impaired immune function (Khosravi 2014, Balmer 2014). Germ-free mice lacking microbiota have smaller haematopoietic stem and progenitor cells with impaired T-cell function. Commensal bacteria that act via interferon signalling pathways are essential for normal haematopoiesis. The use of antibiotics impairs normal haematopoiesis, as observed in the pre-engraftment phase of bone marrow transplantation. The effect is through the depletion of intestinal microbiota rather than a direct suppressive effect on bone marrow, indicating the role of microbes in normal haematopoiesis (Tada 1996), and is largely independent of the duration of treatment, types, and absorption of antibiotics used (Khosravi 2104, Tada 1996, Josefsdottir 2017, Yan 2018).

The role of dietary fibre in haematopoietic function has been demonstrated by Trompette and co-workers (Trompette 2014) as a predominance of Bacteroidetes species noted with a diet rich in fermentable fibres resulting in increased production of short-chain fatty acids (SCFA). In turn, this has both local and systemic effects on the structure and function of the haematopoietic system. Increased levels of propionate, an SCFA, enhance haematopoiesis of dendritic cell precursors from the bone marrow, which exhibits impaired Th (T-helper) cell activation in the lungs by reducing allergic airway inflammation.

1.7 Microbiome and the immune system:

The host immune system is one of the most important determining factors in shaping the configuration of the normal and dysbiotic microbiome (Levy 2017). In some instances, host control systems limit the size of microbial communities and keep microbes away from the epithelium to prevent damage to the mucosal surfaces (Tiffany 2019). Microbes affect host body function through immune pathways, both locally and systemically, and maintain steady-state haematopoiesis (Belkaid and Hand 2014). Mucosal-associated lymphoid tissues present in the lung and gastrointestinal tract play an important role in evoking an immune response to antigen presentation which is closely linked with the microbiome in respective locations. They respond to immune activation through Toll-like receptors and NOD-like receptors (O'Neill 2013) in response to pathogenic microorganisms. Microbes produce chemical substances such as SCFA, GABA-serotonin, indole, and other metabolites which influence host immune function (Kim 2014).

1.8 Gut microbiome:

The gastrointestinal tract is usually colonised by several million commensal bacteria. Metchnikoff first noted an abundant bacterial population and its potential functional role in the gastrointestinal tract (*The Wilde Medal and Lecture of the Manchester Literary and Phi1losophical Society*. Br Med J. 1901;1(2104):1027–8). Microbial diversity results from the co-evolution of the host immune system and microbes (Malard 2018). A harmonious state with commensal microorganisms in the gut is essential to maintain a healthy state and prevent the emergence of potentially pathogenic bacteria. Microbiota plays an essential role in the structural maintenance of the intestinal barrier and regulate immune activation. The gut mucosal barrier consists of epithelial, goblet, and Paneth cells (Belkaid 2014). These tight junctions regulate the interactions between the microbiota and the host immune system. Enterocyte proliferation and wound healing are impaired in germ-free mouse models (Pull

2005). Bacteroidetes and Firmicutes constitute the majority of microbiota in the healthy gastrointestinal tract.

The potential impact of microbial metabolites is not limited to the gastrointestinal tract, as they circulate around and affect the peripheral tissues. Gut microbiota breaks down dietary fibre into metabolic products, especially SCFA which are immune stimulators as they interact with surface receptors on haematopoietic cells, leading to an increase in cytokine levels. Cytokines can induce the production of antimicrobial peptides and mucosal immunoglobulin (IgA). IgA plays an important role in maintaining microbial diversity and mucosal integrity. Butyrate, an important SCFA, is a potential energy source for enterocytes with anti-inflammatory properties (Vinolo 2011). Some commensal bacteria such as *lactobacilli* stimulate mucin production, thereby contributing to the defence barrier (Cheng 2018). Their roles in T-cell proliferation and apoptosis are important, especially in viral infections (Kim 2014, Kurita-Ochiai 1995). Bacterial species such as *Lachnospira* and *Ruminococcus* stimulate secondary bile acid production in the liver which in turn inhibits the growth of certain pathogenic *Clostridium difficile* (Baktash 2018). Any microbial dysbiosis can result in clinically significant outcomes in patients with haematological disorders, such as infections, and a potential impact on the risk of GVHD.

An altered microbial composition is associated with abnormal intestinal function, as demonstrated by the disruption of the gut mucosal barrier and mucosa-associated lymphoid tissue. This results in increased mucosal permeability, leading to an influx of microbial products across intestinal membranes and altered local and systemic immune responses (Takiishi 2017).

1.9 Diet and microbiome:

Dietary factors are important drivers of the microbial composition of the intestinal tract and help maintain a healthy state. Microbial metabolites are key determinants of mutualistic

interactions between the host and microbes and for homeostasis of immune function. Fibre-rich diets play a significant role in shaping and enriching healthy microbiomes. Their roles have been extensively evaluated in various gastrointestinal disorders such as inflammatory bowel disease (IBD), colon cancer, irritable bowel syndrome (IBS), and airway diseases such as asthma. SCFAs, such as butyrate, acetate, and propionate, are produced by anaerobic bacteria from the fermentation of dietary fibres. They stimulate mucin production and regulate immune pathways by reducing cytokine production, thereby enhancing mucosal integrity and suppressing the inflammatory cascade (Yu 2012). In contrast, a high-fat diet is associated with oxygen-free radicle formation, leading to intestinal inflammation, as seen in obesity, type 2 diabetes, and other metabolic syndromes. Processed foods containing potentially carcinogenic materials can be associated with microbial alterations that contribute to the development of certain malignancies. Alterations in dietary factors are associated with bacterial dysbiosis, resulting in reduced biodiversity and the potential overgrowth of pathogenic bacteria.

Microbiome and outcomes of HSCT:

1.10 Microbiome and graft versus host disease

The major causes of mortality after HSCT are relapse, GVHD, and infection. Microbial diversity plays a significant role in the outcome of HSCT, especially in terms of the risk of GVHD and overall survival. Studies have shown that the baseline microbiome of patients with haematological conditions appears similar to that of healthy people in terms of diversity and richness (Liu 2017). Approximately 30% of patients undergoing allogeneic HSCT retain microbial diversity during the process. During haematopoietic stem cell transplantation, there is damage to the gastric epithelium leading to alterations in commensal bacteria and their diversity. Most of the time, a single taxon with pathogenic potential is predominant and affects the disease outcome.

Earlier studies by Taur et al. (Taur 2014) demonstrated that reduced bacterial diversity is associated with increased mortality after haematopoietic stem-cell transplantation which is unrelated to patient and transplant factors. This was also shown by Peled et al. in one of the recent studies (Peled 2020). As a possible mechanism, one of the studies has suggested that there is damage to the intestinal mucosa from treatments like chemotherapy and antibiotics resulting in the loss of commensal bacteria and the overgrowth of certain pathogenic organisms (Peled 2020). Considering that gastrointestinal microbes are potent modulators of the immune system, any microbial dysbiosis can affect immune reconstitution following transplantation (Manzo 2015). Disruptions in the microbiota during the neutrophil engraftment period following stem cell infusions can be related to poor survival (Peled 2020).

GVHD, one of the most common complications of stem cell transplantation, is associated with low bacterial diversity and dysbiosis. As early as in the 1970s, animal studies by Jones et al. demonstrated that germ-free mice receiving HSCT were protected from GVHD, although the exact mechanism behind this was unknown (Jones 1971). The possible mechanisms suggested included the direct effect of microbiota on the induction of inflammation and tissue injury, which is absent in germ-free mice. Another explanation was that mice grown in a germ-free environment have altered immune responses and are less capable of developing GVHD. (Bauer 1963, Hooper 2012). Emphasising the direct role of the microbiota in GVHD, van Bekkum et al. demonstrated that antibiotic decontamination in mouse models was associated with reduced GVHD risk and improved survival after HSCT (van Bekkum 1974). It has also been shown that certain species of lactobacilli exert a protective effect against GVHD development (Jenq 2012).

Recent advances in molecular techniques have enabled a better understanding of the link between the microbiome and GVHD. Patients with GVHD have lower microbial diversity and richness (Jenq 2012). Certain species of *Blautia* are associated with a lower risk of GVHD-

related mortality (Jenq 2015) and improved survival. Golob et al. found that acute severe gut GVHD was associated with reduced alpha diversity at the time of engraftment (Golob 2017), confirming the results of previous studies. (Jenq 2015) In this study, the absence of *Lachnospira* species, especially *Blautia*, and the presence of oral actinobacteria and *Firmicutes* was associated with an increased risk of GVHD. A recent study (Ingham 2019) involving paediatric stem cell transplantation showed that an abundance of lactobacilli is associated with moderate-to-severe GVHD and high mortality, whereas the predominance of obligate anaerobes, such as *Ruminococci*, is associated with a lower risk of GVHD and better survival. *Enterobacteriaceae* species have been implicated in high levels of inflammation, as indicated by high C reactive protein levels.

Various factors affect the risk of GVHD, including the type of graft, previous chemotherapy, pre-conditioning regimens, use of antibiotics, and T-cell depletion of the graft. It is evident that recipient factors play a major role in the development of GVHD, whereas a study by Liu et al. demonstrated that higher bacterial diversity in donors is associated with a lower risk of GVHD (Liu 2017) which would be an interesting area to explore.

Malignancy relapse remains a challenging problem after HSCT. Factors such as HLA compatibility, the intensity of conditioning regime, source of graft, and graft versus host disease affect the risk of relapse or progression of the disease. During HSCT, pre-conditioning treatment exerts an antitumour effect, and this alloimmunity plays a significant role in reducing the risk of relapse. As intestinal bacteria are modulators of the immune response, including antitumour effects, they might have a potential role in the outcomes of HCT, such as relapse of the malignancy. In a retrospective study, Peled et al. (Peled 2017) demonstrated a possible association between certain species of *Eubacterium limosum* and a lower risk of relapse and disease progression. However, no significant association has been found between

overall microbial diversity and the risk of relapse, which has been noted in similar previous studies (Taur 2014).

1.11 Effect of microbial metabolites on GVHD and survival

As mentioned previously, bacterial metabolic products, such as SCFAs, play a key role in immune modulation and HSCT outcomes. Bacterial dysbiosis can alter the levels of butyrate, an SCFA, resulting in decreased histone acetylation and increased risk of gastrointestinal GVHD. It has been demonstrated (Mathewson 2016) that the replacement of butyrate helped improve intestinal mucosal functional integrity, reduce apoptosis, and thereby alleviate the severity of GVHD. Tryptophan metabolites have been implicated in association with GVHD, and lower urinary 3IS (3-indole sulfate) has been noted as a marker associated with a higher incidence of GVHD (Weber 2015). Indole metabolites are associated with reduced intestinal inflammation driven by the interferon pathways, thereby reducing the risk of GVHD. Bile acids and their metabolites are implicated (Haring 2021) in reducing cytokine-mediated inflammation and cell apoptosis, thereby having a positive impact on GVHD outcomes. Recently, Michonneau et al. examined various metabolic signals in the plasma of recipients and noted that they have reduced levels of tryptophan metabolites (indole), compounds of ligands for aryl hydroxy carbon receptor, bile acids, and plasmalogens (Michonneau 2019) compared with patients who developed GVHD. Other potential agents which may require further evaluation include polyamines and other protein metabolic products.

1.12 Antibiotics, microbiome, and outcomes on stem cell transplantation

Antibiotics affect haematopoietic structure and function, and thereby, the outcomes of stem cell transplantation. Bone marrow suppression is an adverse effect of long-term antibiotic use, putting patients at risk of further infections. Antibiotics are widely used following bone marrow transplantation although studies have indicated that there is growing evidence of associated microbial dysbiosis with an impact on engraftment, risk of GVHD, and overall

survival (Staffas 2018, Kim 2004). Bone marrow suppression is an associated side effect of many broad-spectrum antibiotics, especially with prolonged treatment courses that manifest as anaemia, leukopenia, and thrombocytopenia. A study (Josefsdottir 2016) in murine models demonstrated the role of microbial depletion linked to prolonged antibiotic use with changes in progenitor cell development and granulocyte maturation, without any evidence to suggest the direct toxic effects of antibiotics on bone marrow suppression. Promising results have been obtained regarding the restoration of haematopoietic changes with faecal microbiota transfer.

Several pioneering studies have examined the modifying effect of antibiotics on microbial composition and the risk of developing GVHD (Petersen 1986, Buckner 1978) with inconsistent results. Vossen et al. demonstrated that successful total decontamination with nonabsorbable antibiotics in the paediatric population is associated with a low risk of GVHD development (Vossen 2014). Depletion of anaerobic bacteria, as measured by stool culture, with antibiotics such as metronidazole, causes a lower likelihood of developing aGVHD compared to the risk associated with other antibiotics such as ciprofloxacin (Beelan1999, Guthery 2004). Experimental use of rifaximin has been shown to dampen intestinal inflammation and reduce the risk of GVHD by preserving some *lactobacilli* species (Jenq 2012). These studies provide evidence for the use of antibiotics for gastric decontamination before HSCT.

The use of broad-spectrum antibiotics has a negative impact on the microbial ecosystem which has been associated with complications such as GVHD and survival following stem cell transplantation. Antibiotics such as piperacillin-tazobactam have predominant activity against anaerobic bacteria, which negatively affects the levels of beneficial bacteria such as *Lactobacilli* and *Bacteroides* (Shono 2016). These bacteria are well known for their beneficial role in the production of SCFAs such as butyrate, which, in turn, has a significant effect on

the modulation of inflammation and immune response. Changes in the composition of these bacteria affect the risk of GVHD-related mortality post-transplantation.

A study by Weber et al. showed higher transplant-related mortality with the use of antibiotics before HSCT than with antibiotic use post-transplantation or when no antibiotics were used (Weber 2017). Previous studies showed that some anaerobic species like *clostridia* and *Lachnospira* offer a protective effect, while some enterococci and Bacteroides species are associated with disease states. With the use of broad-spectrum antibiotics, an imbalance between these two groups is created, thereby affecting the risk and severity of GVHD. With the use of broad-spectrum antibiotics, anaerobic bacteria are depleted, resulting in the accelerated growth of gram-negative organisms, leading to detrimental outcomes. The exact mechanism underlying antibiotic-induced haematopoietic dysfunction is unknown. Murine studies have shown that microbial dysbiosis causes a reduction in heat-stable circulating microbial products which are important for the promotion of haematopoiesis and inflammatory signalling (Khosravi 2014, Balmer 2014, Yan 2018).

1.13 Mechanisms of microbial dysbiosis:

Microbial dysbiosis refers to the compositional and functional alteration of the microbiota, causing significant disturbance to the homeostatic state between microbes and the host immune system, thereby contributing to many disease states. Several factors affect this disequilibrium, such as infection, inflammation, diet, host genetics, and host environment. In some instances, this maladaptive state of dysbiosis persists chronically with detrimental effects on the host cells. Dysbiosis and reduced microbial diversity can result from the loss of beneficial commensal organisms and from the proliferation of potentially pathogenic organisms. Although the exact mechanisms are unclear, factors such as free radicle oxidative damage and secretion of bacterial toxins can result in rapid changes in the microbial composition, leading to dysbiosis (Weiss and Hennet 2017).

Ecological theories suggest that host control mechanisms in microbial ecosystems shape the composition of beneficial microbiota (Tiffany and Baumler 2019, Foster 2017). Both innate and adaptive immune systems play an important role in constantly checking microbial niches, and vice versa, with functional adaptation. In turn, the microbiota affects immune activation through various signalling mechanisms at local and systemic levels, including epigenetic remodelling and altered gene expression (Levy 2017). A state of physiological hypoxia is maintained in the lower intestinal tract, driving the dominance of obligate anaerobes such as Clostridia and Bacteroides which help break down complex carbohydrates, resulting in the generation of short-chain fatty acids (Zheng 2015). Microbial metabolic products, such as SCFAs, in addition to providing nutrition to the host, stimulate immune education (Tiffany and Baumler 2019). Changes in energy metabolism disrupt anaerobiosis of the colonic epithelium, resulting in the overgrowth of facultative anaerobes such as Proteobacteria which is a hallmark of microbial dysbiosis.

The mechanisms by which the microbiota affect GVHD development have been extensively evaluated. During chemotherapy, there is damage to the intestinal epithelium resulting in the loss of mucus-producing goblet cells and Paneth cells in the lymphoid patches causing disruption of the gut mucosal barrier. This can result in the loss of commensal bacteria and cause the overgrowth of pathogens (Eriguchi 2015, Ara 2018). Microbe-associated molecular patterns (MAMPs) can stimulate the host inflammatory system by crossing the epithelial barrier created by chemotherapy-induced cell injury (Gerbitz 2004). Another mechanism is the production of metabolites such as SCFA, especially butyrate, which influence inflammatory pathways, thereby impacting wound healing and the risk of GVHD development. A recent longitudinal analysis of the faecal microbiome and its metabolites demonstrated that butyrate and indole are potential biomarkers to assess the microbial diversity and risk of aGVHD (Galloway-Peña 2019). In this analysis, members of the *Lachnospira* and *Ruminococcus* families were associated with high faecal butyrate and urinary

indole (3-IS) levels, and this has been associated with a lower risk of developing GVHD after haematopoietic stem cell transplantation. The reverse was noted with the levels of metabolites in patients with predominant enterococci and streptococci species which are associated with a higher GVHD risk.

1.14 Modifying the microbiome/metabolites:

Multiple studies have demonstrated the role of microbial dysbiosis in the outcome of HSCT in terms of the risk of GVHD and overall survival. Modifying the microbiome with beneficial microbial colonies or products will alleviate the impact of complications (Peled 2016). Probiotic formulations can replace beneficial organisms or their metabolic products, such as antimicrobial peptides, in the host. For example, the faecal transfer of *Clostridium difficile* has shown remarkable results in treating persistent infections after transplantation (Taur 2014). The role of lactobacilli in protecting against GVHD risk has been demonstrated in experimental studies in mice (Jenq 2012, Gerbitz 2004). As mentioned previously, a fibre-rich diet plays a major role in reducing inflammation and lowering the risk of GVHD. The replacement of prebiotic products includes the administration of poorly digested absorbable dietary products which can result in an abundance of beneficial bacteria with a lower risk of GVHD (Peled 2016). Bacterial metabolic products such as SCFAs and indole, which can modulate the immune response and inflammation, are under consideration for replacement to reduce the risk of complications associated with HSCT (Mathewson 2016, Peled 2016). Larger studies are required to understand the intricate relationship between microbes and the host immune system and their role as potential therapeutic targets for modifying disease outcomes.

Definitions:

HSCT:

It is a process of replacing all dysfunctional and depleted haematopoietic cells with healthy donor cells, leading to the destruction of residual tumour cells and the replacement of dysfunctional cells.

HLA antigens and MHC :

HLA proteins are expressed on cellular surfaces and play a key role in determining alloimmunity. These genes are coded by highly polymorphic MHC genes present on the short arm of chromosome 6. HLA compatibility is a major factor affecting the success of stem-cell transplantation.

Autologous stem-cell transplantation:

This involves collecting stem cells from the patient and reinfusing them after the purification process. Although it is not associated with any risk of GVHD, it can be associated with a high risk of relapse.

Allogeneic system-cell transplantation:

This process involves the transfer of stem cells from matched relatives (family members) or from unrelated donors. Occasionally, a haploidentical donor (a mismatched family member) can be used depending on the clinical condition.

Pre-conditioning regime:

This consists of high doses of chemotherapy and/or total body irradiation therapy to destroy tumour cells and prepare the haematopoietic system to receive donor cells.

Engraftment:

This involves the establishment of mature peripheral progenitor haematopoietic stem cells in the recipient after the infusion of donor stem cells.

Graft versus host disease:

This is an immunological reaction in which donor T cells consider host cells as foreign and attack different tissues, causing inflammation. GVHD is a major cause of morbidity and mortality after stem-cell transplantation. aGVHD usually develops within the first 100 days after transplantation, whereas chronic GVH is associated with prolonged inflammation involving single or multiple organs.

Microbiota:

The microbial organisms inhabiting the various parts of the body are collectively called microbiota.

Microbiome:

Microbiota along with their genomic components constitute the microbiome.

Chapter 2

Methods and Study Design

2.1 National Bone Marrow Transplant Centre

The study was conducted in the Haematology Department of St. James's hospital Dublin, Ireland's national bone marrow transplantation referral centre. The Haematology Department at St. James's hospital is Ireland's largest which includes the national bone marrow transplantation centre. The Department also incorporates the national blood transfusion and coagulation centres. There are mainly three components for the haematology-oncology services provided and these include:

(i) The Acute Leukaemia/Stem Cell Transplant Service is a purpose-built facility with 21 single rooms for patients undergoing stem cell transplantation or treatment of acute leukaemia and aggressive lymphomas. There is special air filtering in place to minimize the risk of infection. The unit is managed by a clinical nurse manager along with stem cell transplantation-trained nurses.

(ii) Haemato-oncology day ward where the patients requiring less intensive therapy have been admitted. Patients with a wide range of haematological conditions such as lymphoma, myeloma or myelodysplasia are cared for on this service.

(iii) Blood cancer patients are increasingly managed in the haematology day-care setting and treatment is delivered by a day centre team that includes a haematology specialist registrar and haematology trained nurses. Clinical nurse specialists are linked to each service to ensure that patients are educated about their disease and treatment and that each patient's cancer journey is individually planned.

The national stem cell transplant(SCT) service was established in St. James's Hospital Dublin in 1984 and currently is the third-largest centre in the UK and Ireland for SCT. To date over 2500 stem and bone marrow transplantations have been performed with an annual rate of 160-

200 per year. It is affiliated with the European Blood and Marrow Transplantation (EBMT) Registry, and it reports all outcomes to the registry and takes part in EBMT research projects. In 2018 the BMT unit was accredited under the Joint Accreditation Committee ISCT-Europe & EBMT (JACIE).

About 50% of cases are allogeneic transplantations with the rest comprising autologous stem cell infusions. The service is delivered by a Multi-Disciplinary Team (MDT). This consists of medical and nursing specialists and other allied healthcare professionals, including pharmacists and laboratory scientists. The SCT Service is supported by the Apheresis Unit, where stem cells are collected, and a Stem Cell Laboratory that has facilities for cryopreservation and cell storage.

A wide range of patients are referred to the transplant service nationally. After a comprehensive assessment, a decision will be taken into consideration regarding stem cell transplantation. Thereafter patients undergo a range of investigations to assess their suitability for a transplant which include ensuring their disease remission status and analysing their genetic background in relation to risk-stratification. All processes of transplantation are organized by the transplantation coordinator beginning from the patient review in the clinic up to the stem cell transplant, in supporting the patient and the family on the complex transplant journey. Allogenic transplantation sources include peripheral blood stem cells and bone marrow depending on the clinical indications and availability of donors. Depending on the HLA matching and availability both related and unrelated donor sources are used in allogeneic transplantation. Autologous transplantation involving the infusion of a patient's own stem cells is preferred in conditions such as multiple myeloma. In order to identify potential donors, the transplant unit works closely with the Tissue Typing Service and the Irish Unrelated Donor Registry (IUBMR), based at the Irish Blood Transfusion Service. It also

holds joint SCT planning meetings with paediatricians in the National Paediatric Transplant Unit at Our Lady's Children's Hospital, Crumlin, Dublin, Ireland.

The Bone Marrow Leukaemia Trust (BMLT) is a charity founded in 1983 to support the SCT Service. It provides direct support for patients and their relatives and especially recognizes the needs of those coming from outside Dublin. Over the last number of years, the BMLT has provided and managed apartments near St. James's Hospital for patients and their families in the first crucial weeks of adapting to life after transplantation.

Stem cell transplantation involves two phases including a conditioning phase and stem cell infusion. During the conditioning phase, patients undergo vigorous chemotherapy and/or radiotherapy which helps to abolish residual tumour cells. Additionally, this causes an immunosuppressive state preparing the host to accept the new stem cells during the transplantation process. Engraftment is when transplanted stem cells enter the blood, make their way to the bone marrow and start making new blood cells and this usually takes 2-3 weeks. The recovery phase of allogeneic stem cell transplantation is generally complicated and slower compared to autologous transplants and is mostly affected by the engraftment process and the risk of early graft versus host disease. Post-transplant care is delivered through the Haematology Oncology Day Care (HODC) Unit, supervised by a clinical nurse manager and specialist haematology nurses.

The outcomes and survival post-transplantation have improved over the last decade due to further optimisation of treatment regimes and increased availability of suitable donors. The centre has reported a patient 34 years to post their allogeneic transplantation. About 233 patients had been transplanted over two year period between 2018 and 2020. Sixty per cent of these transplantations had reduced intensity preconditioning and the rest were myeloablative.

AML has been the most common indication for transplantation(40%) with a 50% five-year survival rate. This is been followed by myelodysplastic syndrome as the second commonest reason for stem cell transplantation with a 5-year survival rate of 48%. Acute lymphoblastic leukaemia (ALL) and lymphomas are the other indications for transplantation with an associated 5- year survival in excess of 60%.

With regards to this MD research programme, patients undergoing allogeneic stem cell transplantation were recruited over a 2-year period beginning with a small pilot study. After obtaining patient informed consent, study samples including blood samples for genomic DNA extraction and faecal samples for microbial DNA analysis were collected at an early time point shortly after diagnosis and prior to commencing pre-conditioning chemotherapy. Microbial DNA extraction was carried out using standardised protocols. Further genomic sequencing of microbial DNA was carried out in collaboration with Professor Eran Elinav's research group at the Department of Immunology, The Weizmann Institute of Science, Israel.

2.2 Hypothesis:

Based on the literature review, narrowed microbial diversity post-transplantation is associated with increased complications such as GVHD and poor survival. To evaluate this, we analysed the changes in faecal microbial composition of recipients and donors collected 2–3 weeks prior to stem-cell infusion. We hypothesised that a shift in the gut microbial signature occurs weeks before the transplantation process and is a predictor of acute GVHD and overall mortality. Specific functional pathways involving microbial metabolites can potentially drive the pathogenesis of aGVHD. Modifying the microbial composition or replacing beneficial metabolic products would offer a means to alleviate the risk of aGVHD and improve survival following stem-cell transplantation.

