Vitamin D
and
Immune Responses in Haematopoietic Stem Cell Transplantation

Jose Ros-Soto

MBBS

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Author's Declaration

I, Jose Ros-Soto, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Dr Jose Ros-Soto

10th April 2020

Abstract

Among its pleiotropic effects, vitamin D has immunoregulatory properties that help to maintain immune homeostasis. Multiple factors make haematopoietic stem cell transplant (HSCT) recipients at higher risk of vitamin D deficiency, and this (even prior to the stem cell infusion) can impact adversely over the course of HSCT. Owing to the lack of consensus, a cut-off to define vitamin D deficiency has not been established yet and clinical practice may vary across different HSCT units. To address this, one of the chapters of this thesis has examined the current management of vitamin D deficiency in the allogeneic HSCT setting, confirming the highly heterogeneous practice across the EBMT affiliate centres, including those from diverse geographical locations and dedicated to patients from different age.

Graft-versus-host disease (GvHD) is a major cause of morbidity and mortality after allogeneic HSCT. To confirm or rule out this disease, a biopsy result from the affected tissue may be delayed for several days or even weeks. GvHD biomarkers are promising diagnostic tools that can speed up this process, predict outcomes in the early post-HSCT phase and monitor response to immunosuppression, minimising the detrimental effect of this therapy on HSCT recipients. Due to this, an observational study will explore the role of three of these biomarkers (elafin, REG3α and ST2), alongside vitamin D, in the context of patients with acute and chronic GvHD on immunosuppressive therapy.

The final study moves away from vitamin D and biomarkers although it is still linked to the graft-versus-host reaction. Infusion of donor lymphocytes (DLI) is an effective adoptive immunotherapy that approximately 25% of post-HSCT patients have received in the UK[1]. DLI enhances graft-versus-leukaemia effect but its main side

effect is GvHD. This chapter describes a single-centre experience treating 100 patients with DLI after reduced intensity conditioning (RIC) HSCT for mixed chimerism (MC) or relapse of the primary disease. It aims to determine factors implicated in achieving full donor chimerism (FDC) or disease remission, as well as their impact on other outcomes post-DLI, such as survival, relapse post-DLI or GvHD, in order to improve survival and quality of life in these patients. We found that patients with younger donors were less likely to develop acute GvHD and subsequently it contributed to a greater survival, which has been previously reported in HSCT recipients but never in those receiving subsequent DLI.

Impact Statement

Throughout two chapters this thesis explores the impact of vitamin D in the field of stem cell transplantation, and the last chapter focuses on DLI, a type of immunotherapy. The first study is a survey conducted on behalf of the Transplant Complication Working Party (TCWP) of the EBMT. I designed it and elaborated the questionnaire. Feedback was provided by main supervisors and senior members of the TCWP. The survey was circulated across the EBMT affiliate centres by Alenca Harrington (TCWP study coordinator). The study coordinator and I sent the consecutive reminders. Thereafter, I analysed the data. This study highlights the heterogeneity in the management of vitamin D deficiency across international adult and paediatric allogeneic HSCT centres because of the lack of consensus in the HSCT community. These findings have been disseminated internationally as a poster presentation at the EBMT conference in 2019, and a peer-reviewed publication in the journal *Biology of Bone and Marrow Transplantation* was issued in the same year. Recommendations based on the limited evidence-based literature were provided to improve the current approach of this condition in the HSCT population.

The second chapter was an observational study carried out in patients with GvHD, where serial blood samples were taken to measure vitamin D and markers of GvHD. This was a unique study that