Unifying Hypothesis:

Our unifying hypothesis was that there would be a specific microbial readout in the faecal microbiome obtained from these patients prior to transplantation which would be predictive of post-transplant complications and in particular acute GVHD.

Ethics and Consent:

Informed written consent was obtained from recipients of stem cell transplantation and sibling donors involved in the study. This study was approved by the Joint Medical Ethics Committee of St. James's Hospital and Tallaght University Hospital in July 2019 (REC reference 2019-07 List 25(06)) and by the associated General Data Protection Regulation (GDPR) committee.

Funding :

The study was funded by Science Foundation Ireland and the Irish Lung foundation research grants.

2.3 Study Design and Methodology:

Patient Recruitment and Study Cohort

Patients undergoing allogeneic stem cell transplantations from both related and unrelated matched donors were recruited. During the clinical visit for counselling for stem cell transplantation patients are given information leaflets and instructions regarding the study. Participation in the study is entirely voluntary with an option to withdraw their consent at any stage. Participants are given sufficient time to review the study details and fully informed written consent is obtained.

1. Allogenic bone marrow transplant recipients

Patients attending the national bone marrow transplantation centre, Ireland in St. James's Hospital, Dublin who were undergoing allogeneic stem cell transplantation both from related and unrelated donors are contacted in person and given information regarding the project. Patients were recruited for the study prior to starting preconditioning chemotherapy and

samples were obtained. In total 104 patients were recruited who subsequently went on to have a stem cell transplant.

2. Matched Sibling donors – Control Group.

Matched sibling donors were recruited into the study as a control group. Sibling samples were obtained contemporaneously as patient samples. In total 51 sibling donor samples were obtained.

1. Clinical Data:

Clinical data was collected via direct consultation with the patients or from the medical records of participants and collected data were stored in accordance with data protection and GDPR regulations. Recruited patients were assigned a specific study number and no patient identifier data was enclosed in data files. The code linking patient study number to patient name was stored in an encrypted computer in a locked cupboard within the Department of Medicine, Tallaght University Hospital. Clinical information collected included basic demographic data such as age, gender, underlying conditions, body mass index , smoking status, type of transplant, nature of preconditioning therapy, MRSA and VRE status, baseline lung function and radiology imaging results. Follow-up clinical information was collected at 100 days following the stem cell transplantation with regard to the incidence, organ involvement and severity of acute graft versus host disease (aGVHD). The steroid responsiveness and mortality data of aGVHD patients were also documented.

2. Pulmonary Function Test:

Baseline data on pulmonary function tests were collected from medical records which were performed as part of the pre-transplantation workup. As a part of the study pulmonary function tests were performed circa 100 days post-transplantation.

3. Samples:

A. Recipients

Baseline samples prior to pre-conditioning

i. Blood samples; whole blood used for genomic DNA extraction and serum samples for protein metabolic studies were stored at -80 degrees. Genomic DNA extraction from whole blood samples was performed using QIAamp DNA Blood Mini Kit (Catalogue no.: #51106, Qiagen, Manchester, M13 0BH, UK).

ii. Faecal DNA extraction for quantitative microbial DNA analysis using QIAamp PowerFecal DNA kit (Catalogue no.: #51804, Qiagen, Manchester, M13 0BH, UK). Samples are collected in specialized tubes for homogenization and stabilization of samples at the time of collection. (Omnigene gut sample collection kits OM-200, DNA Genotek, 3000-500 Palladium Drive, Ottawa, Ontario, Canada, K2V1C2)

Follow-Up Samples:

Blood and faecal samples for DNA extraction were collected 100 days post-transplantation.

B. Donors

Once off blood and faecal samples were collected from matched sibling donors at baseline prior to the transplantation process in corresponding recipients.

2.4 Sample Analysis:

Initial sample processing was undertaken at Trinity Biomedical Sciences Institute (TBSI) Dublin and Meath Foundation Research laboratory (MRFL) at Tallaght University Hospital. Prior to working in the laboratory, I attended various clinical and biological workshops organized by Trinity College Dublin (TCD) to ensure the safe handling and disposal of the samples. Samples were processed and stored in accordance with the manufacturer's manual

instructions. At each stage of the sample processing and storing, patient information was kept anonymous to ensure confidentiality in accordance with the GDPR recommendations.

- **Blood samples:**

Blood Genomic microbial DNA analysis

The whole blood sample was initially stored directly at -80 degree temperature for future genomic DNA analysis. Genomic DNA purification from blood samples was performed with a Nucleospin Blood extraction kit. The extracted purified genomic DNA sample was stored at -80 degrees. This will be used for future microbial DNA analysis.

a. Protocol for DNA Purification from Whole Blood Genomic DNA purification with NucleoSpin® Blood

Before starting the preparation:

• Check if Buffer B5 and Proteinase K were prepared as per the manufacturer's recommendation.

• Set an incubator or water bath to 70°C.

• Preheat Elution Buffer BE to 70°C

i. Lyse blood sample

iA. Pipette 25 µL Proteinase K directly into 200 µL blood and vortex well. Leave to sit at room temperature for 2 minutes.

iB. Add 200 µL Buffer B3 to the samples and vortex the mixture vigorously (10–20 s). Leave to sit at room temperature for 5 minutes.

iC. Incubate samples at 70°C for 10 minutes and vortex well.

(200 µL blood + 25 µL Proteinase K + 200 µL B3, Mix 70°C 10 min)

ii. Adjust DNA binding conditions

Add 210 µL ethanol (96–100 %) to each sample and vortex again.

(sample + 210 µL ethanol and Mix)

iii. Bind DNA

For each preparation, take one NucleoSpin® Blood Column placed in a Collection Tube and load the sample. Centrifuge 1 min at 11,000 x g at 18°C. If the samples are not drawn through the matrix completely, repeat the centrifugation at higher g-force (< 15,000 x g). Discard Collection Tube with flow-through.

iv. Wash silica membrane

1st wash

Place the NucleoSpin® Blood Column into a new Collection Tube (2 mL) and add 500 µL Buffer BW. Centrifuge 1 min at 11,000 x g. Discard Collection Tube with flow-through.

2nd wash

Place the NucleoSpin® Blood Column into a new Collection Tube (2 mL) and add 600 µL Buffer B5. Centrifuge 1 min at 11,000 x g. Discard flow-through and reuse Collection Tube.

v. Dry silica membrane

Place the NucleoSpin® Blood Column back into the Collection Tube and centrifuge for 1 min at 11,000 x g. Residual ethanol is removed during this step.

vi. Elute highly pure DNA

Place the NucleoSpin® Blood Column in a 1.5 mL microcentrifuge tube (not provided) and add 100 µL preheated Buffer BE (70°C). Dispense buffer directly onto the silica membrane. Incubate at room temperature for 1 min. Centrifuge 1 min at 11,000 x g.

b) Serum samples are extracted after spinning them at 2000g for 10min and collected in 500 microlitres vials and stored at -80°C. This will be utilised for further protein and metabolic studies

Blood genomic DNA and serum samples extracted are stored at -80°C for future research.

- **Faecal sample microbial DNA:**

Microbial DNA was extracted from the faecal samples collected in specialized tubes using the QIAamp Powerfecal DNA kit.

b. Protocol of Extraction of faecal microbial DNA using QIAamp PowerFecal DNA kit

Procedure

1. Add 0.25 g of stool or biosolid to the Bead Tube provided.

For faecal samples that are especially high in lipids, polysaccharides and protein smaller amounts of starting material (~0.10 g) may improve DNA yield and purity.

2. Add 750 µl of PowerBead Solution to the Bead Tube.

3. Add 60 µl of Solution C1 and briefly invert several times or vortex.

4. Heat the tubes at 65°C for 10 min.

5. Secure tubes horizontally using a Vortex Adapter (cat. no. 13000–V1–24). Vortex at maximum speed for 10 min.

6. Centrifuge the tubes at 13,000 x g for 1 min.

7. Transfer the supernatant to a clean 2 ml Collection Tube (provided). Expect between 400 to 500 µl of supernatant.

8. Add 250 µl of Solution C2 and vortex briefly to mix. Incubate at 2–8°C for 5 min.

9. Centrifuge the tubes at 13,000 x g for 1 min.

10. Avoiding the pellet, transfer up to 600 µl of supernatant to a clean 2 ml Collection Tube.

11. Add 200 µl of Solution C3 and vortex briefly. Incubate at 2–8°C for 5 min.

12. Centrifuge the tubes at 13,000 x g for 1 min.

13. Avoiding the pellet, transfer the supernatant to a clean 2 ml Collection Tube (provided).

Do not transfer more than 750 µl at this step.

14. Add 1200 µl of Solution C4 to the supernatant and vortex for 5 s.

15. Load 650 µl of supernatant onto an MB Spin Column and centrifuge at 13,000 x g for

1 min. Discard the flow-through and repeat until all the supernatant has been processed.

Each sample processed will require a total of three loads.

16. Add 500 µl of Solution C5 and centrifuge for 1 min at 13,000 x g.

17. Discard the flow-through and centrifuge again for 1 min at 13,000 x g.

18. Carefully place the MB Spin Column in a clean 2 ml Collection Tube (provided).

Avoid splashing any Solution C5 onto the MB Spin Column.

19. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, you

may use sterile, DNA-free, PCR-grade water (cat. no. 17000-10) or TE buffer.

Note: Eluting with 100 µl of Solution C6 will maximize DNA yield. For more

concentrated DNA, a minimum of 50 µl of Solution C6 can be used.

20. Centrifuge at 13,000 x g for 1 min and discard the MB Spin Column. Store extracted DNA frozen (–20°C to –80°C)

The DNA in the tube was further used for genomic sequencing of microbial DNA

c. Genomic Sequencing of Microbial DNA:

After extraction of microbial DNA from the faecal samples, further genomic analysis was performed with external collaborators at the Department of Immunology, The Weizmann Institute of Science, Israel. After the extraction of microbial DNA the samples were processed to the appropriate concentrations for further genomic sequencing. The samples were transported in biological safe containers and the quality of the samples was maintained at each stage of processing and transportation. The microbial genomic analysis was carried out using 16S sequencing (rRNA genomic sequencing) and whole-shotgun metagenomic sequencing. Microbial readouts in samples were evaluated in relation to outcomes of haematopoietic transplantation such as graft versus host disease and survival. The following is the details of genomic sequencing performed by the immunology team at Weizmann institute.

(i) **Shotgun metagenomic sequencing**:

DNA was isolated from stool swabs using PowerMag Soil DNA Isolation Kit (QIAGEN) optimized for an automated platform. Extracted DNA concentration was quantified with the iQuant assay system and diluted to the same concentration per sample. Subsequently, genomic DNA was fragmented and barcoded using transposase following Illumina Nextera protocol. DNA fragment of correct length was selected using double-sided size selection with Ampure XP SPRI beads. Libraries were pooled together at equimolar concentration and sequenced using Illumina NextSeq.

Shotgun metagenomic sequencing in Detail:

Illumina libraries were prepared using a Nextera DNA Library Prep kit (Illumina, FC-121-1031) according to the manufacturer's protocol and sequenced on an Illumina NextSeq platform with a read length of 75 bp (single-end) for all samples with a read length of 75 bp was used.

Bcl2fastq was used to convert the raw output BCL files to fastq files. Reads were then QC trimmed using Trimmomatic. Bowtie2 alignment against the hg38 host reference was performed (very sensitive preset), and reads mapping to the host reference were removed. Following host removal, reads were mapped against the GTDB using kraken2 and bracken to generate taxonomy profiles. Functional profiles were generated using direct mapping of metagenomic sequences to the unique database using diamond blastx. Pathway enrichment was performed using Minpath1.4.

(ii) Metabolomics

Samples were prepared using the automated MicroLab STAR system. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and recover chemically diverse metabolites, proteins were precipitated with methanol. The resulting extract was divided into four fractions: one for analysis by ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC–MS/MS) with negative ion mode electrospray ionization, one for analysis by UPLC–MS/MS with positive ion mode electrospray ionization, one for LC polar platform, one for analysis by gas chromatography coupled with mass spectrometry (GC–MS) for lipidomics. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities.

(iii) Machine learning:

Random forest (RF) tree-based classification algorithm was used for the predicting assignment in this project. Random forest was chosen for its ability to model non-linear correlations in the data, as the microbiome is such, into meaningful predictions. Then a simple

10-fold cross-validation was devised each time using 90% of the data as a training set and the remaining 10% as a test set, and reported the mean Area Under the Receiver Operator Characteristics (AUROC) of all the results. To achieve a balanced training and validation set, we subsampled the majority group in each iteration to fit the minority group size and then divided it into the corresponding train and validation sets. Random forest was used with the same parameters for each predictions assignments, with 200 trees, max_features = 'sqrt' and a changing random state according to the number of the fold.

After that the top 30 most important features were used that were the most meaningful for the classification to visualize the signal that was captured through the random forest algorithm by principal components analysis (PCA).

(iv) Statistical Microbiome analysis:

PCA was used in order to reduce the high dimensionality of the microbiome samples. PCA is efficient in doing so, as it handles sparsity (which is found in microbiome data) and is able to detect the highest axes of variation in high-dimensional data itself. Data was inserted to PCA following strictly centred log-ratio (CLR) normalization, which is often used for microbiome data and prior to PCA (as data must be scaled and centred). Pseudo-counts of 1 were added for the log transformation, as an error occurs for the log of 0. Eventually, PCA allows us to observe and test the distances between our samples in lower dimensions, while it is capturing the highest variance and differences in the data, unbiasedly.

Similarly, Principal Coordinates Analysis (PCoA) was used as well. The advantage of PCoA is it can use different dissimilarity distances, and not just Euclidean. Here, we have used the commonly used Braycurtis dissimilarity to compute the distances between the samples. Then we employed the PCoA algorithm on the resulting distance matrix. CLR normalization was used as well for the PCoA pre-processing steps.

For the univariate testing of microbiome enzymes, the Deseq algorithm was used, the common pipeline to model count data. Deseq models the data using a generalized linear model and the negative binomial distribution with fitted mean. Data were filtered from low abundant enzymes prior to insertion into Deseq, with enzymes present at least in 90% of the samples with higher than 0.01% relative abundance.

Testing of the univariate species and genera features was done using the non-parametric Mann-Whitney-U test, a common practice for microbiome relative abundance data. Microbial diversity and taxa analysis are calculated using the Mann-Whitney U test with FDR correction (BH) to assess statistical significance.

Mann- Whitney U test: This is a nonparametric test that allows two groups or conditions or treatments to be compared without assuming that values are normally distributed. FDR Correction is being used to conceptualize the rate of type 1 errors.

Power calculation: We used a simulation-based power calculation approach, generating abundance data via a Dirichlet-Multinomial model and Monte Carlo simulations. Assuming that 6 bacteria, in the species level, will display an increase or decrease of 2 FC in their relative abundance, with an alpha of 0.05 (confidence level). This resulted in estimated power with 40 samples (divided into training and validation sets, as described above) in the group who developed GVHD disease and did not survive is 0.89.

Various diversity indices are used to denote the relative abundance and richness of various microbial species. Alpha diversity measures the species diversity in a specific sample whereas beta diversity is a measure of change in the diversity of species between different samples.

Shannon Diversity Index

This is a common diversity index used in ecological literature to characterize species diversity in a community.

OTU (Observed Taxonomic Unit)

The OTU metric counts the number of distinct bacterial taxonomic units, overall measuring the richness of species in a sample.

Evenness Index

Species Evenness is termed as the distribution of abundance across the species in a community. Evenness is high if all species in a sample have a similar degree of abundance.

2.5 Sample Analysis Comparison groups

155 samples (including 104 recipient samples and 51 donor samples) are grouped into 11 groups and 6 comparison analyses were performed according to the clinical relevance.

Comp1: Developed GVHD (Gp1) vs. Did not Develop GVHD (Gp2)

Comp2: mild GVHD (Gp3) vs. severe GVHD (Gp4).

Comp4: GVHD resistant to steroids (Gp5) vs. GVHD responded to steroids (Gp6).

Comp5: GVHD who died (Gp7), GVHD who survived (Gp8), did not develop GVHD (Gp9).

Comp6: Bone marrow donor's faecal samples to patients who developed GVHD and died (Gp10) vs. non-GVHD (Gp 11).

Chapter 3

Stem cell Transplantation – Clinical Characteristics of Recipients and Donors

3.1 Introduction

The eligible participants for the study were recruited at the national bone marrow transplant centre, St. James's Hospital, Dublin. As a part of the national programme, potential transplant candidates are referred to the centre by various haematology teams in the republic of Ireland and parts of Northern Ireland NHS (national health service) trust. During the counselling process for the transplantation process, the details of the research study had been provided and ample time was allowed to make an informed decision for participation in the study. The recruitment process had been occasionally challenging due to the complex nature of patient conditions.

Patients undergoing allogeneic stem cell transplantation from both related and unrelated matched donors have been recruited for the study. 104 stem cell transplant recipients and 51 sibling donors were included in the study. After obtaining informed written consent, patient demographics were collected at the time of clinical consultation and from the medical records. The clinical data included baseline demographics such as age, gender, body mass index (BMI), underlying condition, smoking status, type and intensity of conditioning regime, source of stem cells, and performance status (Karnofsky score) at baseline. The investigation results collected included MRSA and VRE status, renal function(eGFR), baseline lung functions (FEV1 and DLCO), and radiology results. The disease risk and remission status were used as predictive indicators for the outcomes of transplantation. Clinical data collected were stored per GDPR guidelines, and anonymity is maintained throughout the study.

Blood samples were collected for genomic DNA extraction (whole blood) and serum samples were extracted for further metabolic studies. Faecal samples were obtained prior to the preconditioning chemotherapy with a range of 2-10 days prior to the initiation of the

chemotherapy. Generally, the samples were taken approximately 10- 21 days prior to the infusion of the stem cells.

3.2 Recipient characteristics

Out of the 104 participants (n), 75% of patients have received unrelated allogeneic stem cell transplantations whereas sibling donors were the source of stem cells in the rest of the cases (table 3.1). The average age of patients was 47 (ranging from 18 to 67) at the time of the stem cell infusion. Although age is not a limiting factor for transplantation, advanced age and a decrease in functional performance status have been associated with increased mortality (Gupta 2010). There was a slight male predominance in the group of 57.6%. The average BMI of the patients was 26.6 , however about 16% of patients had a BMI over 30. Raised BMI has been associated with increased non-relapse mortality (Doney 2019). The ethnicity of patients varies with mostly Caucasians in about 90% of the cases, Eastern Europeans at 8%, and Others at 2%. Thirty per cent of the patients were either active or ex-smokers. Performance status has been assessed by Karnofsky's score varying from a score of 0-100 (Sorror 2008, Peus 2013, Guilfoyle 2009) and considered a stronger predictor of non-relapse mortality in score <90 (Ref CIBMTR Jan 2009). Eighty-seven per cent of our patients who underwent stem cell transplantation had a Karnofsky score between 70 to 80 whereas 12.5% of the patients had a score over 90.

In our cohort acute leukaemia especially AML was the commonest indication for stem cell transplant(30%) followed by lymphomas (27%) and myelodysplastic syndrome in about 21% of the cases (fig 3.1). HLA matching is strongly associated with a good post-transplant prognosis. Among the unrelated stell cell transplants, 75% of the patients had HLA-matched transplants and 6.7% of the recipients had grafts with one HLA antigen mismatch. Disease remission status and various mutations were used as predictors of outcomes related to stem cell transplantation. Chromosomal and gene mutations are commonly seen in various

haematological malignancies, especially in acute leukaemias. About 50% of primary AML and more than 80% of secondary AML are associated with chromosomal anomalies (Blau 2015). FLT3(Fms Related Receptor tyrosine kinase 3) and nucleophosmin (NPM1) are the two common mutations seen in association with AML. FLT3 mutations are present in about 1/3 of cases of newly diagnosed AML associated with increased relapse rates and poor survival (Kennedy 2020). The presence of mutations can be associated with resistance to treatments and reduces the chances of a cure from about 50% to 20%. NPM1 gene is another commonly mutated gene seen in association with AML which is present in about 20-30% of the cases (Heath 2017) and is actually associated with a favourable prognosis. Forty per cent of our study population had mutations which would place them in a high-risk prognostic group. High-risk diseases are associated with increased complications post-transplantation, including steroid-resistant GVHD and mortality.

Depending on the underlying diagnosis and patient co-morbidities, various intensities of the conditioning chemotherapy had been used either myeloablative or non-myeloablative. About 62% of patients received low-intensity pre-conditioning whereas in the rest of the cases high intensity myeloablative chemotherapy had been used prior to stem cell infusion. The preconditioning agents were used as per disease-specific guidelines and available local policies. Fludarabine, Busulphan and cyclophosphamide were the most common drugs included in the preconditioning for myeloproliferative disorders whereas cyclophosphamide and total body irradiation (TBI) had been used in acute lymphoid leukemias. For various refractory lymphomas, a regime based on fludarabine, Melphalan, and Alemtuzumab was commonly considered.

The sources of the stem cells included bone marrow, untreated peripheral blood, and occasionally cryo-precipitated concentrate of stem cells. Sixty-eight percent of the recipients received stem cells extracted through a process of apheresis from peripheral blood and in

certain high-risk cases, bone marrow harvest had been used as a source of stem cells. Preserved cryoprecipitate was used as a source of stem cells in a very selected cohort of patients (1%). Prophylactic antibiotics and immunosuppressive therapies were used according to the institutional protocol. The most commonly used immunosuppressive agents included calcineurin inhibitors like tacrolimus and cyclosporin and ambisome was used as a prophylactic antifungal agent.

Baseline Demographic Data

Characteristics	
Age (ave)	47.3+/-20
Gender n (%)	
Male	60 (58)
Female	40 (38)
Diseases n (%)	
AML	32 (31)
MDS/Myeloproliferative	21 (20)
HL	9 (9)
NHL	18 (17)
ALL	11 (11)
Myeloma	2 (2)
CMML	4 (4)
Aplastic Anaemia	2 (2)
Others	5 (5)
Allogeneic Tx type n (%)	
Related (SIB)	26 (25)
Unrelated (MUD)	78 (75)
Source of stem cells n (%)	
Bone Marrow	32 (31)
Peripheral Blood	71 (68)
Cryoprecipitate	1 (1%)
Preconditioning n (%)	
High Intensity (Myeloablative)	40 (38)
Low Intensity (non-myeloablative)	64 (62)

Table 3.1 Baseline Recipient characteristics (n-number and percentage)

Baseline MRSA (methicillin-resistant staphylococcus aureus) and VRE (vancomycin-resistant enterococcus) status were determined prior to the transplantation. VRE colonisation is very commonly seen in the pre-HSCT period but does not necessarily always relate to the outcome of transplantation except in some cases can cause bacteraemia in the post-HSCT period and can lead to high mortality and higher incidence of graft versus host disease (Ford 2017). In our cohort, 33% of the patients were VRE positive and of which 56% cases were associated with acute GVHD.

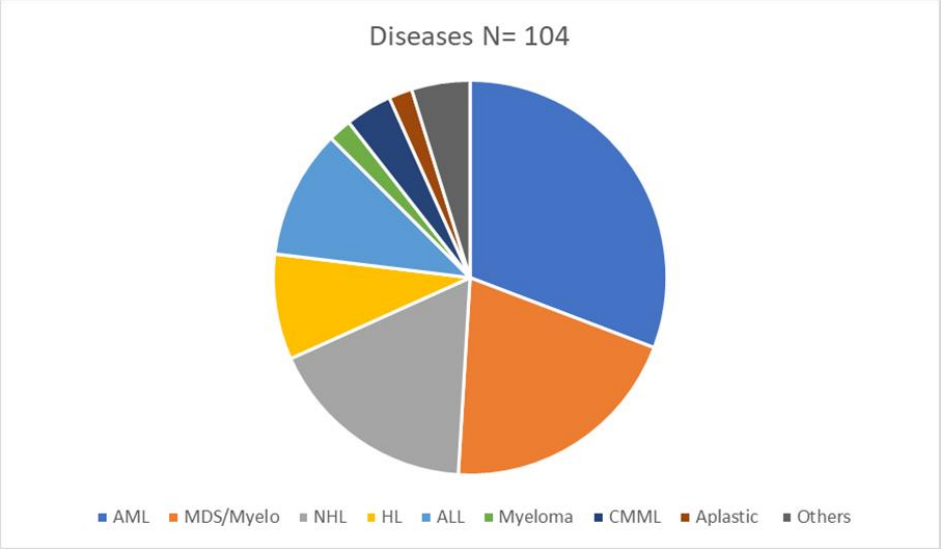


Fig 3.1: Indications for Stem cell Transplantation (Disease percentage)

Other clinical parameters assessed include a history of underlying lung disease and baseline pulmonary function status. Pre-transplant pulmonary function tests are very important in assessing non-infectious pulmonary complications following stem cell transplantation (Chein 2005). Abnormal pulmonary functions at baseline can be associated with increased respiratory complications but their impact on mortality and morbidity has been debatable. A study by Srinivasan et al. (2014) in children has shown that pre-transplant reduction in FEF 25-75% is associated with increased pulmonary complications and this in turn is associated with about threefold risk of mortality. Hence evaluation of pulmonary functions is carefully selected as

one of the pre-transplant fitness criteria. In our cohort, about 15% of patients had abnormal spirometry with reduced FEV1 and 35% of the patients had a reduction in diffusion capacity (DLCO) which are considered to be strong predictors of pulmonary complications associated with transplantation. Busulfan-based chemotherapy is commonly used in the treatment of myeloid leukemias and is one of the early reported agents causing pulmonary toxicity although only about 8% of people develop symptoms (Oliner1961). A study (Kalaycio 2006) in older patients with non-Hodgkins lymphoma treated with busulfan-based chemotherapy has shown mortality related to pulmonary complications in about 3.6% of the cases. The risk factors for pulmonary toxicity include older age, low baseline FEV1, and DLCO. In our study, 55% of the patients had a busulfan-based chemotherapy regime of which about 8 patients (14%) were noted to have low DLCO.

Anti-thymocyte globulin(ATG) is used as a part of conditioning regimes, depletes the host T cells which have survived during the preconditioning chemotherapy as well as causes deletion of newly infused donor T cells thereby reducing the risk of GvHD (Kekre 2017, Siddiqui 2019). At the same time, ATG can be associated with a slightly increased risk of Epstein viral infections and associated lymphoproliferative disorders (van Esser 2001). The use of ATG should be used in balance with its beneficial anti-GvHD effects versus potential harmful effects depending on the underlying disease risk and patient conditions.

3.3 Donor Characteristics

Due to ethical reasons only matched sibling donors were recruited into the study group. Donor recruitment had been limited by challenges in the concurrent recruitment of both recipients and donors within the timeframe before the preconditioning period. The bone marrow transplant centre at St. James's hospital works as part of a large network of a European consortium of bone marrow registries. The donors are available from different European counties which was a limiting factor to the recruitment process and occasionally only the

processed stem cell infusions are available to the transplant unit. After informed written consent faecal samples were collected from donors before the stem cell transplantation.

In total 51 HLA-matched sibling donors have been recruited into the study of which 33 males and 18 females were included. The average age of donors was 46.7 with slight male predominance in our cohort (64.7%). Donor age is carefully selected as the younger age of donors is associated with better long-term survival of stem cell transplants (DeZern 2021) although one of the previous studies didn't show any increased risk of GVHD related to older donor age (Rezvani 2015). Another recent study (Galamidi-Cohen 2020) showed that increased donor age is associated with prolonged neutrophil recovery, which might affect complications and post-transplantation. Donors had an average BMI of 28.2 with 35% being overweight with a BMI above 30. General guidelines include assessment of BMI and avoiding BMI over 35 while selecting donors for stem cell sources (JPAC guideline). A recent study (Abou-Ismail 2022) has noted a non-linear relationship between pre-transplant BMI in recipients with certain donor types like cord-blood sources compared with other sources. Non-relapse mortality and poor survival have been reported in recipients with increased BMI and this should be considered in selecting donors. Donors with high BMI should be assessed for cardiovascular risk factors and access issues for stem cell extraction. In terms of donor-to-recipient gender status, about 37.2% of the cases of stem cell infusions were from a male donor to a male recipient whereas 27.4% of cases were from a male donor to a female recipient. In the rest of the cases, females were the source of the stem cells. Fifty-nine per cent of the related stem cell transplantation cases involved the infusion of peripheral blood stem cells whereas, in the rest of the cases, bone marrow has been the source of stem cells.

3.4 Acute Graft Versus Host disease

Graft versus host disease continues to be a significant problem following stem cell transplantation contributing to high morbidity and mortality. Acute GVHD usually occurs within 100 days of the transplantation and can progress to chronic GVHD if the inflammation persists. GVHD remains as one of the most important predictors of non-relapse mortality at year 1 after stem cell transplantation (Ramdial 2021). HLA compatibility is the most important factor determining the risk for acute GVHD and disease mortality following transplantation (Shaw 2003).

Various mechanisms (Holler 2019) are involved in acute GVHD including tissue damage caused by preconditioning treatment activating the host antigen response system. Subsequent activation of donor T cells evokes an inflammatory response by releasing various mediators such as IL-1(interleukin 1) and TNFα (Tumour Necrosis Factor-alpha) leading to further tissue necrosis.

Acute graft versus host disease is defined as the onset of GVHD within 90 days of stem cell transplantation. The severity of GVHD is calculated by Glucksberg GVHD grading (table 3.2) (Glucksberg 1974, Hatzimichael 2010) depending on the organ involvement and the extent of the disease. This is been further characterized using IBMTR severity index and graded into A to D (Rowlings 1997, Martino 1999).

Glucksberg GVHD Classification

Stages	Skin	Gut/lower GI	Liver (Bilirubin levels μmol /L)
1	Maculopapular rash <20% BSA	Diarrhoea >500mls/day	25-50
2	Maculopapular rash 25-50% BSA	Diarrhoea >1000mls/day	51-102
3	Maculopapular rash >50% BSA	Diarrhoea >1500mls/day	103-255
4	Erythroderma/bullae/desquamation	Diarrhoea >2000mls/day or severe abdominal pain +/- ileus	>255

Table 3.2: Glucksberg Staging Organ Involvement (Glucksberg 1974)

(Note: upper GI involvement nausea/anorexia included in stage 1)

Overall Glucksberg GVHD Grading

Grade 1- up to stage 2 skin with no liver or GI involvement

Grade 2- up to stage 3 skin and /or stage 1 GI or liver involvement

Grade 3- up to stage 3 skin and/or stage 2-3 GI or liver involvement

Grade 4- Stage 4 skin/Gi /liver involvement

International Bone Marrow Transplant Registry (IBMTR) GVHD Severity Index (Rowlings 1997)

Grade A- Stage 1 skin without any GI or liver involvement

Grade B- Stage 2 skin and stage 1-2 GI or liver involvement

Grade C- Stage 3 skin or GI tract or liver involvement

Grade D- Stage 4 skin or GI tract or liver involvement

There are several factors such as patient and disease factors, and the source of stem cells that also affect the risk of graft versus host disease. For example advanced age, female donor status and increased BMI are some of the patient-related factors contributing to the risk of GVHD. Other factors include the type of GVH prophylaxis, the CMV(cytomegalovirus) positivity, and donor age (Flowers 2011) Considering GVHD has been linked with chronic inflammation, the myelodysplastic syndrome which is linked to persistent inflammatory process has a high association with GVHD compared to other myeloid diseases.

3.5 Characteristics Of Acute Graft Versus Host Disease

In our cohort myeloproliferative diseases, especially AML and refractory lymphomas were the main indications for transplantation and hence most of the graft versus host diseases were associated with these conditions (fig 3.2)

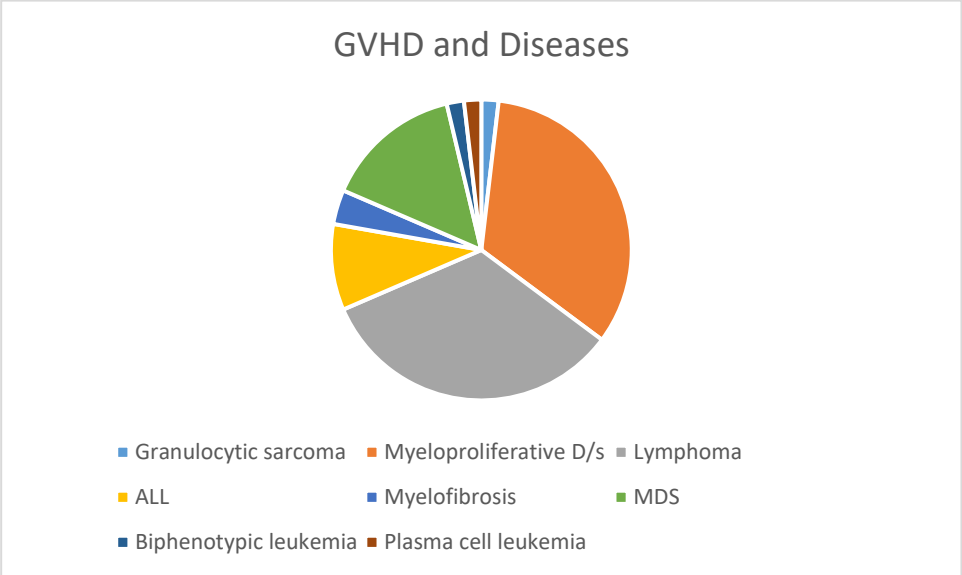


Fig 3.2: aGVHD risk and Diseases (percentage)

The incidence of GVHD was 52% with slight male predominance (table 3.3) The number of patients older than 60 years of age was comparable in both groups (20.3% Vs 20%). We have observed only a slightly higher BMI in the graft versus host disease group (16.6% Vs 16%) although this could be related to the small sample size.

Unrelated allogeneic transplantations were the commonest in both groups, although there is a lesser incidence of GVHD in sibling transplant groups as expected. Consistent with previous study results (Anasetti 2012) infusion of peripheral blood stem cells is associated with a higher risk of GVHD compared to bone marrow source of stem cells. Previous studies (Couriel 2004) have shown that non-myeloablative chemotherapy is associated with a lower risk of acute and chronic graft versus host disease. In our study, the myeloablative chemotherapy numbers were comparable in both groups but didn't show much difference in terms of risk of GVHD, but this could be related to the sample size.

	GVHD (N=54)	Non-GVHD (N=50)
Age >50 years n(%)	26 (48)	27(54)
Gender n (%)		
Male	32 (59)	28 (56)
Female	22 (41)	22 (44)
BMI >30 n(%)	9 (16.6)	8 (16)
Type of Allogeneic Tx n (%)		
Sibling	11 (20)	15 (30)
Unrelated	43 (80)	35 (70)
Sources of Stem Cells n (%)		
Bone Marrow	14 (26)	18 (36)
Peripheral Blood	43 (72)	32 (64)
Cryoprecipitate	1 (2)	
Preconditioning n (%)		
High Intensity/Myeloablative	20(37)	20 (40)
Low Intensity/non-myeloablative	34 (73)	30 (60)

Table 3.3 baseline characteristics of GVHD and non-GVHD groups

Although acute GVHD can affect many organs in the body, the most commonly involved organs include the skin, gut, and liver. Occasionally acute GVH can evolve into a persistent inflammatory state leading to chronic GVH which causes a significant burden on the physical and emotional health of the patient and is a major cause of morbidity associated with stem cell transplantation.

i. Organ Involvement in Acute Graft Versus Host Disease

The skin and gut are the common organs affected by GVHD in the majority of the cases with multiorgan involvement in more than 35% of patients (table 3.4). Most of the skin and gastrointestinal GVHD had a tissue diagnosis Oral mucositis was one of the commonest manifestations of GVHD involving the upper GI tract. Ocular GVHD was one of the rare presentations in about 2% of acute GVHD cases although this had been mostly underdiagnosed.

Organ involvement	Percentage (Total N=54)
Skin	**78**
Gut	**46**
Liver	**13**
>/=2 organs	**35**

Table 3.4 Acute GVHD and organ involvement

ii. Acute Versus Host Disease Grade and severity

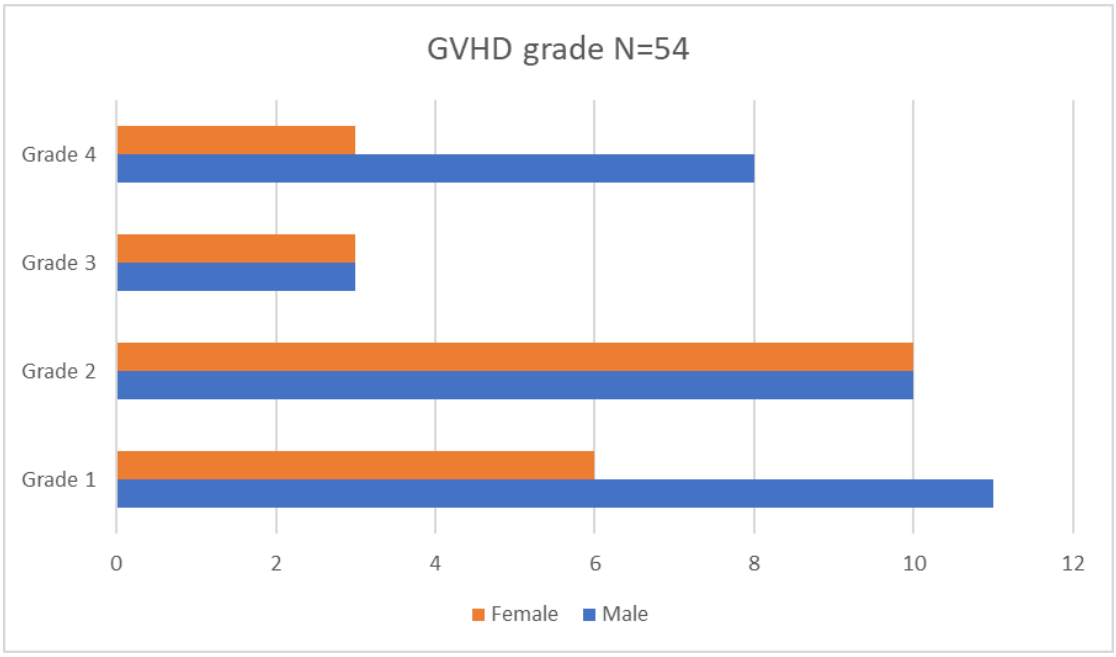


Fig 3.3: GVHD grade and Gender

A slight male predominance was noted in most of the GVHD grades, especially in the severe group (fig 3.3). The figure below shows gender differences in terms of severe GVHD grade, treatment response, and mortality risk (Fig 3.4). In the patient cohort, about 31.4% had severe aGVHD (grade3-4) of which 20% of cases were associated with steroid resistance adding a significant burden to patient care. Compared to previous studies (80) the mortality rate in the

acute GVHD group was 20% whereas GVHD cases accounted for 50% of all-cause mortality. (overall mortality in the study group was 20%)

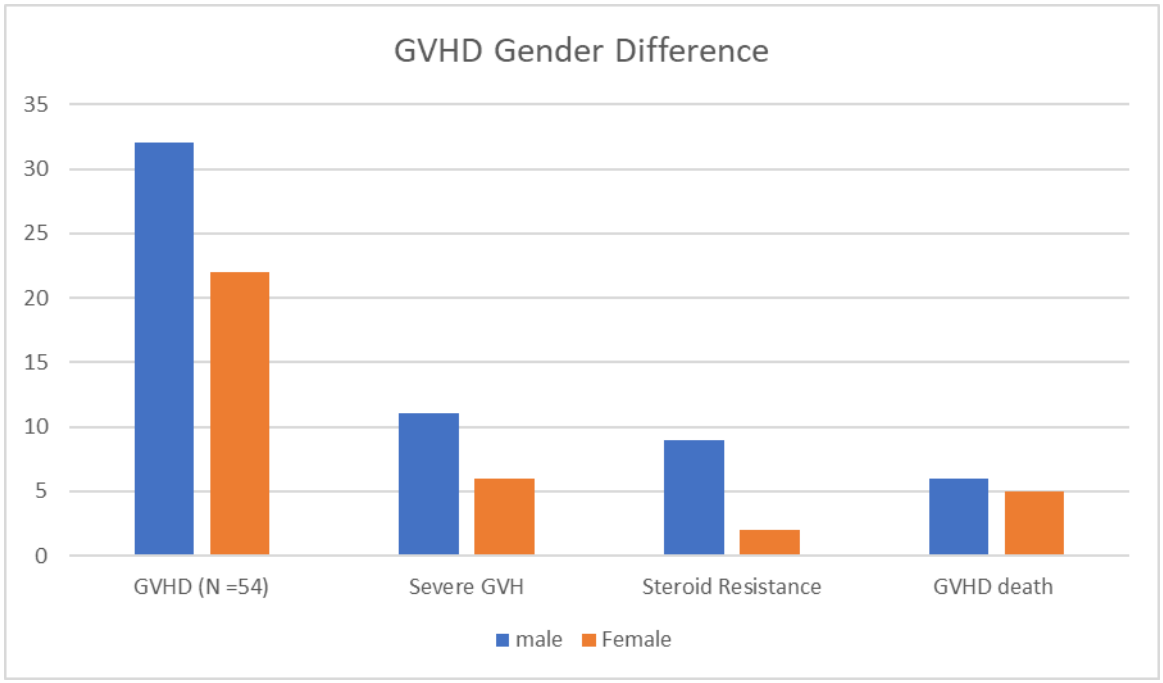


Fig 3.4: GVHD Characteristics

iii. Acute GVHD and steroid Resistance

Acute GVHD is generally treated with corticosteroids in a 1-2mg/Kg dosage. In resistant cases, other immunosuppressants are used as steroid-sparing agents. Steroid resistance continues to challenge the management of GVHD in terms of patient morbidity, poor quality of life, and increased mortality. Steroid refractory GVHD usually requires specific treatments like ECP (extracorporeal photopheresis), anti-TNF agents, targeted rapamycin kinase inhibitors, or immunosuppressive agents like methotrexate or mycophenolate mofetil (Dignan 2012). Extracorporeal photopheresis has been used as one of the main therapies for steroid-resistant GVHD in our patient cohort.

In our cohort, about 20% of acute GVHD cases were associated with poor response to steroid treatment adding complexity to managing these patients, increasing hospital admissions, and poor patient quality of life.

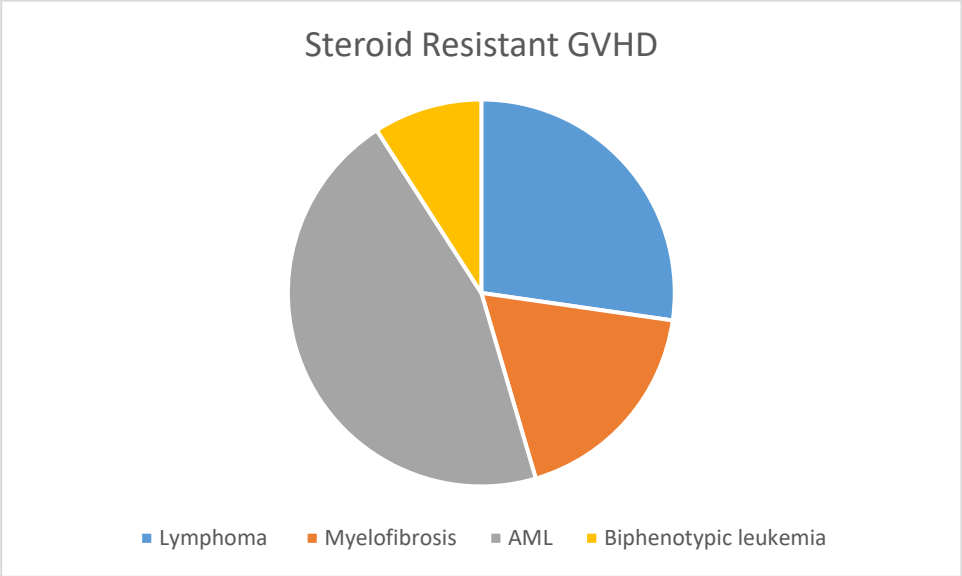


Fig 3.5: aGVHD Steroid resistance and Diseases

There are several factors associated with the risk of steroid resistance in acute GVHD including high-risk disease index, unrelated donor status, absence of in-vivo Tcell depletion, and use of reduced intensity conditioning (Pagliuca 2021). Steroid refractory cases involved a male predominance of 81% and in 90% of the cases, unrelated donors were the source of stem cells. With regards to other risk factors for steroid resistance, about 45% of cases were associated with a high disease index but notable that only 18% of the cases received low-intensity pre-conditioning in our cohort. Myeloproliferative diseases like AML were the commonest indication for stem cell transplantation; hence, the highest incidence of GVHD and steroid resistance(45%) has been observed with AML followed by conditions such as lymphomas and myelofibrosis with 27% and 18% respectively (fig 3.5). Organ involvement and severity at the onset of GVHD could predict the outcomes as noted to have a poor response in association with lower GI tract and liver involvement (Ho 2008). About 73% of the steroid-refractory GVHD cases involved the gastrointestinal tract and skin. The mortality rate

associated with steroid-refractory GVHD in our cohort was about 10% although the results can't be generalized due to the small sample size.

iv. Lung GVHD and Bronchiolitis Obliterans

Bronchiolitis Obliterans (BO) is a form of chronic GVHD and is one of the late-onset non-infectious complications following stem cell transplantation. BO can present from subclinical impaired pulmonary functions to varying degrees of respiratory symptoms including respiratory failure (Hildebrandt 2011). The overall prevalence of BO in transplanted patients is about 5.5% and 14% in patients with chronic GVHD (Brandon 2011). Bronchiolitis obliterans remains underdiagnosed as patients develop symptoms only at a later stage and regular monitoring of pulmonary functions is required for early diagnosis and management of this condition. The common presentations include shortness of breath and cough. Organising pneumonia on the other hand causes restrictive lung defects by involving the lung parenchyma. This can present as impaired diffusion capacity on lung function tests. High-resolution CT thorax (HRCT) is required for the evaluation of lung GVHD and the different manifestations include ground glass changes, nodularity and fibrotic changes. The condition is usually managed with high-dose corticosteroids and occasionally may need steroid-sparing immunosuppressants.

In our cohort, we have evaluated the risk of pulmonary complications through symptom review, pulmonary function tests 100 days and one year after stem cell transplantation. Out of the 104 allogeneic transplant recipients, about 7 patients (6.7%) developed lung GVHD mostly diagnosed based on the symptoms. There was a slight male predominance and 70% of the patients had an unrelated transplant. Regarding the underlying conditions 3 patients had a history of MDS, 2 patients had AML and a case of granulocytic sarcoma and biphenotypic leukaemia. Twenty-eight per cent of the patients had a history of smoking and abnormal

pulmonary functions before the transplant. 86% of these patients received busulfan-based chemotherapy which is well known for its pulmonary complications. 50% of the BOOP cases

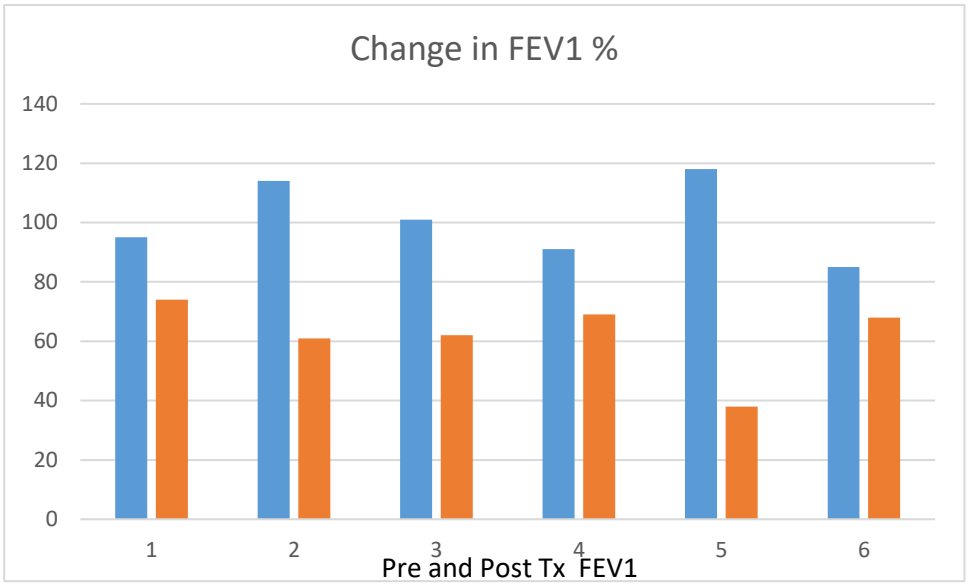


Figure 3.6: Changes in pre and post-Transplant FEV1 with BOOP

had a history of acute and chronic graft versus host disease. All of the cases of BOOP had developed 100 days after the transplantation and within one year. Figure 3.6 shows the changes in FEV1 during the pre and post-transplant pulmonary functions. The cases were treated with a high dose of corticosteroids 1-2 mg/kg /day with good clinical response.

v. Donor Status and Acute GVHD

Out of the 51 sibling donors recruited into the study, 22 of their corresponding recipients developed acute GVHD within 100 days of transplantation (43%). There was a slight male predominance noted both in the GVH and a non-GVH group of donors but this could be related to the sample cohort as opposed to association with GVHD risk. The average age of the donors was 44 years. In our cohort, 59% of the GVHD cases had male donors as a source of stem cells. About 45% of cases of sibling transplants were associated with moderate to severe graft versus host disease. A male donor predominance had been noted in the recipients who developed severe GVHD.

vi. Mortality related to acute GVHD and Stem cell Transplantation

Transplant-related mortality is one of the major limiting factors for allogeneic bone marrow transplantation. Many factors affect the risk of mortality including infectious complications, treatment-related toxicity, and graft versus host disease (Styczyński 2020). With the increased availability of matched donors and improved treatment strategies, there has been a progression in the outcomes of transplantation and better patient quality of life. Overall a 5-year survival rate of more than 80% has been reported in association with allogeneic stem cell transplants and most of the early mortality occurs within 100 days of transplantation mainly due to infectious complications. In our cohort, bacterial and fungal infections and associated multiorgan failure had contributed to the majority of the deaths. The all-cause mortality rate in our cohort at 100 days following transplantation was 20%. A slight female predominance was noted in association with all causes of death including graft versus host disease. More than 60% of deaths were associated with high-risk AML and lymphomas. In about 52% of the cases, acute graft versus host disease was the major contributing factor leading up to mortality. Lymphoproliferative disorders were associated with the highest number of GVH and non-GVH-related mortality (figure 3.7)We haven’t noticed any correlation between baseline abnormality on pulmonary functions and risk of mortality.

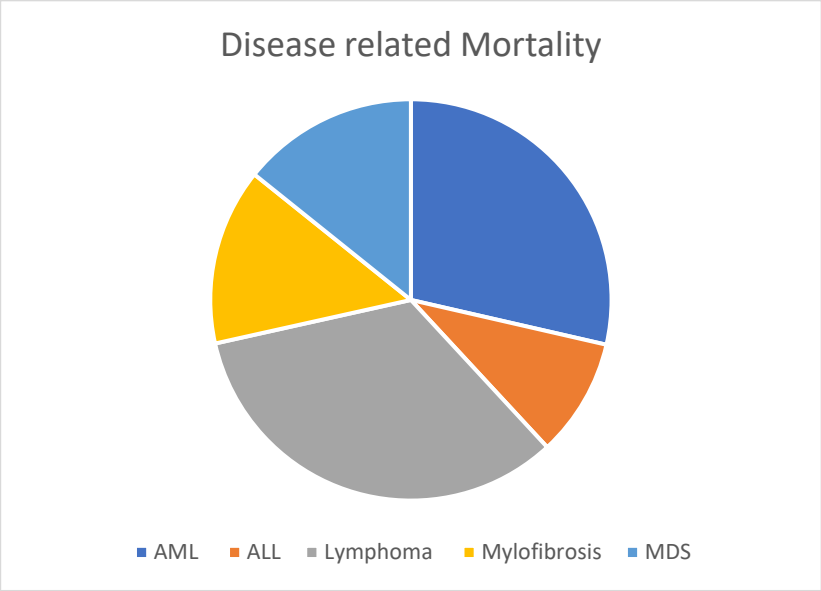


Fig 3.7: Disease-related all-cause Mortality

Chapter 4

Investigating Gut Microbiome Expression on the risk of acute Graft Versus Host Disease Following Stem Cell Transplantation

4.1 Introduction:

The role of the microbiota in various inflammatory and metabolic disorders has been extensively evaluated especially of the microorganisms in the gastrointestinal tract, a diverse and rich microbial niche that accounts for maintaining a homeostatic state of health in the host. Through their direct action on mucosal and epithelial surfaces and through their metabolic products, microbes influence various inflammatory processes and immune pathways in the host cells. The role of beneficial microbial organisms in maintaining effective haematopoiesis plays a significant role in stem cell transplantation (Khosravi 2014, Balmer 2014, Tada 1996). Altering this microbial signature will have an impact on the outcome of transplantation including the risk of graft versus host disease and overall survival.

Various factors affect the microbiome diversity during the transplantation process including recipient factors, diet, antibiotic use, intensity, and type of preconditioning regime used. Several studies (Taur 2014, Peled 2020, Ilett 2020, Montassier 2015) demonstrated the shifts in microbes with loss of diversity and predominance of single taxa resulting in an increased risk of graft versus host disease and survival following stem cell transplantation. Lower microbial diversity pre-transplantation is associated with poor survival. This alteration in microbial composition can even precede the transplantation process but very limited information is available on the same (Peled 2020)

Our data is unique in that the microbial analysis is carried out over a 2-4 weeks period prior to the stem cell infusion looking for a specific microbial signature predicting the risk of acute graft versus host disease (aGVHD), steroid responsiveness, and survival. The microbiome

changes can precede even weeks prior to the cell stem transplantation leading to changes in the clinical outcomes.

4.2 Methods and Comparisons

The faecal samples were collected from recipients undergoing related and unrelated stem cell transplantation. The samples were analysed through different comparisons with regard to incidence and severity of aGVHD, steroid responsiveness, and survival following transplantation.

Comparison 1 was carried out between patients who developed aGVHD (group 1)and those who didn't develop aGVHD (group2). The occurrence of acute GVHD in our sample cohort was 52%.

Comparison 4 was carried out based on the steroid responsiveness of aGVHD.(group5) 20% of patients had steroid-resistant (group 5) aGVHD adding complexity to the clinical management of this condition.

Comparison 5 was carried out between aGVHD patients who died (group 7), GVHD patients who survived(group 8), and those who did not develop GVHD (group 9). The mortality rate associated with GVHD in our cohort was 13%, comparable to the previous study results(80). Overall mortality associated with stem cell transplantation in our cohort was 15.4%.

Microbial DNA extraction was carried out from faecal samples using the QIAamp PowerFecal DNA kit. 32.6% of patients had a history of VRE colonization (vancomycin-resistant Enterococci) at the time of stem cell transplantation. After extraction of microbial DNA to appropriate concentrations, genomic analysis has been carried out with shotgun metagenomics sequencing, using both taxonomic annotations to genus and species profiles and using functional mapping to the Uniprot database enzyme commission (EC) numbers.Using shotgun sequencing a detailed analysis has been carried out, first by computing diversity metrics, such

as Shannon diversity, observed taxonomic units (OTU) and evenness, on the taxonomic profiles of the genus and species level. Alpha diversity is measured by the richness of a species and its relative proportion among other groups. Then, we used principal component and principal coordinates analysis for dimensionality reduction of the microbiome composition. We measured the distances between microbiome composition using beta-diversity metrics and tested for clustering of samples according to the different comparisons. Finally, we have also used a random forest algorithm to predict rather a patient that developed aGVHD had recovered or deceased.

4.3 Results:

Random Forest:

While using random forest and genus information for sample analysis we were able to accurately predict with a specificity of 0.73 on average in most of the comparison groups (fig 4.1)

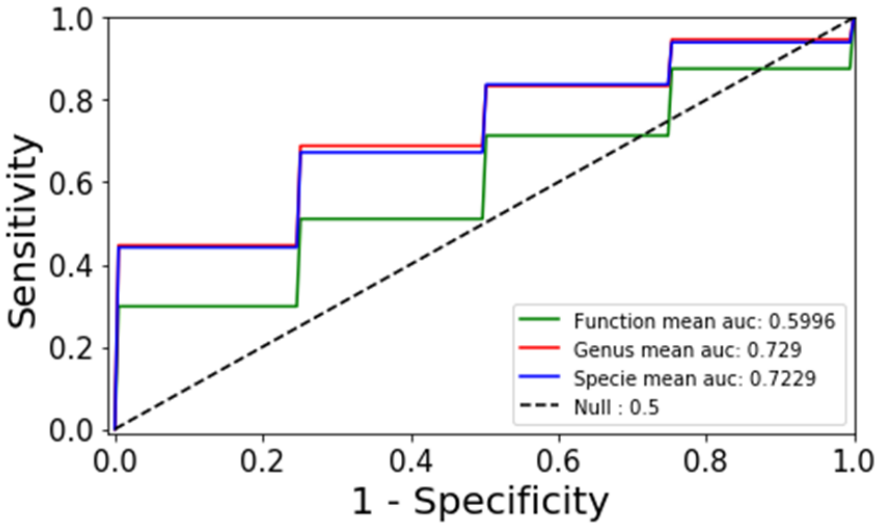


Fig 4.1: Random Forest Prediction Model

Alpha Diversity :

Various diversity indices such as Shannon diversity, evenness, and OTU were evaluated between various comparisons, especially to the risk of developing acute GVHD and its severity (Comparisons 1 &2). In comparison 5 microbial diversity is examined with regards to survival following aGVHD.

At the genus level, microbial diversity and richness didn't change significantly between most of the groups (comparison 1, 2, and 5). (Fig 4.2a)

Similarly, at species levels, no significant difference in diversity and richness parameters has been observed between most of the comparison groups Fig (4.2b) Significance or lack of it might be due to the sensitivity of unbalanced design

Interestingly a difference in diversity and evenness was observed between the steroid-resistant and sensitive groups(comp 4), as a slightly higher genus, alpha diversity index has been observed in association with steroid responsiveness although, at the species level, no significant difference has been observed between these two groups (fig 4.3).

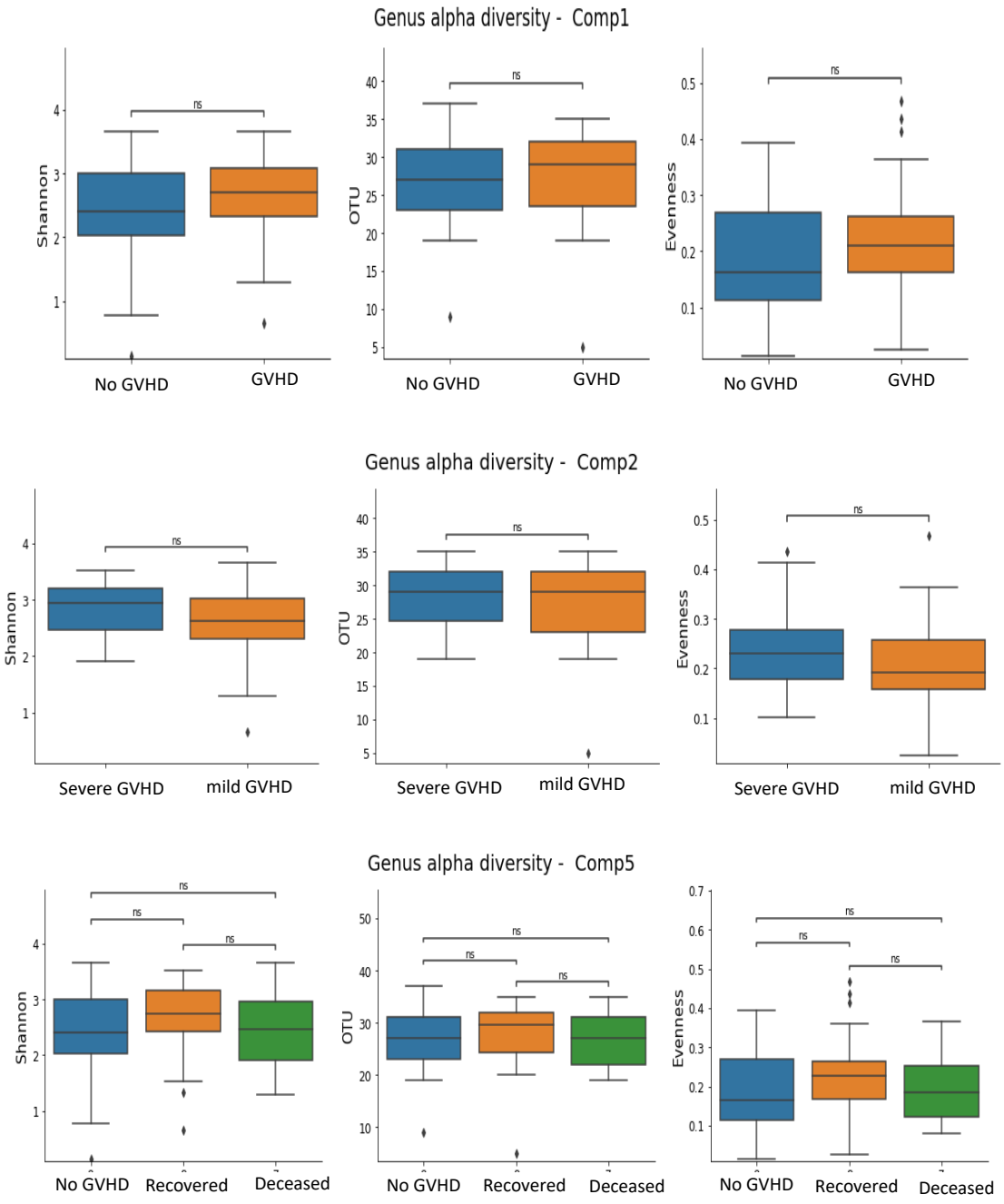


Fig 4.2a Comparison of Genus alpha diversity in GVH Vs Non-GVH group

The analysis of alpha diversity at the genus level didn't show any significant difference between the microbiome of patients in terms of risk of developing aGVHD and severity and mortality related to aGVHD

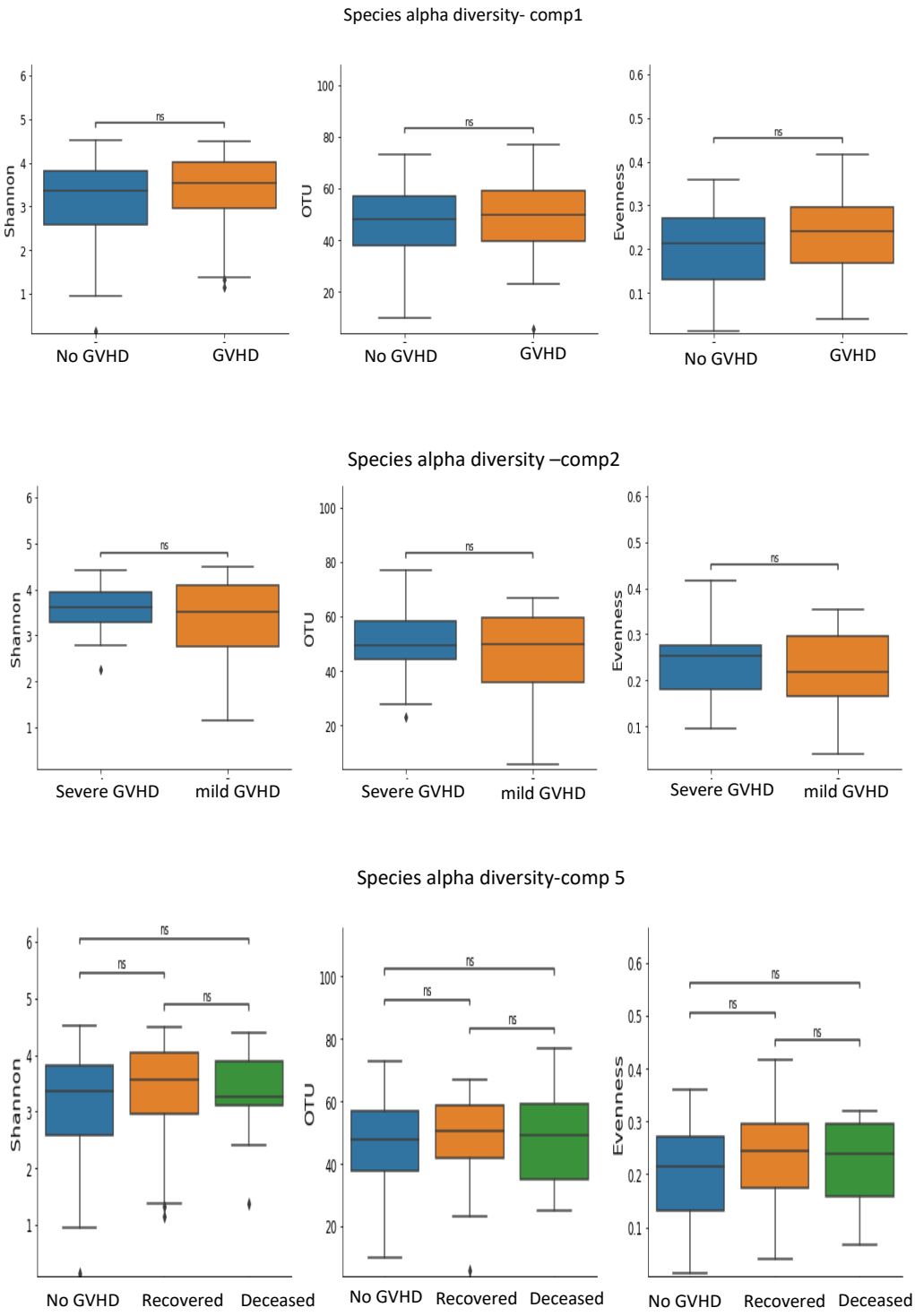


Fig 4.2b Comparison of Species alpha diversity in GVH Vs Non-GVH group

Analysis of microbial species diversity hasn't shown any significant difference between the various groups with regard to the development and severity of aGVHD and the survival following aGVHD.

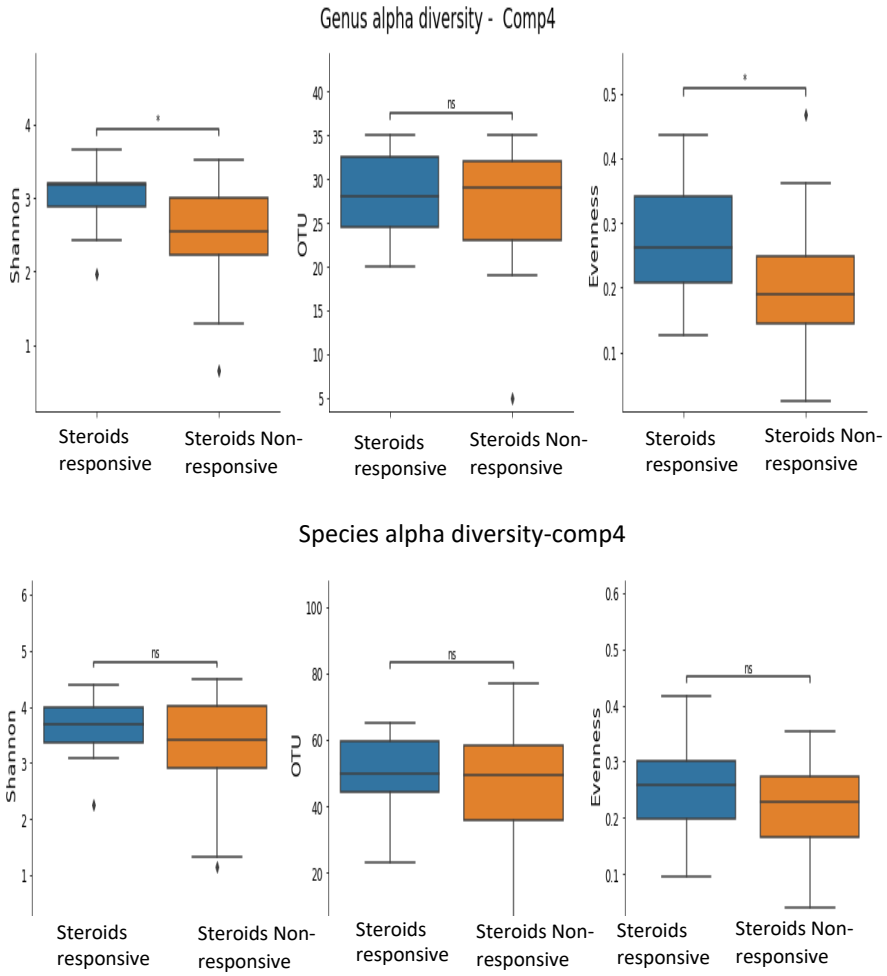


Fig 4.3: Alpha Diversity and Steroid Responsiveness

Microbial analysis showed a slightly higher alpha diversity in the steroid-sensitive group in comparison to the steroid-resistant group in the patients with aGVHD.

Further subset analysis based on gender showed similar results to the unstratified analysis, but at the genus level, we observed a stronger separation in alpha diversity and richness with male patients having higher scores in the steroid-sensitive group (fig 4.4). We observe that the steroid-responsive group has a significantly higher score, especially on the evenness diversity measure at the species level as well.

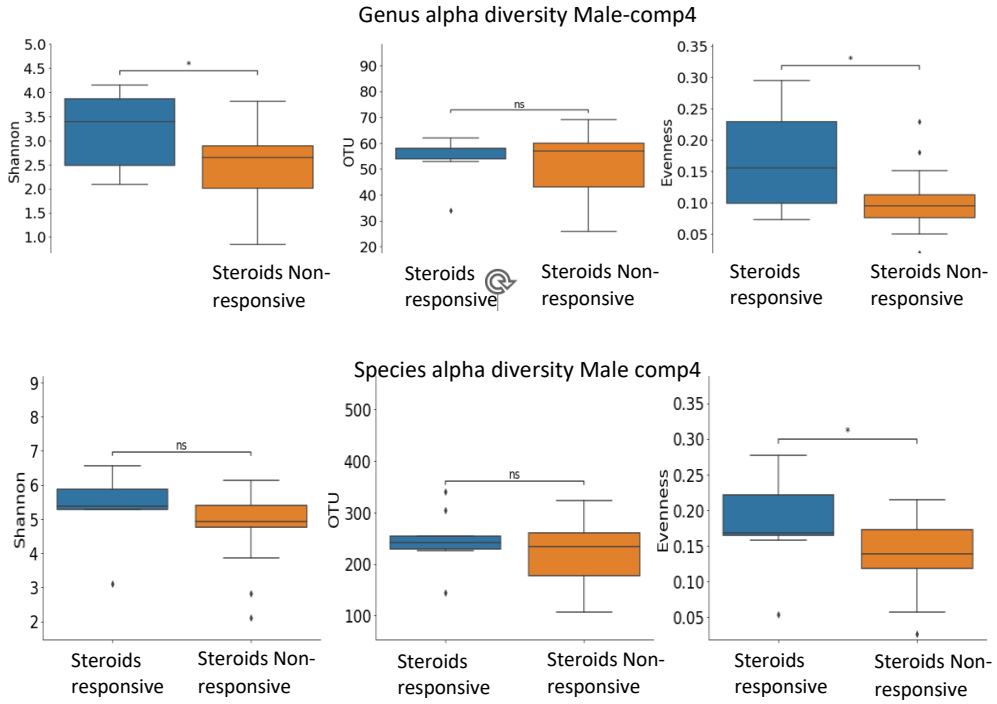


Fig 4.4: Alpha Diversity and Steroid Responsiveness -Males

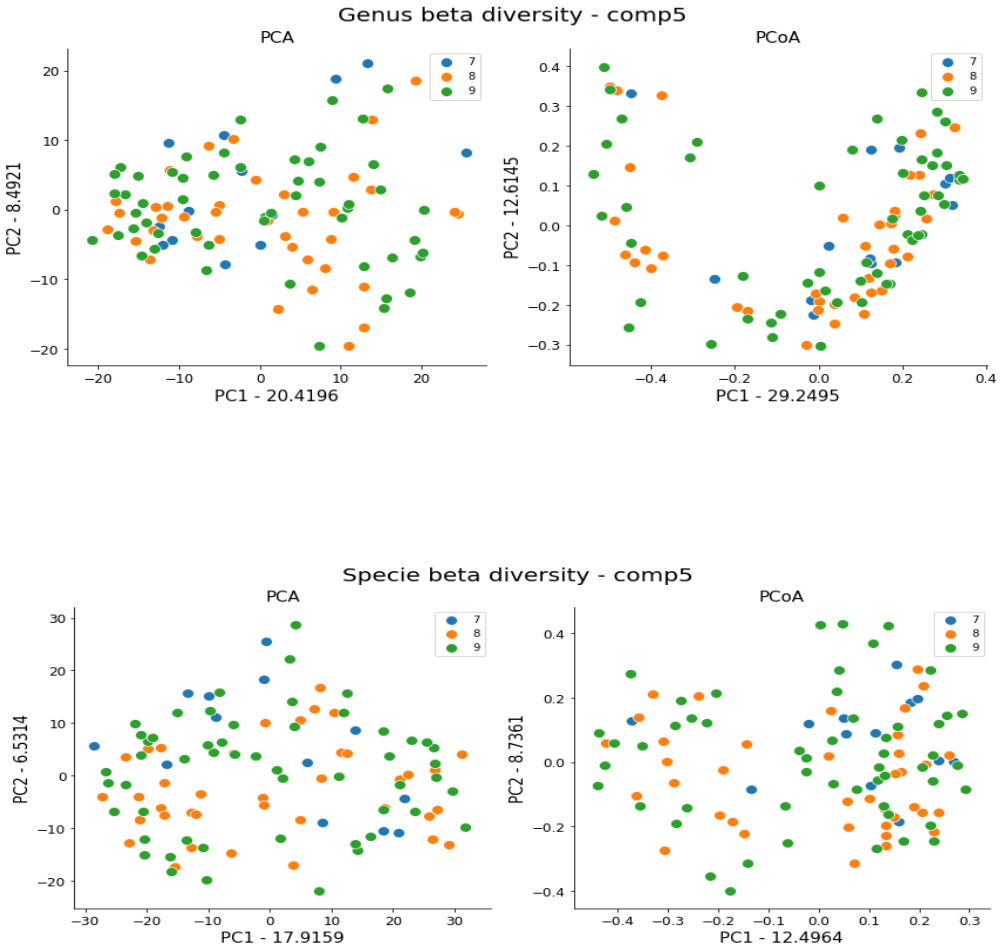
Microbial analysis of male patients with aGVHD shows a striking difference in various diversity indices at the genus level as well as a higher evenness index at the species level in the steroid-responsive group compared to the steroid-resistant group.

Beta Diversity:

Beta diversity is a measure of the similarities and dissimilarities of microbial profiles between different samples. Beta diversity has been calculated by PCA (principal components analysis) and principal coordinates analysis (PCoA).

In PCA each dot represents the microbiome profile of each patient. PCA has been commonly used in exploratory data analysis and for making prediction models. PCoA is a statistical method that converts the data on the distance between the items into a map-based visualization of those items. The analysis mainly looks for maximum variants and clustering of the different points.

For comparison 5 at the genus level has shown some clustering of points between non-survivors(group 7)and survivors (group 8) of acute graft versus host disease whereas, at the species level, no significant relationship has been noted (fig 4.5)

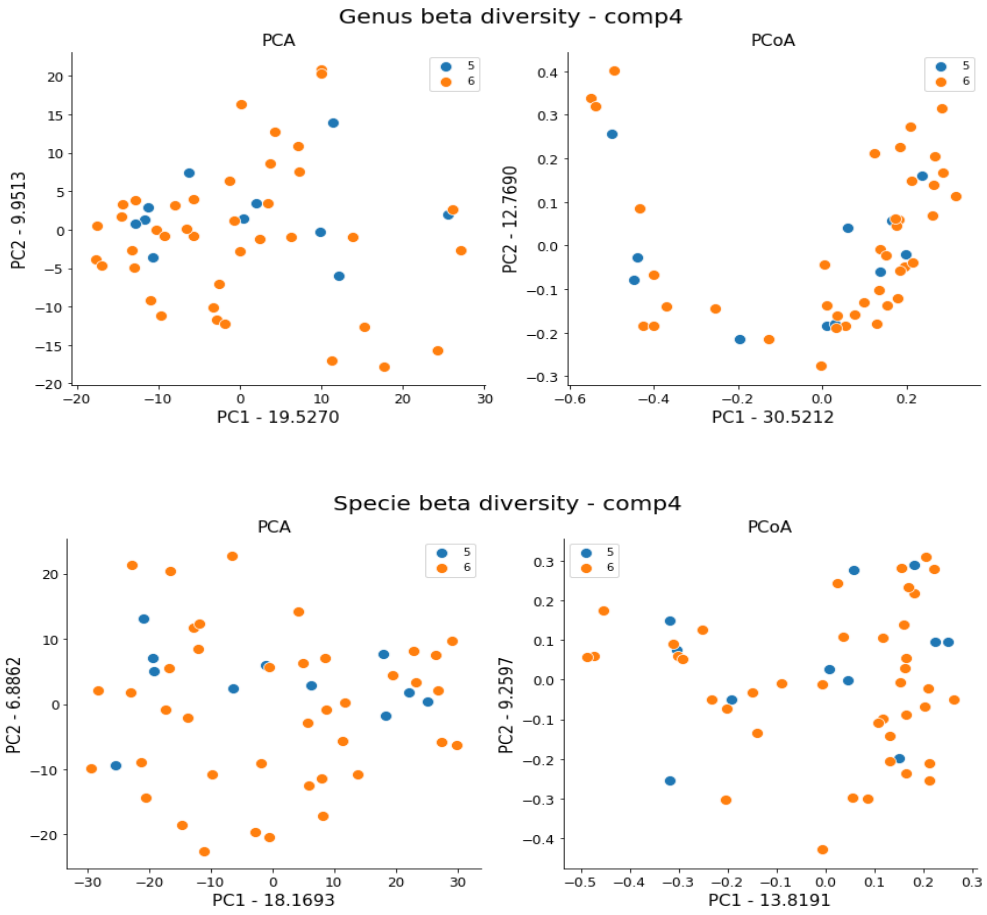


Group 7- GVH non-survivors, Group 8- GVH survivors, Group 9- non-GVH

Fig 4.5: Beta Diversity and GVHD survival

Some clustering of dots (microbiome of each patient) was noted with genus beta-diversity but not at species levels. With regards to the aGVHD survivors and non-survivors, there is some clustering of microbiome noted for genus alpha diversity between the two groups.

For comparison 4 there has some clustering noted in the steroid-responsive patients (group 6) compared to the steroid-resistant GVHD (group 5) at species levels (fig 4.6). This is a significant finding indicating changes in microbial composition in response to treatment.



group 5- steroid-resistant group 6- steroid-sensitive

Fig 4.6: Beta Diversity and Steroid Responsiveness

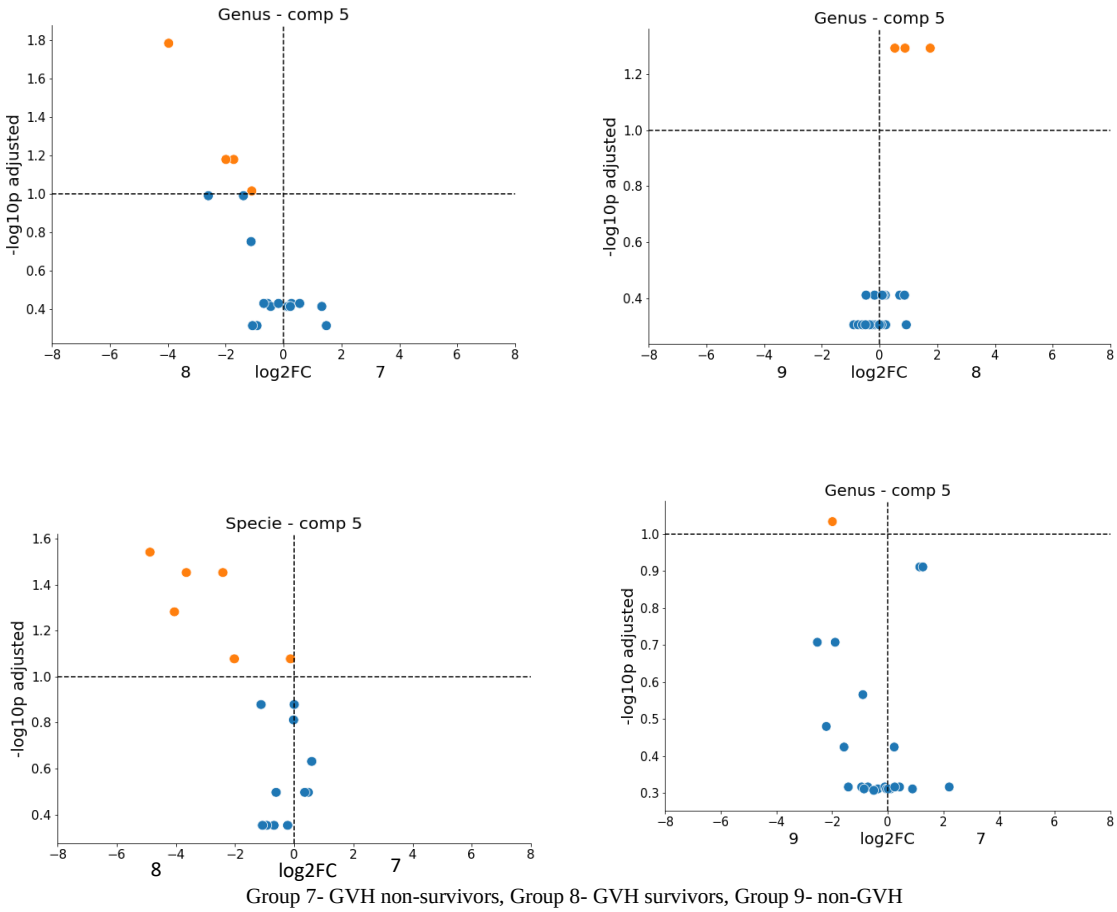
Clustering of the microbiome was noted at species levels in the steroid-responsive aGVHD group. No significant difference in genus beta diversity in response to steroid treatment. This suggests that some specific microbial species could be driving steroid responsiveness in patients who develop aGVHD.

Univariate- Taxonomy Analysis:

Univariate analysis of different groups has shown some significant findings in comparison 5 where non-survivors (group 7) of graft versus host disease are compared with survivors(group 8)and those who didn't develop graft versus host disease(group 9). There was some taxonomic

predominance of microbial organisms observed between the different groups in comparison 5 (Fig 4.7). The recovered (Gp 8)group has shown a predominance of microbial species including *streptococcus*, *Veillonella*, *eubacterium*, and *Roseburia* compared to the group who died after GVHD likely indicating their potential role in GVHD survival. There has been a predominance of *streptococci*, *Lachnospiracae* and *Dorea* spp in the patients who recovered from GVHD

compared to patients who didn't develop GVHD. This could be an observation as opposed to an association. The differences could be related to an unbalanced design. *Veillonella* was the only predominant spp noted in the non-GVHD group(Gp9).



Group 7- GVH non-survivors, Group 8- GVH survivors, Group 9- non-GVH

Fig 4.7: Univariate Taxonomy analysis and GVHD survival

There was a predominance of certain specific taxa in the GVHD survival group especially butyrate-producing microorganisms, predicting the role of certain microbiota associated with survival and aGHVD

These differences in species could be predictive of a potential protective role against the risk of GVHD and the abundance of certain species might be associated with increased survival after stem cell transplantation.

Univariate Functional Analysis:

Univariate analysis of the functional profile of genomic components has shown very exciting differences in possible metabolic pathways driving the GVHD risk and survival. Functional studies involving enzymatic activities (DNA fragments coding for enzyme action) which is referred to as metagenomics showed an explosion of significant gene counterparts, especially between GVHD survivors and non-survivors.

There were small differences observed in gene counts between people who developed graft versus host disease (group 1) and who didn't (group 2) in comparison 1. (Fig 4.8)

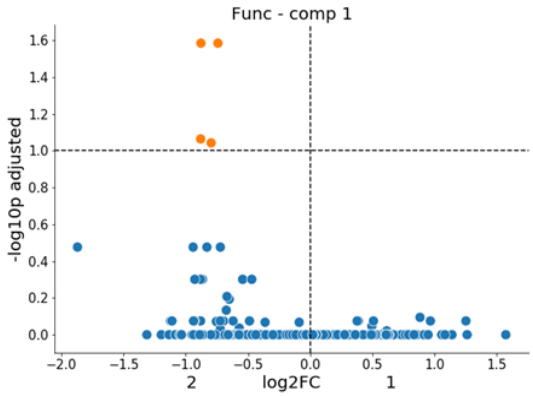
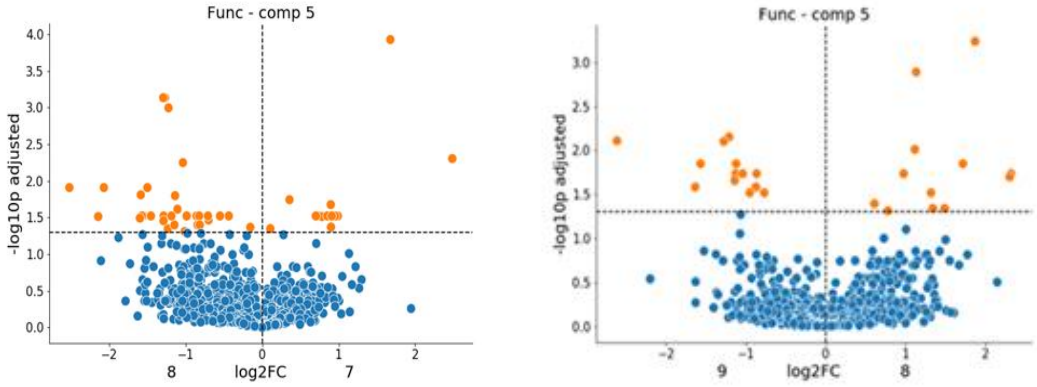


Fig 4.8: Univariate Functional analysis and Risk of GVHD

Functional analysis of genomic counterparts of the microbiome has shown some predominance in the group that didn't develop aGVHD, although not statistically significant.

The small species differences observed in comparison 5 (Gp7,8 and 9) seemed to be more significant at genomic levels. Interestingly higher expressions of certain enzymes were found in association with GVHD survivors compared to non-survivors (Fig 4.9)



Group 7- GVH non-survivors, Group 8- GVH survivors, Group 9- non-GVH

Fig 4.9: Univariate Functional Analysis and GVHD survival

Microbial genomic analysis has shown higher expression of enzymatic actions in the aGVHD survivors compared to the group that didn't survive and to those who didn't develop aGVHD

When using the random forest for comparison of groups 7 and 8, the differences noted were better than random with regards to genus, species information, and functional analysis with a prediction accuracy of more than 70%. (fig 4.10)

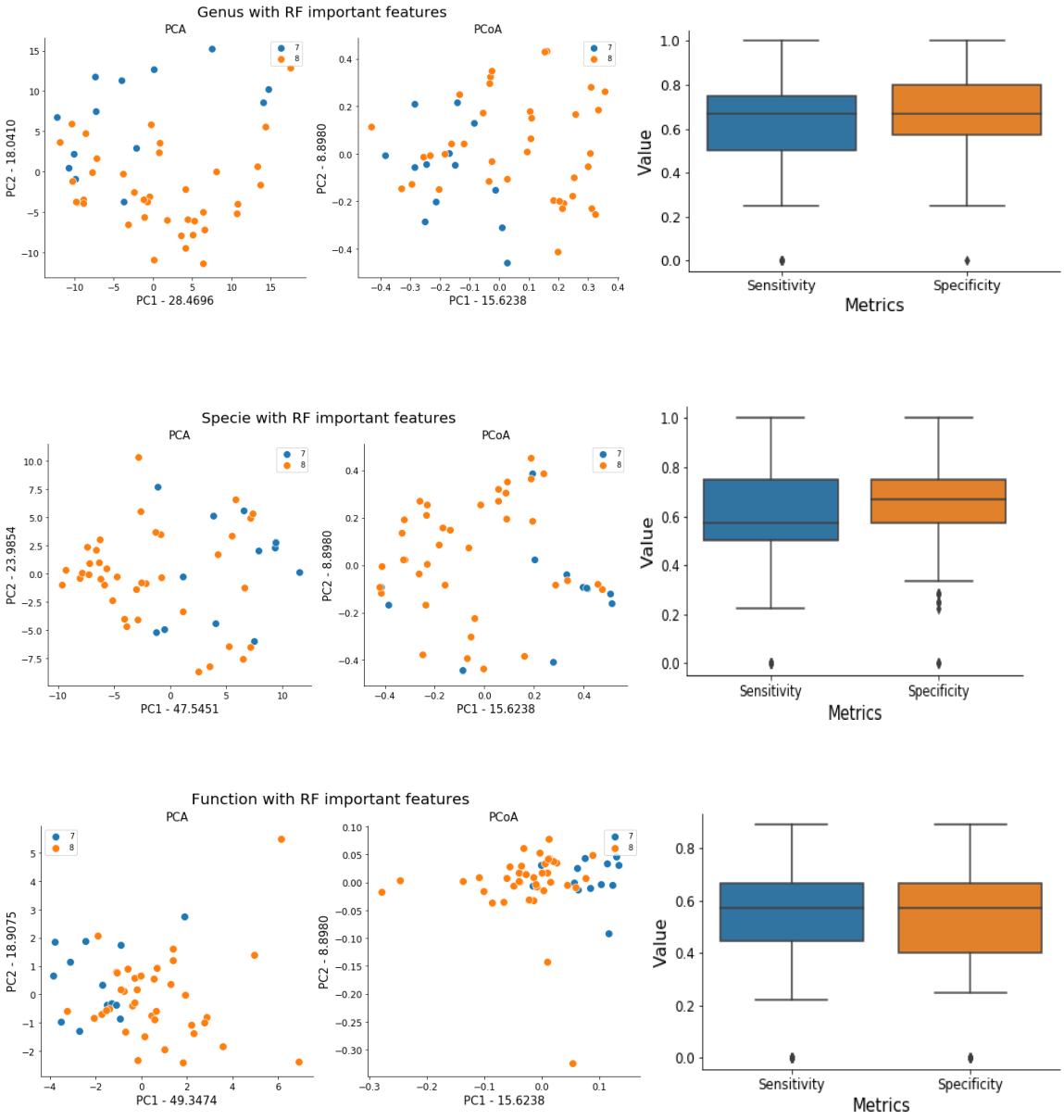


Fig 4.10: Random Forest Prediction and GVHD survival

Using RF prediction models the microbial differences noticed between aGVD survival and non-survival groups were more than a random chance with a prediction accuracy more than 70%

4.4 Discussion:

Despite advancements in treatment options and the availability of matched donors, graft versus host disease continue to pose challenges to stem cell transplantation. It remains one of the major causes of mortality and morbidity, independent of infectious complications and risk of relapse. HLA matching would certainly be an important parameter in determining the risk of GVHD. In addition to disease and graft factors, the host environment also plays a significant role in affecting the outcomes of transplantation. The role of microbiota in various disorders has been increasingly identified recently with advanced molecular techniques. The knowledge of various microbial organisms and their genomic components sheds light on their potential role in the pathogenesis of the disease. Gut microbiota predominates in their numbers by millions and is one of the easily accessible microbial niches for sample collection.

Studies looking into post-transplantation gut microbes (Taur 2014, Peled 2020) demonstrated that lower bacterial diversity is observed in the post-transplantation period compared to pre-transplantation. Moreover, lower diversity is predominated by single taxa including *Enterococci*, *Lactobacilli*, *Enterobacteriaceae*, and *Streptococci* (Taur 2014). The lower diversity is followed by the predominant growth of potentially pathogenic bacteria like *Proteobacteria* increasing the risk of bacteraemia (Taur 2012).

Our study was looking at microbial diversity on samples collected up to 2-4 weeks prior to stem cell infusion on the risk of acute graft versus host disease and survival. We hypothesize that the microbial shift occurs even weeks prior to the engraftment which could be predictive of the risk of acute graft versus host disease and survival following stem cell transplantation. In our cohort, no significant difference was observed in alpha and beta diversity between recipients who developed GVHD and those who didn't, but univariate functional analysis of enzymatic studies has shown slightly more encoding gene counterparts in the non-GVHD group. Previous studies have shown (Golob 2017, Jenq2015) association of a lower bacterial

index after transplantation is associated with a higher incidence of acute GVHD after the engraftment period.

Studies have shown that changes in microbial composition are associated with survival following acute GVHD. A study by Jenq et al (2015) demonstrated *Blautia* species were associated with GVHD survival whereas *Veillonella* spp with increased GVHD-related morality following stem cell transplantation. Another study cohort (Golob 2017) in the post-transplantation period has noted the predominance of *Firmicutes* and *Actinobacteria* in association with increased incidence of aGVHD and *Lachnospiraceae* with reduced risk of acute GVHD. Few other studies (Holler 2014, Sten-Thoeringer 2019) have shown an abundance of *Enterococcus* in association with increased incidence of GVHD and the role of lactose fermentation in driving *enterococcal* growth(Stein-Thoeringer 2019) Han et al have developed(Han et al 2020) gut microbiota score(GMS) to predict the risk of aGVHD following myeloablative HSCT. The authors have demonstrated the role of microbiota associated aGVHD is linked with increased cytokine production and through Treg/Th17 pathway activation (Han 2018). It has been noted that GMS at neutrophil engraftment can predict the risk of aGVHD and can be used as a potential tool for manipulating the GVHD risk and improving the outcome of HSCT.

In comparison 5 although there has been no significant difference in alpha and beta diversity, GVHD survivors have been noted to have a predominance of certain species compared to GVHD non-survivor group. With the univariate taxonomy analysis, GVHD survivors had a higher diversity index with a predominance of *streptococci*, *Veillonella*, *Eubacterium*, and *Roseburia* compared to non-survivors. Compared to the non-GVHD group, GVHD survivors are noted to have a predominance of certain species like *Streptococci*, *Lachnospira*, and *Dorea*. Although these findings couldn't reflect a causative association, this was a significant observation. *Eubacterium*, *Roseburia* and *Lachnospira* belong to the intestinal colonic niche

which ferments various micronutrients into short-chain fatty acids especially butyrate which has anti-inflammatory properties (Canani 2011). Our study findings with GVHD survivors were supportive of a recent study (Payen 2020) which showed a negative correlation between aGVHD and the prominence of anaerobic bacteria belonging to *Lachnospiraceae* and *Ruminococcaceae* families. In the intestine, Butyrate plays a significant role in reducing mucosal inflammation and free radicle injury, Butyrate affects gene expression of various pro-inflammatory cytokines thereby reducing inflammation and maintaining a homeostatic state of the immune system in host cells. *Dorea* which is a gram-positive bacteria belonging to the *Lachnospira* family is the only predominant species noted in the non-GVHD group. The beneficial effects of short-chain fatty acids in reducing inflammation and maintaining mucosal integrity play an important role in the pathogenesis of acute graft versus host disease.

The difference in diversity index in the survivors could certainly shed light on the understanding of the association between higher diversity and reduced risk of mortality following transplantation. A recent study by Peled et al (Peled 2020) using 16S rRNA gene sequencing looking at post-transplantation microbial diversity in patients from multiple centres has shown higher GVHD-related deaths in the lower diversity group compared to the higher bacterial diversity group. Our findings indicate that microbial shift happens even weeks preceding transplantation which can be used as a marker to predict the risk of aGVHD.

One of the recent studies (Ilett 2020) looking at the microbial diversity and risk of acute graft versus host disease has shown decreased gene richness in the early post-transplantation period in patients who developed aGVHD. However, they haven't noted any significant association between pre-transplant microbial diversity and risk of acute graft versus host disease. Univariate functional analysis of comparison 5 cohorts showed striking differences at genomic levels coding for enzymatic pathways with significantly higher expression of genomic counts

noted in the GVHD survivor group. This could indicate potential enzymatic pathways driving inflammation and the risk of GVHD-related survival which is affected by microbial diversity.

Translating this to clinical practice means a potential modulation of the specific microbiome could affect the risk of GVHD and thereby the outcome of stem cell transplantation.

4.5 Potential Microbiota Determining Risk of acute Graft Versus Host Disease and Survival

The microbial shift has been implicated in the pathogenesis of graft versus host disease and other complications associated with stem cell transplantation. Interestingly, the microbial composition changes precede even weeks before stem cell infusion. From our analysis, there are few bacteria identified which could be potentially linked to the risk of acute GVHD. Applying this information to clinical practice means potential modulation of this microbial composition could offer improved outcomes to the risk of acute GVHD and overall survival. The following is the information on some of the key microbial agents identified in our study which might have an association with the risk of acute GVHD and steroid responsiveness through their involvement in various metabolic pathways.

***Roseburia*:**

Roseburia is gram-positive obligate anaerobic bacteria that belong to the phylum *Firmicutes*. They are motile with multiple flagellate-type structures and are rod-shaped. The genus has been named in the honor of Theodor Rosebury who had been pioneering the work of oral microbiome. *Roseburia* belongs to the class clostridia and the family *Lachnospiraceae*(Stackebrandt 2014) and is part of the intestinal commensal bacteria that are associated with the production of SCFA, especially butyrate. The important species in this group include *R.intestinalis, R.faecis, R.hominis, R.inulinivorans, and R.cecicola*(Tamanai-Shacoori 2017) Several inflammatory and metabolic disorders that have been associated with *Roseburia*

species such as diabetes, allergic disorders, irritable bowel syndrome, and various biliary diseases and the beneficial role of these microbes has been well studied.

As one of the most abundant gut bacteria, *R.intestinalis* has been first isolated from the faecal samples based on an M2 culture system (Louis 2004), and based on its phylogenetic similarity to other *Roseburia* species it has been coined as *R.intestinalis* (Duncan 2002). *Roseburia* species have a significant role in various inflammatory and metabolic disorders and are essential for maintaining intestinal barrier function, and regulating cytokine-mediated inflammatory response through butyrate production (Nie 2021). *Roseburia* is well known to be involved in the regulation of gut inflammation, atherosclerosis, and immune response, mediated through butyrate (Kasahara 2018).

Microbial dysbiosis has been identified as a cause of many inflammatory and metabolic disorders and the role of the gut microbiome merits special attention. A large number of studies have looked at the role of *Robesuria* and other commensal bacteria in maintaining the epithelial barrier function and anti-inflammatory action mediated through their metabolites. Reduced numbers of *Roseburia* species are seen in association with inflammatory bowel diseases as shown in Crohn's patient cohorts (Gevers 2014, Morgan 2012). A study by Shen et al. using genomic sequencing of faecal samples has revealed decreased levels of *R. intestinalis* in association with Crohn's disease. *Roseburia* has anti-inflammatory action mediated through Treg cells and cytokines like IL 10, TGF-beta, and TSLP. Mice model studies have demonstrated an abundance of *Roseburia* species has been linked with signs of reduced intestinal inflammation such as decreased epithelial cell injury, lesser mucosal lymphocytic infiltration, and overall improvements in disease outcome (Shen 2018).

Similarly, ulcerative colitis has been also associated with a shift in microbial composition in the gut including *Roseburia* species associated with reduced butyrate levels(Kumari 2013), mediating the inflammatory process. The abundance of *Roseburia* is shown to reduce hepatic

steatosis and inflammation. Seo et al. have demonstrated the role of the Flagellin component of the bacteria in upregulating the levels of tight junction proteins likely through action via Toll-like receptors thereby maintaining the epithelial integrity (Seo 2019) The dysbiosis of *R. intestinalis* has been implicated in alcoholic fatty liver disease.

Although there are different mechanisms by which *Roseburia* exerts its beneficial effects on inflammation and immune response, its unique butyrate-producing properties remain one of the key mechanisms. Butyrate is one of the essential short-chain fatty acids, along with acetate and propionate, produced by the metabolism of dietary fibre by intestinal bacteria. Butyrate causes enhancement of Treg cells (regulatory T cells) in extra-medullary sites leading to the production of anti-inflammatory cytokines such IL- 10, IF- gamma, and TGF -beta. (Arpaia 2013). The effect of butyrate on immune response is not limited to T cell regulation but involves the B cell population and its memory cells. Recent studies have shown that butyrate stimulates the production of 5-hydroxy indole acetic acid(5-HIAA)which in turn can bind to the aryl hydrocarbon receptors(AhR) of regulatory B cells, exerting anti-inflammatory effects (Michaudel and Sokol, 2020). Butyrate has an additional role in regulating macrophage function through inhibition of acetone deacetylase and reduced butyrate levels which can evoke a pro-inflammatory response(Michaudel and Sokol 2020). The regulatory role of butyrate on type 2 innate lymphoid cells (ILC2) has been associated with anti-inflammatory effects such as shown in asthma and airway hyperactivity (Lewis 2019). The SCFA can regulate oxidative phosphorylation and glycolytic pathways in ILC 2. Germ-free murine models have shown that butyrate-mediated suppression of ILC2-driven airway hyperactivity and inflammation (Lewis 2019)

Roseburia species affects the gut barrier function possibly via flagellin /Toll-like receptors-5 (TLR-5) thereby leading to increased expression of T regulatory cells (Treg) and maintaining the epithelial cell integrity (Patterson 2017). Activated TLR-5 can cause stimulation of ILC3

(type 3 innate lymphoid cells) leading to the production of anti-inflammatory cytokines (Vijayan 2018). Nie et al. have shown alteration of tryptophan metabolism related to Flagellin-TLRS 5 signalling and stimulation of ILC through indole metabolites leading to anti-inflammatory properties both locally in the intestine and systemically Nie 2021).

Eubacterium:

Eubacterium is a gram-positive organism belonging to the family of *Eubacteriaceae* with a thick peptidoglycan cell wall. They can be motile with a flagellum or immotile and are generally non-sporing bacteria. They are normal flora in the intestine and occasionally can cause opportunistic infections. The genus *Eubacterium* have been first proposed by Prevot in 1938 (Wade 2006) and since then a large number of phylogenetically different species have been described. They are found in the oral cavity and intestine of mammals and are well known for their butyrate-producing properties.

Many species under the genus *Eubacterium* differ phenotypically and phylogenetically, making it one of the complex taxonomic groups. They are included under the phylum *Firmicutes* and recent 16S rRNA sequencing helped the identification and reclassification of a large number of species The genus has a large number of sub-species about 40-44 depending on the taxonomic classification(Mukherjee 2020). Based on the detailed information on genomic structure these species have been reclassified as *Anaeributyricum* hallii (Shetty 2018). A few of the functionally important species include *E.hallii and E.rectale* which are butyrate producers.

Functions**:**

Various *Eubacterium* species have been beneficial as producers of SCFA such as butyrate and propionate. The type of diet is a major determining factor for the presence of these beneficial bacteria in the gut. The presence of *Eubacterium* in the gut is been increased by fibre rich diet and their levels are depressed with high fat and protein-containing diet (Duncan 2007).

They play a significant role in the intestinal microbial niche due to unique butyrate-producing properties from intermediary products of glucose metabolism such as lactate and acetate (Engels 2016). One of the species *E.hallii* can convert glycerol into one of the esters of propionate, an SCFA which is been utilized for the formation of cobalamine (Engels 2016) Thus this species act as a major SCFA producer maintaining a metabolic balance between the immune system and gut microbiota. SCFA acts as an immune regulator of the T cell population and controls inflammation (Louis 2014).

The lactate fermenting action of *E.hallii* is beneficial to maintaining a metabolic balance by clearing lactate from excess accumulation. This has been shown in associated with several gastrointestinal disorders (Duncan 2004). The authors also described the ability of *E.hallii* to produce butyrate from glucose.

Lachnospira:

Lachnospiraceae is one of the most abundant taxa in the human gut well known for its butyrate production. They are obligate anaerobes belonging to the order *clostridial*, phylum *Firmicutes*, and can be spore-forming or not. One of the earliest identified species was *L.multipara* named by Bryant and Small in 1956 (NCBI, National Centre for Biotechnology Information). The species can be present in the gut from birth and increase in richness and become one of the prominent bacteria with age (Odamaki 2016). With new molecular techniques, several genera have been identified in the *Lachnospiraceae* family including *Blautia, Dorea, Lachnospira, Roseburia, Ruminococcus, Coprococcus, and Oribacterium* (Vacca 2020).

Many members of the *Lachnospiraceae* family are essential for health being one of the major Butyrate producers in the gut. They ferment complex indigestible starches such as cellulose and hemicellulose into beneficial short-chain fatty acids like acetate, propionate, and butyrate which are absorbed and used as energy sources by the host(Biddle 2013, Louis 2017) and are involved in several immune reactions. In addition to butyrate, the species of *Lachonospira*

produces formate, lactate, and H2(Tamanai 2017). There are two main pathways involved in the production of butyrate from butyryl CoA, (Louis 2017) by actions of butyryl kinase and butyryl CoA: acetate CoA transferase. As predominant species in the gut, *Blautia* and *Roseburia* are two major butyrate producers which are associated with healthy states (Koh 2016).

Intestinal epithelial cells derive their energy from various metabolites, of which SCFA plays a major role (Sun 2017). The enhanced fatty acid metabolism mediated through *Lachnospira* is associated with reduced levels of inflammatory mediators (Kasahara 2018). The levels of SCFA depend on the diet with more abundant levels associated with a fibre-rich diet and lower levels have been reported in association with several gastrointestinal disorders. (Fusurawa 2013). Treg cells (T regulatory Cells) are important in developing immune tolerance and maintaining homeostasis and SCFA plays a significant role in Treg cell differentiation and accumulation in the intestinal tract (Steinmeyer 2015, Furusawa 2013, Smith 2013).

Blautia belongs to the *Lachnospiraceae*, one of the important species involved in colonic inflammatory disorders including malignancy and liver cirrhosis and their abundance is associated with better disease outcomes (Chen 2012, Bajaj 2012). Jenq et al. have demonstrated an abundance of *Blautia spp* in reducing the severity of graft versus host disease and improving survival following stem cell transplantation (Jenq 2015)

On contrary, to the beneficial effects mediated through butyrate, *Lachnospiraceae* species are involved in many metabolic and inflammatory disorders. The abundance of *Firmicutes* phylum where *Lachnospiraceae* belongs, with a reduction in *Bacteroides*, is associated with high BMI and possible correlation with diabetes and lipid metabolic diseases (Ley 2006, Lippert 2017). *Lachnospiraceae* is associated with impairment in glucose metabolism, resulting in persistent inflammation and the development of Diabetes (Kostic 2015). Mice model studies have shown certain *Lachnospira* species are associated with the hyperglycaemic

state, obesity, and Diabetes (Kemeyama 2014). In another study, there were decreased levels of butyrate-producing bacteria noted in the faecal samples of Type 2 DM patients indicating a protective role of butyrate on carbohydrate metabolic disorders (Qin 2010). It appears like *Blautia* species have their beneficial effects mediated through butyrate from dietary fibre fermentation (Sheridan 2016. The role of gut bacteria in carbohydrate metabolism plays a significant impact on glucose levels in diabetic patients (Koh 2016). It's been noted that certain *Lachnospira* species including *Blautia spp* have been involved in accelerating the fibrotic process in association with Non-alcoholic steatohepatitis(NASH) (Shen 2017) and the abundance of *Lachnospira spp* noted with the condition. There are a significantly large number of *Lachnopsiraceae* seen in relation to primary sclerosing cholangitis associated with IBD (Quraishi 2017). Several *Lachnospira* species have been linked with inflammatory bowel disease including Crohn's and ulcerative colitis. (Gevers 2014). Higher levels of butyrate producers have a better disease outcome in relation to IBD (Sasaki 2019).

Veillonella:

Veillonella is a gram-negative anaerobic bacteria found commonly in mammals' oral cavities and intestines. They are first described by Veillon and Zuber in 1898(Kolenbrander 2006) and belong to the phylum *Firmicutes* and are non-motile bacteria. They are well known for their lactate fermenting properties to acetate and propionate, the beneficial SCFA. Most of the *Veillonella spp* are considered harmless and even beneficial, for example, *V. dispar*, the species commonly found in the mouth reduces nitrate production (Mitsui 2018). *Veillonella spp* especially in the oral cavity utilizes lactate and converts nitrate into NO2, which is shown beneficial in reducing the growth of pathogenic bacteria and improving general health (Wicaksono 2020). With the production of NO from nitrite molecules, which has (González-Soltero 2020) vasodilatory properties these species have a potential role in improving blood flow to the tissues.

These groups of bacteria usually co-exist with streptococci and utilize the lactate formed by sugar fermentation by Streptococci. With their unique lactate fermenting properties, *Veillonella spp* are seen abundantly in high-performing athletes where they coverts lactate into propionate thereby improving exercise performance and reducing exhaustion related to lactate accumulation. Murine inoculation of these strains has been shown to reduce exhaustion time related to exercise (Scheiman 2019). Loomba et al. have shown (Loomba 2021) that aldafermin-mediated (analogue of gut hormone FDF 19) enrichment of *Veillonella spp*. results in a reduction of toxic bile acid products resulting in a beneficial, role in the pathogenesis of NASH. This is likely mediated through their lactate fermenting capacity and improving overall efficiency. These bacteria as producers of SCFA especially propionate has a beneficial role in reducing inflammation and maintaining gut microbial homeostasis.

In summary, we have observed specific microbiota in association with patients group who have developed GVHD and survived suggesting a protective role of the certain microbiome in survival following stem cell transplantation. These are mainly butyrate-producing organisms of the gut possibly driving anti-inflammatory responses through butyrate production. These findings support our hypothesis as shown by the pre-transplant microbiome might be helpful in predicting the risk of aGVHD and mortality following the transplantation. Modifying these microbiomes in allogeneic transplant patients could be a potential therapeutic target in order to reduce the risk of aGVHD and improve survival.

Key Points:

- Higher alpha diversity was noted in male patients with aGVHD who have responded to steroid treatment.
- Predominance of certain tax especially butyrate producing bacteria were noted in patients with aGVHD who have survived indicating protective role of these microorganisms.
- Using Random Forest prediction models , the association of microbial diversity and survival with a GVHD can be predicted with more than >70% rather than a random finding.

Chapter 5

Influence of the microbiome on functional enzymatic pathways driving steroid responsiveness in graft versus host disease

5.1 Introduction:

Despite advancements in treatment regimes, graft-versus-host disease (GVHD) continues to pose significant challenges in the success of stem-cell transplantation. Steroid responsiveness is a key determinant of the morbidity and mortality associated with GVHD. Several other factors influence the risk of GVHD and response to treatment, including disease severity, patient comorbidities, human leukocyte antigen (HLA) compatibility, and graft source. aGVHD usually develops in the first 100 days of transplantation in approximately 50% of cases with HLA-matched donors and has a higher incidence with unmatched donors (Ferrara 2009). This carries a mortality rate of 10% (Mac Milan 2015), greatly burdens the patient's quality of life, and increases hospitalisation rates.

In our cohort, the incidence of GVHD was 52% with a slight male predominance. The number of patients aged >60 years was comparable in both groups (20.3% vs. 20%). We observed a slightly higher BMI in the GVHD group (16.6% vs. 16%), although this could be related to the small sample size.

Unrelated allogeneic transplantations were the most common in both groups, and there was a lower incidence of GVHD in the sibling transplant groups, as expected. Consistent with results of a previous study (Cutler 2001), infusion of peripheral blood stem cells is associated with a higher risk of GVHD than the bone marrow source of the graft. The risk of GVHD varies with the intensity of the pre-conditioning regime, either myeloablative or reduced-intensity, and depends on other factors such as disease severity, donor factors, and source of graft used (Nakasone 2014). In our study, the intensity of chemotherapy did not show any significant

difference in terms of the risk of GVHD, but this could be related to the small sample size. Total body irradiation has been used to reduce the risk of GVHD, as shown by results of a previous study (Nakasone 2014)

5.2 Methods and Comparisons:

Faecal samples were collected from recipients undergoing related and unrelated stem cell transplantation. The samples were analysed in different comparisons based on clinically relevant information, such as the incidence and severity of acute GVHD, steroid responsiveness, and survival following transplantation.

Comparison 4 was performed in patients with GVHD based on their responsiveness to steroids. GVHD severity is classified based on the Glucksberg classification system. This is based on organ involvement, mainly the skin, gastrointestinal tract, and liver, and the severity is graded from 1 to 4 based on clinical manifestations. aGVHD is associated with high mortality, especially severe GVHD, with a survival rate of less than 25%. With advancements in conditioning regimens, availability of HLA-matched grafts, and prophylactic therapies, the incidence of GVHD has been reduced. In our cohort, the incidence of severe GVHD was 32%, of which 65% of cases were related to steroid resistance, adding significant mortality risk.

Faecal microbial DNA extraction was performed using the QIAamp PowerFecal DNA kit. After extraction of microbial DNA and dilution to appropriate concentrations, genomic analysis was carried out using shotgun metagenomics and KEGG pathway analysis. Samples were analysed using a random forest classification for alpha and beta diversity, specific taxa, and univariate functional analysis.

5.3 Results

Alpha diversity:

As described already in chapter 4 changes in diversity and evenness were mainly observed between the steroid-resistant and steroid-sensitive groups (comp 4); with a slightly higher genus alpha diversity index in association with steroid responsiveness (refer to fig 4.3). However, no significant differences were observed between the two groups at the species level.

Further subset analysis based on sex showed similar results as those obtained by unstratified analysis; however, at the genus level, we observed a stronger separation in alpha diversity and richness with higher scores in steroid-sensitive male patients (refer to fig 4.4). We observed that the steroid-responsive group had a significantly higher score, mainly in the evenness diversity measure at the species level.

Beta diversity:

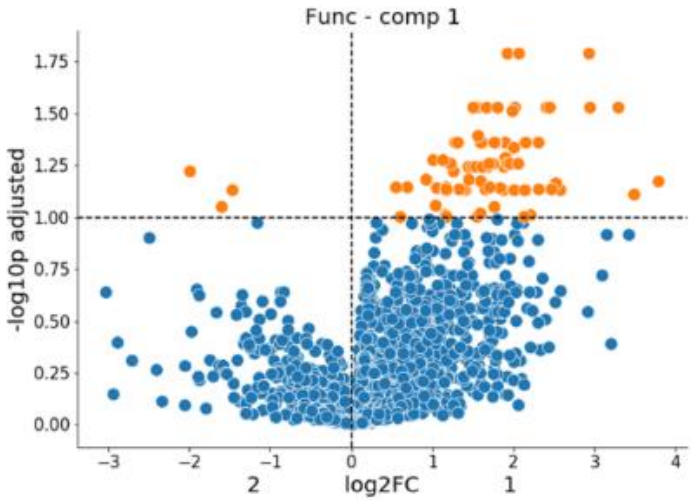
For comparison 4 species beta diversity, some clustering of points (refer to fig 4.6) was noted in the steroid-responsive group (group 6) compared with steroid-resistant cases (group 5). This could be an observation rather than an association.

Univariate functional analysis:

Further subset-stratified functional analyses of male patients showed a significant difference in enzymatic actions in comparison 1 (GVHD vs. non-GVHD) and comparison 4 (steroid-resistant vs. responsive).

In comparison 1 large number of enzymes were observed in the GVHD group compared to the non-GVHD group although no significant upregulation of any enzymatic pathways was seen (Fig 5.1) Interestingly a significant enzymatic upregulation was observed (fig 5.2) in the steroid-sensitive group (group 6) in comparison 4. Enrichment of the L arginine degradation

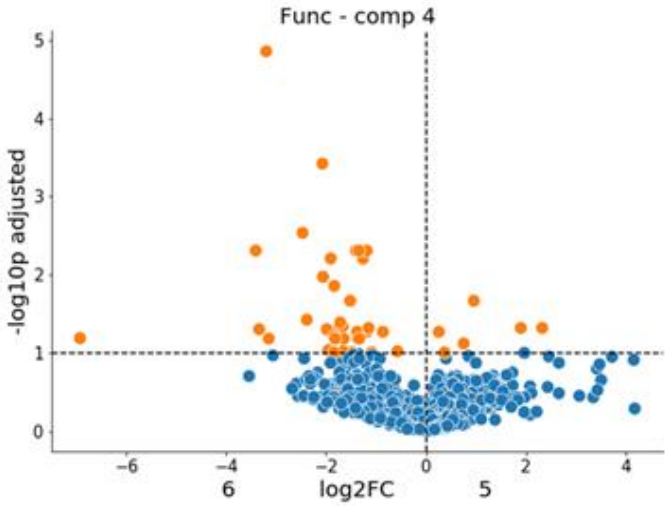
pathway seems to be associated with steroid responsiveness. Although numerous enzymes were noted to be associated with the steroid-resistant group, a significant pathway involving L arginine–proline degradation was found to be a significant one in the steroid-responsive group.



- Steroid resistant
- Steroid sensitive

Fig 5.1 Microbial enzymatic upregulation and aGHVD

Comparison 1 shows upregulation of microbial enzymatic components in patients with aGVHD (group 1) compared to those who didn't develop aGVHD (group 2)



- Steroid resistant
- Steroid sensitive

Fig 5.2 Microbial enzymatic upregulation and steroid responsiveness

Significant upregulation of enzymatic pathways with the steroid-sensitive group (Top left-hand side of comparison 4) compared to steroid-resistant group in patients with aGVHD.

5.4 Discussion:

GVHD is caused by the interaction between donor T cells and host antigens, evoking an immune response. This can lead to inflammatory reactions that can cause tissue damage and organ dysfunction. Unfortunately, GVHD remains the leading cause of mortality and morbidity. Several factors are involved in the pathophysiology of GVHD, such as host and donor factors and immune reactions. Preconditioning chemotherapy/radiotherapy intended to eliminate the underlying tumour cells can cause mucosal and tissue damage by direct toxic effects. One of the major determining factors is HLA compatibility, which is necessary for successful engraftment after stem cell transplantation. However, in many cases, donor T-cells interact with host antigens, leading to an immune response and inflammation. Several pro-inflammatory mediators such as cytokines and chemokines are involved in this inflammatory cascade.

Microbial metabolites and GVHD:

Most recently, the role of microbiota and their metabolites (Levy 2016) has been highlighted in the regulation of immune-cell development and differentiation and maintenance of a homeostatic state. The lack of microbiota-derived metabolites can result in the underdevelopment of the immune system, as previously demonstrated in germ-free mouse models (Smith 2007). Several microbiota-derived metabolites have been implicated to play a role in immune and inflammatory responses, among which short-chain fatty acids (SCFAs) merit special attention. These are produced by fermentation of dietary fibres by colonic bacteria, especially Firmicutes and Bacteroides (Morrison & Preston 2016), and the commonly found SCFAs include acetate, propionate, and butyrate. Evidence suggests the role of SCFAs in regulating myelopoiesis (Balmer 2014) and in reducing systemic infection (Khosravi 2014) and allergic responses (Trompette 2014)

Another key metabolite produced by the metabolism of tryptophan includes ligands for aryl hydrocarbon receptor-like indole-3 aldehyde (Zelante 2013) which plays a major role in maintaining the epithelial barrier and homeostasis of intraepithelial lymphocytes (Li 2011). This plays a significant role in the activation of the host immune system by producing antimicrobial peptides that help prevent microbial invasion. Decreased levels of urinary indole sulfate are associated with an increased risk of GVHD and higher treatment-related mortality (Weber 2015). Further studies have demonstrated (Swim 2018) the role of indole metabolites in reducing intestinal inflammation and damage through interferon 1 signalling pathways with a potential role in the pathogenesis of GVHD.

Several microbial metabolites influence the host immune response, thereby driving the pathogenesis of GVHD (Michonneau 2019, Lin 2021). In addition to immune factors related to transplantation, the host microenvironment and microbiota play (Jenq 2012, 2015) a significant role in the pathogenesis of graft versus host disease. The role of SCFAs, especially butyrate, has been demonstrated to reduce the inflammatory response and risk of developing GVHD (Mathewson 2016). Reduced butyrate levels result in decreased histone acetylation, and the replacement of butyrate can improve epithelial barrier integrity, reduce apoptosis, and alleviate the risk of developing GVHD mouse models. Previous studies (Riwes 2018) examining various other microbial metabolic products, including bile acids and polyamines, did not show any convincing effects on GVHD risk.

Recently, Haring et al. examined the role of bile acids and their metabolites in GVHD risk. GVHD is mediated through pro-inflammatory cytokine activation, leading to a cascade of inflammatory processes and cell destruction. Bile acids have been observed to reduce cytokine-induced damage involved in the pathogenesis of GVHD. Bile acid derivatives, such as tauroursodeoxycholic acid, have been shown to reduce GVHD severity following transplantation in murine models (Haring 2021). The role of bile acids in reducing intestinal

cell apoptosis has been shown to be cell-specific through their action on antigen-presenting cells without affecting the anti-tumour effects of the graft. The metabolites of bile acids exert their influence on the host cells without altering the microbial diversity of the intestine.

With high-output metabolomics techniques, a wide range of microbial metabolites have been identified in stem cell transplantation. Changes in host metabolism and microbial dysbiosis resulting from transplantation result in significant changes in circulating metabolites, causing an altered immune response, which has been implicated in the pathogenesis of GVHD. A study by Michonneau et al. in HLA identical recipient and donor cohorts demonstrated significant changes in recipient microbial metabolites in the post-transplantation period, including tryptophan metabolites (indoles), which are compounds acting as ligands for aryl hydroxy carbon receptors, bile acids, and plasmalogens (Michonneau 2019).

Our study cohort on faecal microbial DNA analysis demonstrated upregulation of the L arginine degradation pathway in association with steroid responsiveness. This pathway is involved in the metabolism of polyamines, especially putrescine and spermidine. Polyamines play a major role in regulating inflammatory and immune responses and reducing apoptosis. Because GVHD is associated with acute and chronic inflammation, the potential role of polyamines in modifying inflammation will have an impact on the morbidity and mortality associated with GVHD.

Polyamines:

Polyamines are low-molecular-weight polycationic compounds found in eukaryotic cells that are involved in various cellular functions. Putrescine, spermidine, and spermine are the most commonly known polyamines involved in various biological processes. They are derived from dietary sources and are produced by microbial action in the intestinal tract. The main functions of polyamines include allergies, immune responses, the regulation of inflammatory reactions, and antioxidant properties (Muñoz Esparza 2019).

History of polyamines:

Pioneering studies on polyamines have been conducted since spermine crystals were identified in human semen by Antoni van Leeuwenhoek in 1678. The name spermine was coined by Ladenburg and Abel in 1888. The detailed molecular structure of this compound was identified several years later by Rosenheim in 1924 (Bachrach 2010). Furthermore, putrescine and spermidine were synthesised by Rosenheim, remarking early studies of polyamines. Several other studies have been conducted to identify the role of spermine in the growth of bacteria and aid in the regeneration of liver cells (Bachrach 2010). One of the earliest indications of the role of spermine in curing disease was noted by von Poehl 1898 (Walter 1900).

Structure:

Polyamines are organic cations with multiple amino groups that can be naturally present and synthesised. Polyamines are produced by the metabolism of amino acids, mainly ornithine and arginine. The molecular structures of the polyamines are largely similar but differ in the number of amino groups present. Putrescine is a diamine in which spermidine is a triamine and spermine is a tetramine. The important enzyme in the polyamine metabolic pathway is ornithine decarboxylase, which converts ornithine to putrescine and spermidine (Pegg 2009). Spermidine is synthesised from putrescine by the action of spermidine synthase, which is further metabolised to spermine by spermine synthase. These products are subsequently degraded to form putrescine, which restarts the metabolic cycle (Pal 2015).

Function:

Polyamines are required for maintaining the structural stability of DNA and RNA, protein synthesis, growth, and differentiation of cells (Igarashi 2020). They play a key role in regulating the expression of genes involved in cell growth and tissue repair and are implicated in colonic and prostatic malignancies (Gerner 2004). Therefore, polyamines play multiple physiological roles. For example, polyamines suppress inflammation by inhibiting

inflammatory cytokine synthesis in macrophages, exert anticarcinogenic effects, and inhibit aberrant gene methylation. Some natural polyamines exhibit antioxidant properties that affect mitochondrial permeability and regulate apoptotic pathways (Agostinelli 2010). They act as free radical scavengers (Belle 2004) and as membrane stabilisers. Lovaas et al. showed the antioxidant effect of polyamines by chelation of oxidised metals and reduction of the formation of hydroperoxides and oxidised compounds (Lovaas 1997). Polyamines affect the functioning of multiple ion channels. For example, intracellular spermine can cause intrinsic gating of potassium channels, which in turn affects the excitability potential of various muscle and neuronal cells (William 1997). They also affect calcium-permeable glutamate and NMDA receptors in the central nervous system and increase the permeability of ions and voltage across the membranes (William 1997).

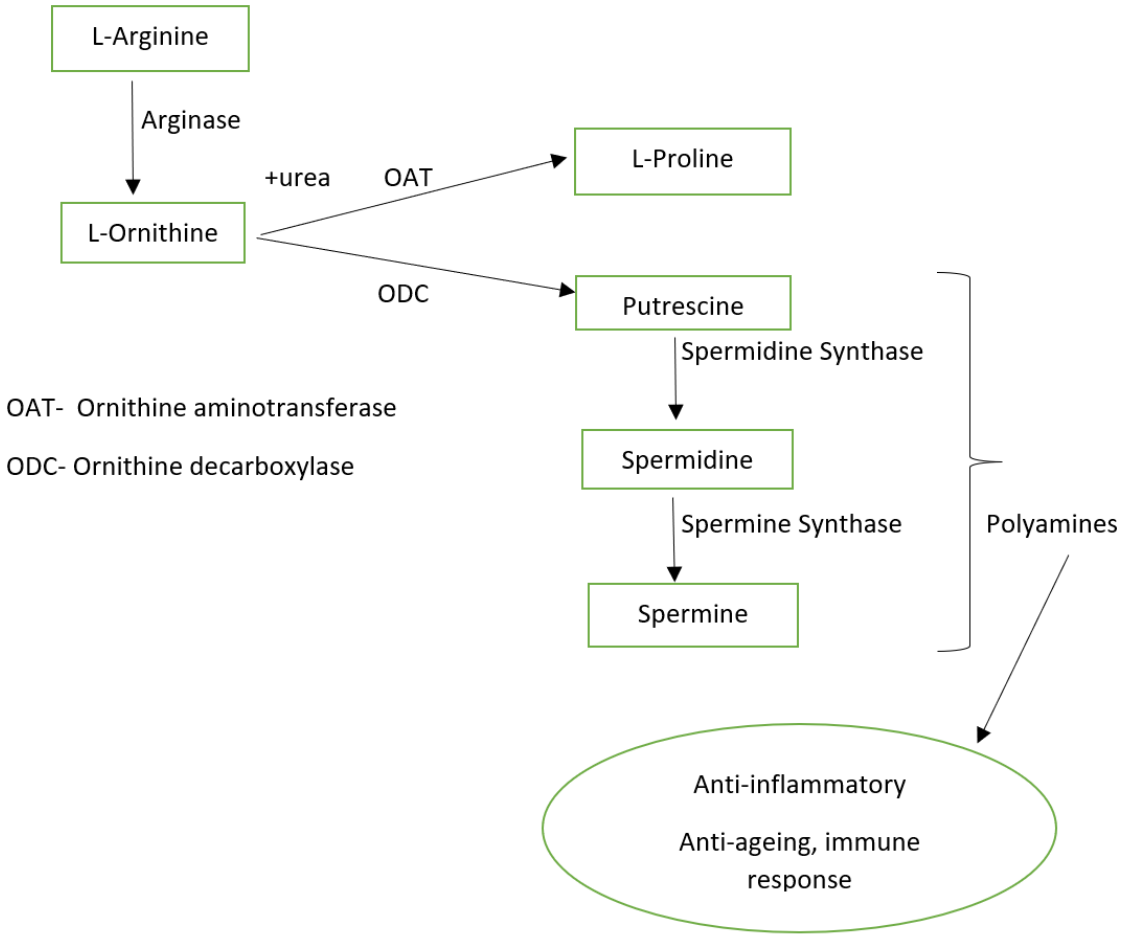


Fig 5.3 Synthesis and Metabolism of Polyamines

Metabolism of polyamines and influence on the gut microbiome

Intrinsically, the first step in polyamine synthesis is putrescine formation from ornithine by ornithine decarboxylase (ODC). Subsequently, spermidine is produced by the addition of propylamine to spermidine synthase. Spermine synthase converts spermidine into spermine through the addition of a further propylamine group (Larque 2006, Moinard 2005, Minois 2011) (fig 5.3). The catabolism and turnover of these polyamines are mainly mediated by the acetylation of the by-products through the N-acetyl transferase enzyme with the addition of acetyl CoA. Oxidative deamination through diamine and polyamine oxidase helps eliminate these metabolites from the body.

Diets rich in proteins and breast milk are good sources of polyamines (Larque 2007). Several foods are rich in polyamines, including green leaves, soya, meat, wheat, and mushrooms. They are primarily absorbed from the duodenum and first part of the jejunum (Munoz-Esparza 2019). Polyamines are also produced by intestinal microbes through various metabolic pathways. They are mainly produced by colonic microbes and are absorbed into the bloodstream through the colonic mucosa (Tofalo 2019). The concentration of polyamines and their metabolites in the healthy human intestine varies from 0.5 to 1 mM (Matsumoto 2012). New molecular techniques have identified many non-culturable species that are involved in various metabolic functions. As mentioned previously, several metabolites produced by gut microbes affect health and disease, polyamines of which require further evaluation. Pugin et al. (2017) identified several bacterial species that are involved in the production and degradation of polyamines at varying levels. These microbes belong to several genera, including *Bifidobacterium*, *Clostridium*, *Enterococcus*, *Lactobacillus*, *Pediococcus*, *Streptococcus*, *Enterobacter*, *Escherichia, Klebsiella*, *Morganella*, and *Proteus*.

Another study demonstrated that the production of luminal putrescine by gut microbes involves collective biosynthetic pathways (Nakamura 2019) involving arginine, ornithine, and

citrulline metabolism. Intestinal bacteria belonging to *Bacteroides* and *Fusobacteria* are associated with the production of putrescine and spermidine in in vivo and in vitro pectin-fed gnotobiotic mouse models (Noack 2000). These polyamine metabolites are involved in various cell signalling and differentiation pathways and regulate inflammation and immune responses. The production of polyamines in the gut depends on various transport mechanisms, enzymatic activities, and pH levels. The presence of fermentable carbohydrates in the intestinal lumen is associated with an increased production of polyamines (Tofalo 2019). The activity of various enzymes involved in the polyamine synthesis pathway is influenced by the microbial species in the gut. For example, various *E. coli* species utilise S-adenosylmethionine decarboxylase and spermidine synthase, which are involved in spermidine biosynthesis (Sugiyama 2017) with the involvement of various transporters and genes, whereas *Bacteroides* species use analogues of carboxyspermidine decarboxylase (Hanfrey 2011). The interaction of various microbes can alter polyamine levels, as shown by Kitada et al., as increased polyamine production from mixed bacterial cultures (Kitada 2018), and can affect gut environmental factors such as pH levels (Richard 2004). Hence, the microbial composition of the intestine will have an impact on the level of polyamines and thus on the anti-inflammatory response.

Polyamines in inflammation:

Polyamines are known to regulate inflammatory and immune responses through various cell signalling mechanisms. They are involved in cell differentiation and growth and development of the immune system (Gomez-Gallego 2017, Pérez-Cano 2009). The abundance of these polyamines is essential during the neonatal stage and drives maturation of the intestinal tract. In intestinal epithelial cells, polyamines play a role in 94mmune94ng protein synthesis and stability, regulate E-cadherin transcription and apoptosis, and increase cell proliferation. Furthermore, spermidine enhances longevity in bacteria, yeast, worms, and flies by promoting autophagy.

Microbiota-derived polyamines are involved in intestinal epithelial cell proliferation and macrophage differentiation. Putrescine has been shown to increase anti-inflammatory macrophages in the colon and decrease dextran sulfate sodium-induced colitis in murine models (Nakamura 2021). The reduction in polyamines levels is attributed to the exacerbation of inflammatory bowel diseases (IBDs) and the potential reversal by polyamine supplementation. The upregulation of spermine oxidase which converts spermine to spermidine has been implicated in the pathogenesis of IBD. In contrast, polyamines are essential for maintaining intestinal mucosal integrity, promoting mucosal healing, and reducing oxidative stress, thus, polyamine play a key role in the regulation of intestinal inflammation. Polyamines exert their effects on DNA and protein synthesis and stabilisation. Local homeostasis of spermidine and spermine through spermine oxidase (SMOX) is a critical regulator of colonic inflammation (Gobert 2018). The role of spermidine and spermine in IBD has been noted in in vitro studies of colonic epithelial cells (Bailey 2017). It has been suggested that spermidine overexpression is associated with dysregulated epithelial cell proliferation and that spermine deficiency causes increased apoptosis, resulting in persistent chronic inflammation.

Polyamines are implicated in the growth of cancer cells (Casero 2018) due to the increased activity of ornithine decarboxylase (ODC). Increased polyamine levels stimulate the proliferation of tumour cells, and the use of drugs to inhibit enzymatic activity has potential therapeutic effects in cancer therapy (Casero 2018, Gerner 2004). High polyamine levels are associated with tumour cells and autoreactive B and T cells in autoimmune diseases. In the tumour environment, polyamine catabolism by tumour cells and suppressive myeloid cells is associated with the dampening of cytotoxic T cell function, indicating the role of polyamines in immune function (Hesterberg 2018).

The anti-inflammatory and antioxidant properties of polyamines confer cardiovascular protection. Increased polyamine intake is associated with reduced endogenous polyamine production, thereby increasing the availability of arginine for nitric oxide production. Nitric oxide is a vasodilator that plays a protective role in maintaining vascular integrity (Soda 2010) and free radical scavenging. Spermidine has been associated with reduced cardiovascular morbidity, reduced blood pressure, and heart failure (Madeo 2018). The anti-inflammatory properties of polyamines are mediated by two mechanisms: the direct action of leucocytes and the anti-inflammatory protein vasoregulin. Experimental studies have shown that increased polyamine level causes negative regulation of inflammation by their action on various cells including lymphocytes and neutrophils. . Furthermore, the involvement of spermine in the inhibition of macrophage cytokine synthesis has also been reported. In a mouse model, exogenous administration of polyamines exerted anti-inflammatory effects through antioxidant activity and lysosomal stabilisation (Lagishetty & Naik 2008). Another mechanism by which polyamines exert anti-inflammatory properties is through the inhibition of lipopolysaccharide (LPS)-mediated nitric oxide release and maintenance of mucosal integrity. In murine models, spermine inhibits LPS-induced nitric oxide synthetase expression, inhibits the release of pro-inflammatory mediators like NO and TNF, and mediates the release of anti-inflammatory mediators such as IL 10.7 (ter Steege 1999).

The anti-aging effects of spermidine and other polyamines have been well-documented for decades. The association of polyamines with inflammation is mediated by triggering the production of anti-inflammatory cytokines and decreasing the production of pro-inflammatory cytokines. As the aging process is associated with chronic inflammation, the indispensable role of spermidine-like molecules is vital for slowing the aging process. Although autophagy seems to be the main mechanism associated with the anti-aging properties of spermidine, other mechanisms such as suppression of inflammation, regulation of lipid metabolism, cell proliferation, and growth might also play a significant role (Minios 2014).

Choi et al. showed that LPS-exposed microglial cells in the CNS showed decreased production of pro-inflammatory mediators and cytokines after treatment with spermidine. In addition to the reduction of nitric oxide and prostaglandin E2 production, spermidine treatment is associated with reduced mRNA expression of proinflammatory cytokines like IL6 and TNF, thereby strengthening the anti-inflammatory properties of polyamines (Choi 2012). Paul and Kang showed that polyamine treatment reduces neutrophil infiltration and oedema associated with inflammation. At the cellular level, MAPK pathways trigger autophagy mediated by spermidine (Paul and Kang 2013). The addition of spermidine results in enhanced autophagy influx through activation of a microtubule-associated protein (MAP1S), reducing oxidative stress, suppressing liver fibrosis, and prolonging lifespan. This effect is absent in knockout mice and is a potentially promising tool for treating liver fibrosis and preventing the development of hepatocellular carcinoma.

The arginine degradation pathway was upregulated in the steroid-responsive group. In turn, this has a positive impact on the outcome of stem-cell transplantation. The anti-inflammatory properties of polyamines have potential therapeutic applications in alleviating the burden of GVHD and in predicting the response to treatment.

Polyamines in organ transplantation

In recent years, organ transplantation has advanced and offers promising results in the management of advanced irreversible organ failure. Despite advanced treatment regimens and better donor availability, there are challenges to the long-term outcomes of transplantation such as GVHD and relapse. GVHD poses significant challenges to mortality following transplantation despite newer prophylactic therapies. The role of microbiota and their metabolic products has been studied in various organ transplantations and their associated complications. Modifying the microbial composition and metabolites may offer potential therapeutic targets for the management of organ transplantation.

Polyamines play important roles in reducing inflammation and promoting cell growth and differentiation. Historical studies have demonstrated the role of polyamines as markers of immune activation, and urinary putrescine levels have been used to predict graft rejection following heart transplantation (Carrier 1988). Polyamines are essential for liver-cell regeneration and for protection against liver damage (Okumura 2016). A study examining the role of polyamines in ischemia/reperfusion injury and liver regeneration in a living donor partial liver transplantation mouse model showed that the administration of polyamines is associated with reduced expression of pro-inflammatory cytokines, which was histologically evident by lower necrosis and apoptosis in the transplanted liver cells. Additionally, the reperfusion time was improved in the group treated with polyamines, indicating their role as a potential therapeutic target in improving the outcomes of liver transplantation. Both biosynthetic and degradative polyamine pathways are activated following liver transplantation, as indicated by elevated transaminase levels and activation of ODC and spermidine/spermine N 1-acetyl transferase, causing increased putrescine production. Putrescine supplementation is associated with decreased cold ischaemic time and improved graft survival (Terakura 1995)

Bone marrow-derived mesenchymal cells are a promising treatment option for reducing inflammation and the effects of ischaemia, improving functional recovery following ischaemic stroke. Evidence suggests that mesenchymal cells migrate to the brain and differentiate into neuronal and glial cells, which are affected by several neurorestorative factors (Dharmasaroja 2009). Easy availability, pluripotency of differential nature (Jiang 2002), and anti-inflammatory properties (Iyer 2008) make mesenchymal cells a potential treatment option for reducing ischaemic injury and aiding in cellular repair. Metabolic profiling has shown that polyamine metabolism is altered following ischaemic stroke, as noted by reduced levels of natural polyamines such as putrescine, cadaverine, and spermidine (Shin 2016). The authors

have demonstrated that the replacement of polyamine levels by treatment with mesenchymal cells reduces ischaemic time and helps in tissue repair.

Polyamines in bone marrow transplantation :

There is compelling evidence that polyamines are associated with reduced inflammatory mediators and modified immune responses. Microbial metabolites play a significant role in determining the outcomes of stem-cell transplantation and risk of GVHD. The role of SCFA and butyrate-producing bacteria is associated with a reduced risk of GVHD and improved survival, as shown by early signals in our study cohort. Other metabolites, including tryptophan, bile acids, and amine derivatives, have been studied in stem-cell transplantation.

The role of polyamines in myelopoiesis and immune function may account for their effects on GVHD and stem cell transplantation outcomes. Erythrocyte spermine and spermidine levels were low immediately after stem cell transplantation and increased to normal levels by engraftment. This can be used as an index of early successful engraftment and points toward the essential role of polyamines in haematopoiesis (Bergeron 1997). The effect of putrescine on the growth and differentiation of human mesenchymal cells has been evaluated (Chen 2015). Putrescine accelerated osteogenic proliferation and differentiation in a dose-dependent manner compared with that in the control group. Osteogenesis is evident by the increased cellular expression of Runx 2 (transcription factor involved in osteoblast differentiation) as well as increased levels of alkaline phosphatase and phosphatase activity, indicating bone formation. The differentiation of mesenchymal cells to osteoblasts without any evidence of adipocyte formation was noted through oil-O staining (Chen 2015), indicating the selective role of putrescine in promoting osteogenesis. Another interesting study in the area of osteoporosis noted that exposure to warmth increases microbiota-derived polyamine levels. These metabolites, in turn, promote periosteal bone formation and reverse the transcriptomic changes caused by ovariectomy (Chevalier 2020). Hence, spermine and spermidine

supplementation improves bone growth and offers a potential treatment option for osteoporosis.

A study involving metabolic profiling of recipient and donor fresh frozen plasma samples was performed at aGVHD onset or day +90 in patients without GVHD. In patients without GVHD, increased levels of microbiota-derived polyamine metabolites were noted, including N-acetyl putrescine and N-acetyl spermidine, which indicated the potential protective role of polyamines on gut integrity in patients without GVHD. In GVHD patients, metabolites of amino acid pathways were significantly reduced, notably host- and microbiota-derived tryptophan metabolites such as 3-indoxyl sulfate, indole acetate, indole propionate, and N-acetyl kynurenine (Michonneau 2018). Spermine dialdehyde (SDA), an oxidised product of spermine, has been evaluated as an ex vivo purging agent for GVHD prevention. SDA preferentially suppresses T-cell and NK cell activity compared to myeloid cells. Mice transplanted with SDA-treated stem cells survived for approximately 100 days compared to control mice, which died at days 25–35 from GVHD (Wang 1990). When used in phosphate-buffered saline (PBS), SDA preferentially inhibits T-cell proliferation without affecting myeloid cells. The authors have also demonstrated that SDA in saline has shown more inhibitory effects on leukaemic cells than on normal myeloid and T cells. Lethally irradiated mice receiving bone marrow cells treated with SDA in saline ex vivo did not show any evidence of leukaemia, proving the potential benefit of polyamines and their metabolites as purging agents in allogeneic and autologous stem cell transplantation (Lau 1992).

As discussed, polyamines are implicated in various inflammatory processes and are closely linked to immune mediation in association with various organ transplantations, including HSCT. The potential anti-inflammatory properties of polyamines would have a beneficial effect in reducing the risk of GVHD and enhancing treatment response. The activation of

polyamine pathways at the cellular and genomic levels drives steroid responsiveness and can be a potential therapeutic target for modifying the outcomes of HSCT.

The findings are supportive of our hypothesis that there are specific microbiome signatures predicting the response to treatment in aGVHD. Identification of specific microbiota and potential metabolic pathways driving the risk of aGVHD is a crucial finding which will have an impact on the outcomes of stem cell transplantation. Furthermore, the identification of upregulated Arginine degradation pathway suggests the role of polyamines in the risk of aGVHD. With the upregulation of this pathway increased concentrations of polyamines are available especially putrescine and spermidine with significant anti-inflammatory effects. As we understand from previous studies polyamines are multiple roles in inflammation and organ transplantation. The anti-ageing effect of spermidine is well established in animal models. Considering aGVHD is associated with an inflammatory response, polyamine supplementation would be a key consideration in order to alleviate the impact of aGVHD and improve the treatment responsiveness.

We propose to carry out mouse model experiments to assess the impact of polyamine supplementation such as putrescine and spermidine on the risk of aGVHD. It is expected to see a lesser risk of aGVHD in the stem-cell transplanted mice supplemented with polyamines. If proven, this information would be really valuable in the management of aGVHD associated with bone marrow transplants in terms of alleviating the burden of aGVHD and improving quality of life and survival.

Key Points:

- Higher alpha diversity is noted in association with steroid responsiveness especially in male patients with aGVHD. There was also some clustering of points (microbial beta diversity) in steroid responsive aGVHD patients.
- Significant upregulation of L-arginine degradation pathway in association with steroid responsiveness.
- Upregulation of arginine degradation pathway results in increased availability of polyamines such as putrescine and spermidine.
- We postulate that the anti-inflammatory and anti-ageing properties of polyamines accounts for the better steroid responsiveness and improved survival in aGVHD patients.

Chapter 6

Role of donor faecal microbiome on the Severity of Graft versus Host disease following Stem Cell Transplantation

6.1 Introduction

Graft versus Host Disease continues to pose challenges to the outcomes of HSCT in term of functional morbidity and mortality for the patients. Donor T cells recognise the host antigens as foreign, evoking an immune-inflammatory response accounting for the pathogenesis of GVHD (Billingham 1996, Ghimire 2017) Although HLA matching is the key determinant factor for GVHD, the incidence of GVHD remains high as 35-45% even in matched sibling donor transplantation (Ferrara 2009). There are complex immune pathways involved in the pathogenesis (Magenau 2016) of GVHD involving the host and donor innate and adaptive immune systems accounting for the pathophysiology of GVHD. In addition to host and donor conditions, various other factors such as the host microenvironment might play a role in the pathogenesis of GVHD. The role of the microbiome has been extensively evaluated in allogeneic HSCT revealing that healthy diverse microbiota is essential for maintaining a homeostatic state with the host immune system and improving graft survival.

6.2 Methods and Comparisons:

Fifty-one HLA-matched sibling donors to patients receiving allogeneic HSCT were recruited for our study. Due to ethical and GDPR regulations, unrelated donors weren't included in the study. Donor faecal samples to those recipients who developed GVHD and died and those who didn't develop GVHD were compared with regard to the information on genus, species, and functional enzymatic pathways involved.

Further on faecal samples of bone marrow transplant recipients who had developed severe graft versus disease were compared to recipients who haven't developed any GVHD. The corresponding donor samples were also analysed with regard to the severity of GVHD in the recipient cohort.

6.3 Results:

In the donor sample analysis, no significant difference was observed in the bacterial taxonomy in terms of alpha diversity, beta diversity, and univariate functional studies for enzymatic activity (Fig 6.1a, 6.1b & 6.2)

Donor Microbial Alpha Diversity:

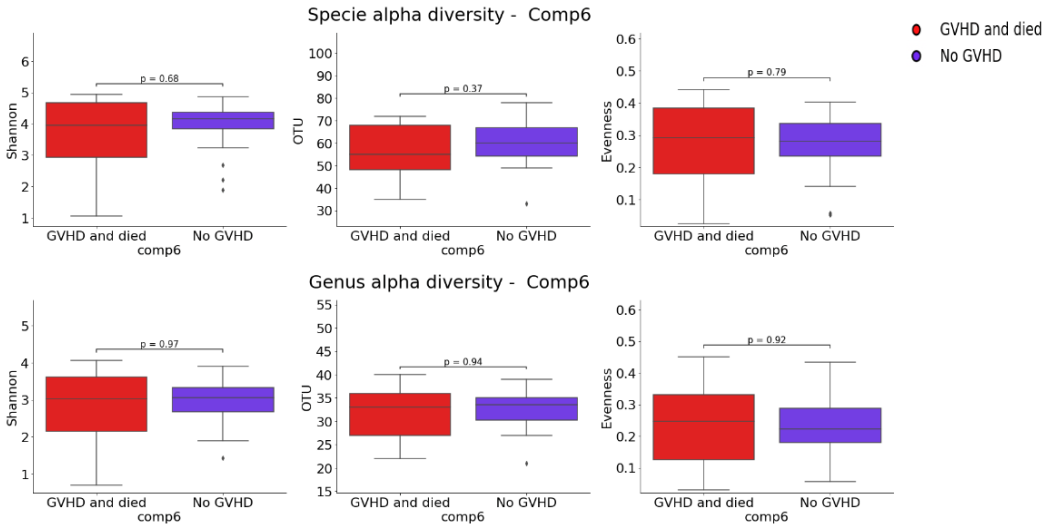


Fig 6.1a: Donor sample analysis with the risk of GVHD and non-GVHD in Recipients

No significant difference has been observed in microbial alpha diversity in donors to the patients with aGVHD and to those who didn't develop aGVHD

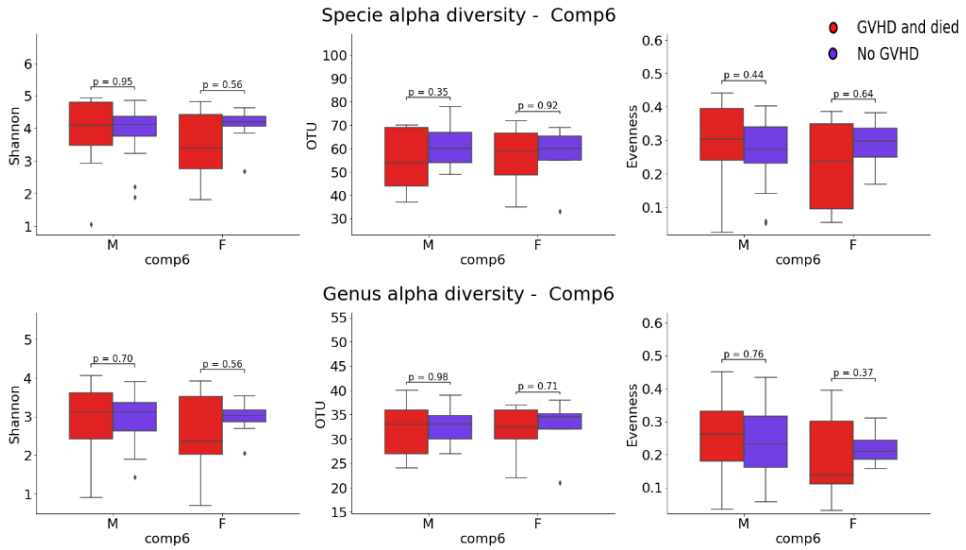


Fig 6.1b: Donor microbiome analysis on the risk of GVHD and non-GVHD- Gender basis

On the basis of gender analysis, no significant difference in microbial diversity has been observed in the different donor groups (where their recipients developed GVHD or Not)

Donor microbial beta diversity :

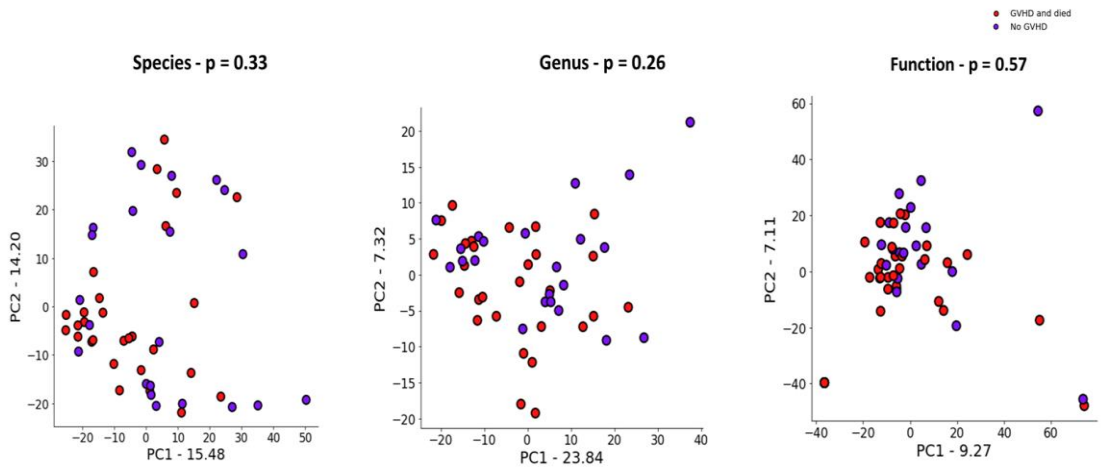


Fig 6.2 Donor microbiome beta diversity

No significant difference in genus and species beta diversity has been observed between the donor groups where the recipients had aGVHD or not

Microbiome linked to severe GVHD in Donor and Recipient cohort

Alpha Diversity

Interestingly, significantly lower alpha diversity was observed in donors to patients who developed severe GVHD compared to those who did not develop GVHD at all (Fig 6.3). This was observed in species and genus alpha diversity. Species alpha diversity had a Shannon index of 0.01 and evenness of 0.02. Similar trends were observed with genus information (p-value of 0.09 and 0.12 respectively). These findings implicate the potential protective role of certain donor microbiomes against developing severe graft versus host disease and improved outcomes following stem cell transplantation.

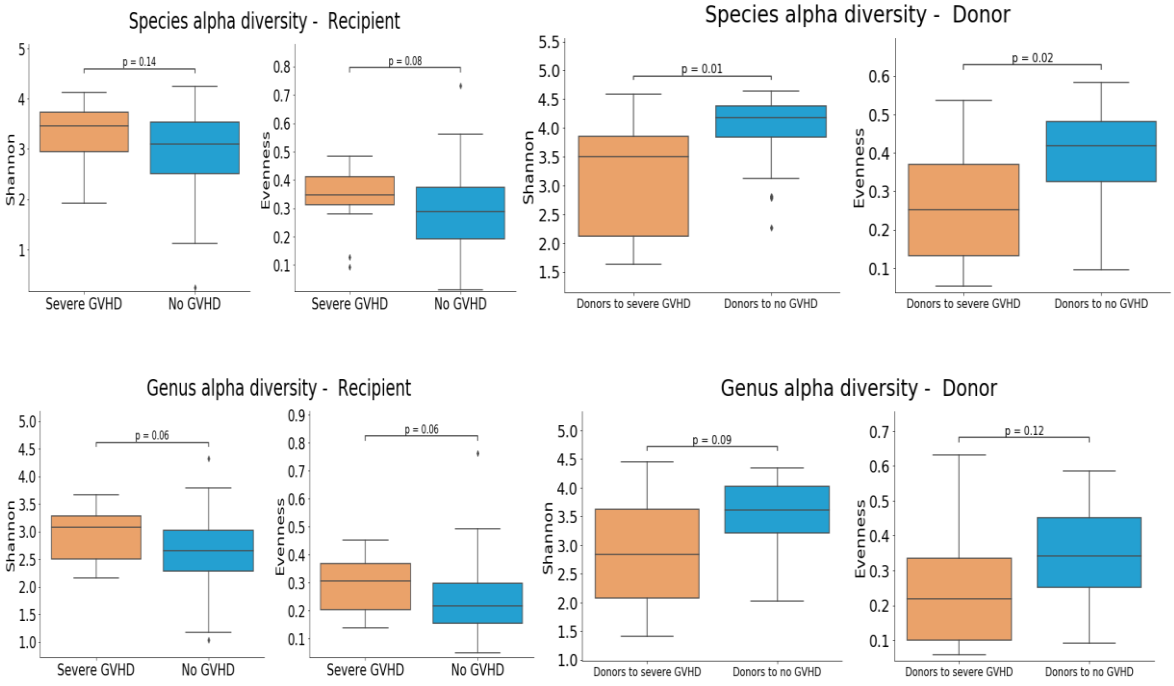


Fig 6.3: Microbial Diversity on the risk of Severe GVHD in Recipient and Donor Cohorts

Lower microbial diversity has been observed in the donor group where their recipients groups developed severe aGVHD. In contrast a higher microbial diversity was observed in the severe aGVHD recipient group.

In contrast, nearly opposite trends in alpha diversity were observed in the recipient group with higher diversity indices in the severe GVHD group (Fig 6.3)

Beta Diversity

Although we weren't able to identify any significant differences between the groups (severe GVHD Vs non-GVHD) on various comparisons, we have observed slight differences in some PCA between these groups (fig 6.4). The differences could be related to sample size and can be further enhanced by a larger sample.

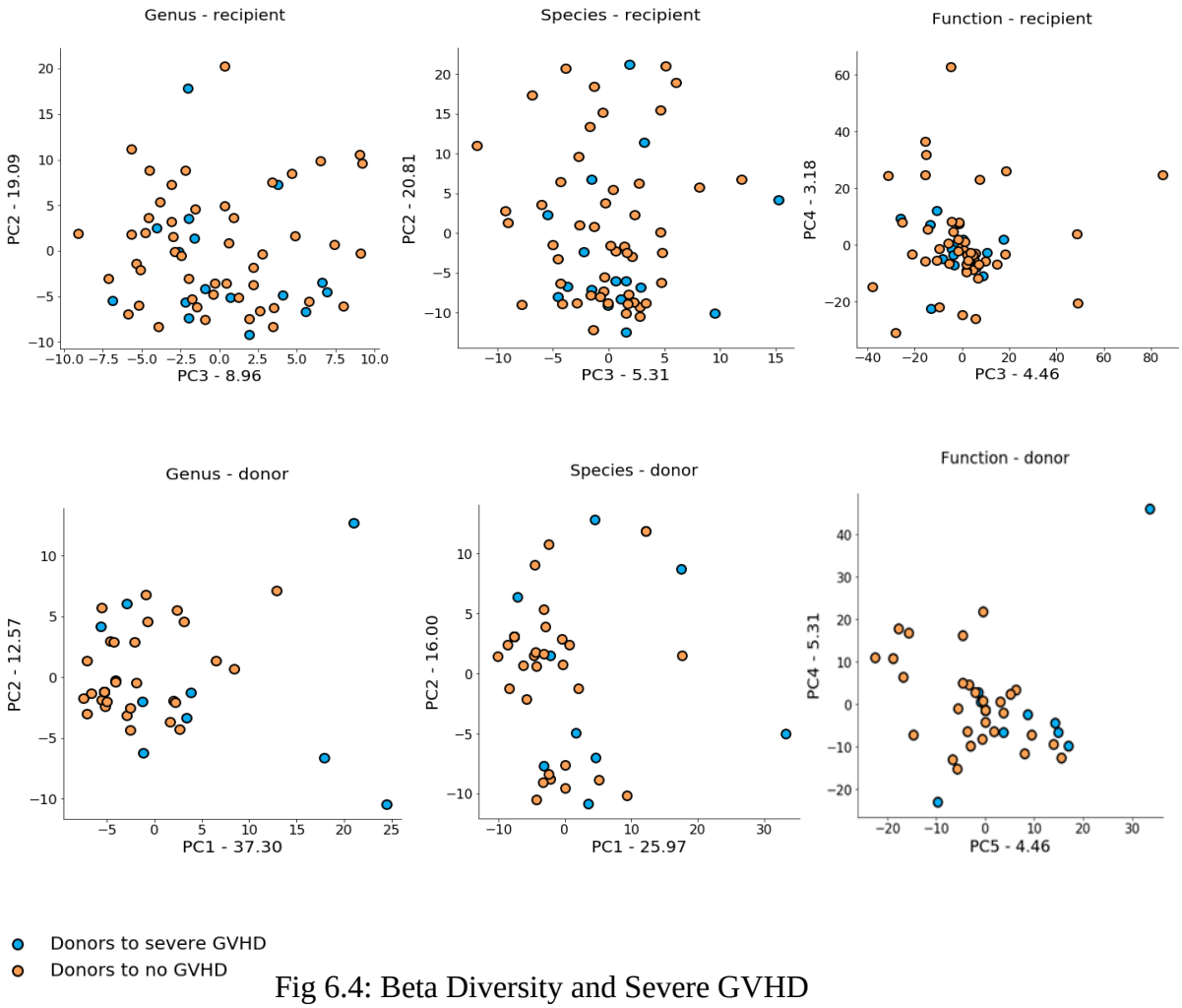


Fig 6.4: Beta Diversity and Severe GVHD

No siginificant difference in microbial beta diversity was observed between different donor groups.

Univariate Function

On further functional analysis of microbes, we were able to identify a single genus 'Dorea' as significant in the comparison between No GVHD and Severe GVHD (Fig 6.5) Although this is a significant finding, can't be considered a causative association.

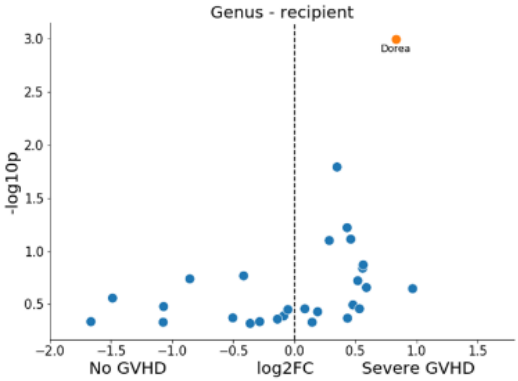


Fig 6.5: Univariate Function and Severe GVHD

Dorea was the genus identified as significant in the severe aGVHD recipient group.

6.4 Discussion:

Donor Microbiome and Risk of Acute Graft Versus Host Disease

Several studies have shown the influence of recipient microbiota and their metabolites on the risk of GVHD and outcomes of transplantation. The close interaction of microbiota with the immune system from the neonatal period onwards helps to develop immune tolerance and maintain a homeostatic state at the mucosal and epithelial surfaces (Lathrop 2011, Cebula 2013). Although immune tolerance develops against self-microbiota, the external microbes play a role in evoking an immune reaction as identified as foreign by the host immune system. The donor microbiota may play a role in contributing to the risk of GvHD following HSCT although very few studies have evaluated the same. Studies have shown that microbiota induces immune response and differentiation of Treg (T regulatory) cells thereby influencing the risk of GvHD (Furuswa 2013). This effect is independent of the HLA compatibility and can affect wide ranges of tissues including bone marrow(Khosrawi 2014, Trompette 2014). Liu et al have suggested the role of donor microbiota affecting the composition of transplanted donor immune cells and triggering alloreactivity and GvHD risk (Liu 2017) Previous murine studies haven’t shown any association between donor microbiota on the T cell differentiation

and the risk of GvHD(Tawara 2013). The study has evaluated baseline faecal microbiota composition in 22 sibling donors in terms of diversity and richness through 16s rRNA genomic sequencing. The donor cohort had a relative abundance of obligate anaerobes like Bacteroides, Lachnospira, and Ruminococci. A lower risk of aGvHD has been noted with a higher microbial diversity in donors although no specific taxa have been implicated with the occurrence of GvHD (Liu 2017). Recipient microbial composition was predominated by enterococci, Lactobacilli, Streptococci, and Enterobacteriaceae species although no correlation has been noted between baseline recipient microbial diversity and risk of aGvHD. Similarly, no correlation had been found between the baseline recipient-donor microbial dissimilarity and the risk of aGvHD. Interestingly the team has found a relative abundance of bacteria with anti-inflammatory properties in higher diversity donor cohorts such as Bacteroides spp and Bifidobacterium(Liu 2017). These species had been shown previously in associated with Treg cell differentiation and regulation of inflammation (Troy 2010, Lopez 2011). The immune tolerance to microbial species can be affected by various factors such as host genetics and the environment and a constant interaction throughout life to develop a diverse microbiome and immune response. Although the study by Liu et al in donor cohorts has evaluated some of the donor risk factors such as CMV positivity, and gender status it hasn't included other factors like BMI (body mass index), and age of the donors which might have an impact on the risk of GvHD. A small pilot study involving transplantation of healthy donor faecal microbiota (FMT) in allogeneic bone marrow recipients showed increased gut alpha diversity with supplementation, especially of butyrate-producing bacteria and successful remission of aGvHD (van Lier 2020). The FMT has shown promising benefits in terms of steroid-resistant and sensitive GvHD and improved survival at 2 weeks of the study duration.

In our study, no significant difference has been found in faecal microbial taxonomy in donors whose recipients had GvHD and those who hadn't. This could be related to the small sample size and the power of the study could be increased by larger sample size. It is a natural

expectation that healthy donors might have a healthy diverse microbiome. But the fact that GvHD occurs even in severe forms in sibling stem cell recipients, indicates the role of environmental factors such as microbiome beyond the HLA compatibility to the risk of GVHD. But our comparison of donor microbiome in terms of severe GvHD and no GvHD risk in recipients has shown very interesting findings. Donor microbial alpha diversity has been significantly lower in those whose recipients developed severe GvHD. Species alpha diversity showed a significantly low Shannon diversity index (p=0.01) and Evenness index(p=0.02). Similar findings were seen with genus alpha diversity (p=0.09 and P=0.12) as well. PCA of the two groups showed some separation in terms of beta diversity although no significant differences have been noted. Our analysis of recipients showed exactly opposite trends with higher alpha diversity in the patients with severe GvHD. These findings shed light on the importance of the microbiome in the selection of suitable donors which might have an impact on the outcomes of HSCT along with other clinical factors.

Dorea

Dorea was the only taxa found to be significant in patients with severe aGVHD. *Dorea* is a gram-positive non-spore-forming bacteria that belong to the *Lachnospiraceae* family. They are seen abundantly in the lower intestine of humans and are classified under the phylum *Firmicutes*. In 1974 the *Eubacterium Formicigenerans* is reclassified as *D.Formicigenerans* by Holdeman and Moore which are rod-shaped, obligate anaerobic bacteria (Taras 2002). *D.longicatena* is another species isolated from the human faecal samples. This group of bacteria ferments glucose and other carbohydrates to make end products of formic and acetic acids, ethanol, H2, and carbon dioxide (Comprehensive gut microbiota 2022 vol2).

Role of Microbiome on the Severity of Acute Graft Versus Host Disease

Acute graft versus host disease usually develops within 100 days of post-transplantation and skin and intestinal tracts are the main target organs involved followed by the liver. The severity of GVHD is determined by the extent of organ involvement and its impact on the patient's functional status. Acute GVHD is graded into 1-4 depending on the organ involvement and severity of GVHD. Grade 1 and 2 are usually mild in nature and generally well respond to immunosuppressive therapy. Grade 3-4 is classified into the severe GVHD group which is associated with higher mortality and morbidity and is resistant to treatment. The mortality from severe GVHD is an independent predictor of poor transplant outcomes when compared to mortality related to infection and relapse. Involvement of the lower Gi tract and liver are common in severe GVHD cases.

In our cohort incidence of severe GVHD was about 31% mainly involving the skin and lower GI tract. 24% of severe GVHD cases had liver involvement and there was a male predominance of 65% (Fig 6.6). The average age of the patient in the severe GVHD group was 49 (range 23 to 67) and an average BMI of 26. Steroid resistance was present in 65% of those severe GVHD cases. Severe GVHD causes significant functional disability to the patient and a huge economic burden to the hospital system in terms of recurrent hospital presentations and the need for specialized therapies. There are several risk factors implicated in steroid-refractory severe GVHD including unrelated donors, female donor status, and absence of the use of ATG in the pre-conditioning regime (Calmettes 2015)

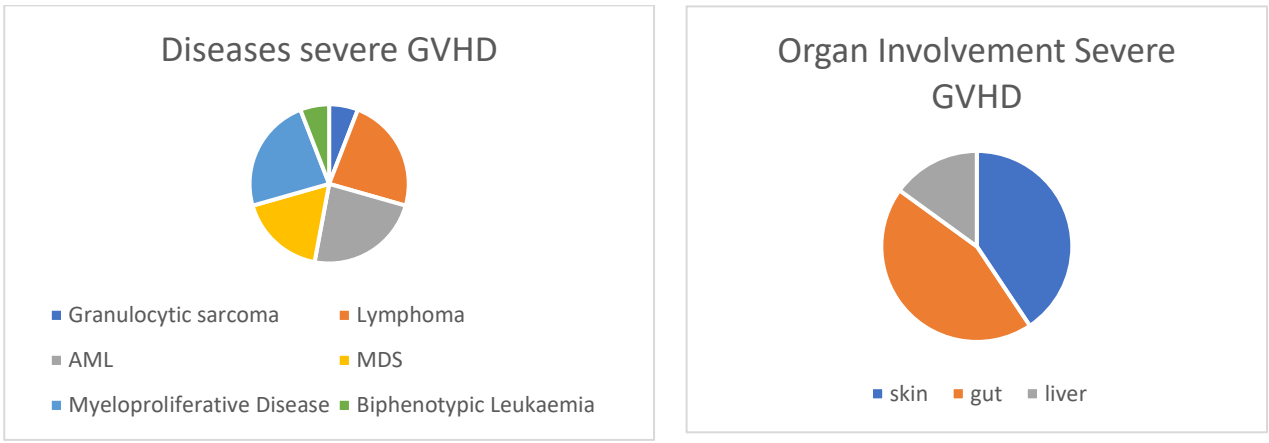


Fig 6.6: Severe GVHD Characteristics

Consistent with previous study results (Pagliuca 2021, Camettes 2105) we have identified unrelated donor status, use of low-intensity pre-conditioning and absence of ATG was associated with a severe GVHD grade. In our cohort, 82% of the patients with severe GVHD had stem cell transplants from an unrelated donor. In the severe GVHD group, there was a slightly higher proportion of patients (about 53%) who received low-intensity pre-conditioning chemotherapy compared to high-intensity conditioning (47%).

ATG (anti-thymocyte globulin) has been widely used to reduce the risk of allograft rejection through its immunomodulatory properties through T-cell depletion and inducing apoptosis of B-cell lineage (Mohty 2007, Siddiqui 2019, ATG is a polyclonal IgG produced from animals like rabbits and horses hyper immunised with human thymocytes and is used as part of the pre-conditioning to reduce the risk of acute GVHD and in the treatment of certain aplastic anaemias (Baron 2017). In our cohort, we didn't find any significant difference in severe GVHD risk with the use of ATG (53% had ATG VS 47% had no ATG) although the sample was small.

Apart from treatment resistance, severe GVHD adds to significant healthcare costs requiring multiple immunosuppressive therapies and adding to patient morbidity. Severe acute GVHD can progress to a chronic stage if it persists beyond 100days after stem cell transplantation.

This leads to chronic inflammation and tissue damage and causes scarring of different organs, of which skin and lower GI tract are the commonest ones involved. Pulmonary complication like BOOP is also considered part of chronic GVHD and can cause pulmonary insufficiency and significant morbidity.

It is interesting to see the growing evidence of microbial dysregulation in association with acute GVHD in both animal and human studies (Hong 2021) and the severity of GVHD is linked with the degree of dysbiosis. Our study showed higher alpha diversity in the patient cohort with severe GVHD although there were no specific taxa have been identified for this. It can be postulated that microbial dysbiosis especially of the beneficial bacteria could have resulted in the overgrowth of potentially pathogenic bacteria linked to severe GVHD risk. The exciting finding is that poor microbial diversity in donors was linked with a severe GVHD risk in recipients indicating potential manipulation of the microbiome would impact the outcomes of HSCT in terms of risk of mortality and morbidity. There are several factors considered during donor selection including HLA compatibility, disease risk, and patient co-morbid conditions which are known to affect the outcomes of HSCT. Unfortunately, the modification of these factors is limited to a certain extent. The research on the microbiota and related genomic components has shed new avenues for therapeutic targets and there is growing evidence to suggest a healthy diverse microbiota is been linked with better outcomes of HSCT. Faecal microbial Transplantation has been known to reduce the severity of recurrent c. difficile infection in patients with chronic colonisation and has improved patient quality of life. Its role has been explored in other conditions like IBD, metabolic syndrome and functional gastrointestinal disorders. Similarly modifying the microbial composition, especially of gut microbiota in the recipients and donors of HSCT could result in a better outcome in terms of reducing the risk of GVHD and improving survival. Further research is required to explore the potential metabolic and genomic pathways linked with the risk of GVHD and mortality and modulation of this microbiome as treatment targets.

Although we have not observed any significant association between baseline donor microbiome and the incidence of aGVHD in the recipients, a lower microbial diversity in donors was associated with more severe GVHD and poor transplant outcomes in the patients. This highlights the importance of a healthy donor microbiome as a factor in determining the success of HSCT and a potentially modifiable tool while considering donor selection.

Key Points:

- No significant difference was observed in microbial diversity of donors to those recipients who developed aGVHD and those who didn't develop it.
- Interestingly a lower microbial diversity of the donor microbiome was associated with severe aGVHD risk in the recipients.
- The finding points out the importance of the microbiomes during donor selection for stem cell transplantation.

Chapter 7

Conclusions

Haematopoietic stem cell transplantation remains one of the life-saving modalities for treating refractory haematological malignancies. Over the last decade, there has been significant progress in the field of stem-cell transplantation in terms of newer treatment options and improved survival. More patients are becoming suitable transplant candidates with the increased availability of donors and improvements in treatment strategies. In addition to HLA compatibility, which is still the key determinant of successful transplantation, patient and donor factors play an added role in improved transplant-related outcomes. Notably, various environmental factors have been increasingly identified as the key players crucial to the success of bone marrow transplantation of which the microbiota and microbiome merit special attention. The microbiota in various body parts contribute to immune and inflammatory regulation and help in maintaining a healthy homeostatic state with the host cells, in which the intestinal microbial organisms play a critical role. The genomic components and microbiota are collectively known as the microbiome which affects various functional and immunological pathways in the host cells. Trillions of microbial organisms in the gut exert their beneficial role through direct mucosal action and by the production of several microbial metabolic products. The metabolic products are essential anti-inflammatory gadgets, among which SCFAs and polyamines have been implicated in various inflammatory and metabolic disorders.

Acute GVHD continues to affect the short and long-term outcomes of transplantation with an incidence of 52% in our cohort and has contributed to more than 50% of all-cause mortality. Acute GVHD is one of the encounters limiting the success of stem cell transplantation resulting in a non-relapse mortality rate of 12% in our cohort. It is also associated with significant long-term morbidity with the development of chronic GVHD. As observed in our study, unrelated transplants and high-risk mutations associated with the diseases are major

contributing factors to the high mortality rates. However, 20% of aGVHD cases were associated with HLA-matched sibling donors indicating the role of external factors including the role of microbiota in the development of GVHD and long-term outcomes of transplantation.

Investigating the role of the microbiota and genomic components on the risk of acute GVHD and treatment response has revealed exciting results, which will be further evaluated in animal mouse models. Previous studies revealed the presence of certain bacteria in the human gut which have been linked with various inflammatory bowel diseases and metabolic syndrome. The implicated mechanism included direct mucosal action to augment the local immune response and production of various metabolic products such as SCFA with anti-inflammatory properties. SCFAs such as butyrate and propionate have been linked with the enhanced production of anti-inflammatory cytokines such as IL-10, IF-gamma, and TGF-β. Moreover, they suppress the production of TNF, which has been linked with the inflammatory cascade. The evaluation of butyrate producers with the risk of acute GVHD indicates the potential beneficial role of these microorganisms in reducing the impact of inflammation linked with aGVHD and improving survival.

Microbial diversity is a key determining factor linked with the risk of aGVHD and a diverse microbiome is essential for maintaining a homeostatic state of health. Previous studies have focussed on the changes in microbial composition following stem-cell transplantation. A shift in the microbial diversity occurs following stem-cell transplantation from various factors including the toxicity related to pre-conditioning chemotherapy and the use of broad-spectrum antibiotics causing changes to the intestinal microbial environment. The intestinal tract is one of the predominant sites in which microbial flora exert their constant check on immune regulation and systemic inflammation through local mucosal actions and the production of antimicrobial products. Our study is unique and highlights the distinct changes in the microbial

signature related to transplantation which can occur days to weeks prior to stem-cell infusion. An analysis of faecal samples of stem-cell transplant recipients revealed the presence of certain bacteria in association with GVHD survival, especially butyrate producers. The cohort samples were collected days before the conditioning treatment and stem-cell infusions, indicating the effects of microbiota independent of the changes related to chemotherapy and transplantation on the risk of aGVHD. Assessing microbial diversity and its potential modulation would be a key therapeutic target to reduce the risk of GVHD. Moreover, the presence of microorganisms such as *Eubacteria*, *Veillonella*, *Lachnosira* and *Roseburia* in the GVHD - survivor group indicates their potential beneficial effects through the production of SCFAs such as butyrate. These metabolic products can be used as components of formulations to reduce inflammation, which is one of the key mechanisms leading to the development of aGVHD. The shift in microbial diversity occurs due to the abolition of beneficial bacteria due to various therapeutic interventions resulting in the overgrowth of pathogenic bacteria. The presence of these anaerobic bacteria in the intestine suggests the alleviated risk of severe GVHD and potential survival benefits. The overexpression of certain enzymes observed through genomic sequencing in the survivor group indicates the potential metabolic pathways driving inflammation and GVHD risk. Using random forest prediction models, the association between the microbiome and the risk of GVHD can be predicted with an accuracy of over 70%.

As shown earlier, microbial metabolic products such as SCFA are well known for their anti-inflammatory properties, which is one of the potential mechanisms associated with the beneficial role of the bacteria in our GVHD survival group. Other than SCFA, other metabolic components have been implicated to play a role in inflammation and organ transplantation. Among these components, polyamines merit special consideration. Sixty-five percent of cases in our severe GVHD cohort were associated with treatment resistance, making the management of aGVHD further complex and decreasing longevity. The mortality rate

associated with the severe GVHD group was 30%, mostly because of steroid resistance. The response to treatment is crucial in determining survival; thus, factors contributing to the response to treatment become more relevant when managing severe cases of acute GVHD.

Severe GVHD is associated with reduced alpha diversity of various microbial species and occasional overgrowth of potentially pathogenic bacteria. A higher genus diversity and richness have been observed in association with the steroid-responsive cohort, indicating the presence of potential microbes and their metabolic products driving the treatment response in aGVHD. Wider species diversity was also observed in the steroid-responsive group. Furthermore, a stronger correlation has been observed with functional enzymatic pathways in steroid-responsive male patients, especially upregulation of the L-arginine proline degradation pathway.

Polyamines have been well known for their anti-inflammatory properties as well as regulation of cell differentiation and tissue repair. They are involved in the differentiation of mesenchymal cells and are essential components promoting successful haematopoiesis following engraftment. Spermine and spermidine are well-known polyamines that have been implicated for their potential anti-oxidant and anti-inflammatory capabilities and are promising targets for the antiaging process because they regulate apoptosis. Upregulation of the degradation pathway for L- arginine results in increased availability of putrescine and spermidine which are potent anti-inflammatory agents. It can be postulated that through the availability of these agents, the harmful effects of GVHD especially, the inflammatory response could be alleviated thereby enhancing the treatment response. The increased production of polyamines driving steroid sensitivity depends on the presence of certain microbiota in the intestinal lumen and is closely linked with the wider microbial diversity. The anti-ageing properties of these polyamines mainly, through the regulation of apoptosis could be linked with the potential survival of aGVHD patients.

Further in-vitro studies are planned to investigate the role of polyamines in animal models with the risk of inflammation and GVHD. As we have demonstrated there was upregulation of the L- arginine degradation pathway in the steroid-responsive group. With this upregulation, an increased amount of polyamines such as putrescine and spermidine will be available with potent anti-inflammatory properties. Their actions on various cytokine and metabolic pathways regulate inflammation and cell proliferation. This finding will have an impact on the pathogenesis of GVHD because putrescine can reduce the inflammatory cascade and leading improved survival following stem-cell transplantation. As a potential stimulator of the apoptotic process, spermidine plays a crucial role in the anti-oxidation and ageing processes. The enhanced steroid responsiveness linked with polyamine activation will be a critical step in managing graft versus host disease and reducing the mortality and morbidity associated with severe GVHD. The anti-oxidant properties of polyamines exert cardiovascular protection, thereby reducing mortality resulting from the complications associated with stem-cell transplantation.

We propose to evaluate the risk of aGVHD in stem cell transplanted mouse models which have been supplemented with putrescine. With our study findings, the polyamine supplementation will be helpful in reducing the risk of inflammation with associated lower risk of aGVHD and improved survival compared to knock-out mice. Identification of specific bacteria involved in polyamine metabolism and enhanced butyrate production will be very crucial in the outcomes of HSCT. Replacing these specific microbiotas or their beneficial microbial metabolites to patients undergoing stem cell transplants would be crucial in reducing the risk of GVHD and would be potential therapeutic targets in the field of stem cell transplantation.

The effect of the recipient and donor microbiome on the risk of developing severe GVHD has shown striking differences in the donor cohort. A significantly lower microbial diversity in the

donors was linked with the development of severe GVHD in recipients when compared to that in patients who did not develop GVHD. Nearly opposite trends with a higher alpha diversity have been observed in recipients with severe GVHD compared to the non-GVHD group. As an observation, *Dorea* spp. have been noted to be predominant in the recipients with severe GVHD.

The success of stem-cell transplantation depends on the availability of a matched healthy donor. Although the risk of GVHD and mortality is higher in unrelated donors, recipients of matched sibling donors also suffer from complications, including severe GVHD, suggesting the role of factors other than HLA-matching on GVHD risk. As has been already described, environmental factors such as recipient microbial niches have a critical role in determining the transplant outcomes and GVHD risk. Furthermore, we evaluated the significance of the donor microbiome on the incidence of acute GVHD in recipients, and no significant differences were observed in terms of alpha and beta diversity. The non-significance of the results could be attributed to the small sample size. However, an interesting association was noted in the microbial diversity of the donors and recipients who developed severe aGVHD versus those who did not develop GVHD. The significantly lower alpha diversity in the donor microbiome was linked with severe GVHD in the corresponding recipients. This further highlights the sympathetic role of a healthy donor microbiome essential for a successful transplant outcome. A diverse donor microbiome at baseline is associated with a reduced risk of severe GVHD and reduced mortality and morbidity risk.

In summary, we identified distinct changes in the microbial diversity related to stem-cell transplantation with a predominance of certain microbial populations in association with survivors of GVHD compared to non-survivors and patients who did not develop GVHD. We believe that the shift in the microbial signature occurs weeks before stem cell infusion, indicating the role of the microbiome on the outcomes of HSCT independent of disease and

treatment-related factors. The enhancement in the polyamine degradation pathway associated with steroid responsiveness would be a crucial finding to guide treatment for resistant GVHD and improve outcomes of HSCT. Further studies are required to evaluate the effect of polyamine supplementation on the risk of GVHD and treatment responses and could be a potential treatment target for the future. Lower donor microbial diversity associated with severe GVHD in the recipients is a key finding when considering the donor selection processes for stem-cell transplantation. Future studies are required to confirm the effects of polyamines and apply the results to clinical practice, which will have an impact on the risk of GVHD and treatment response and could improve the risk of mortality related to stem-cell transplantation.

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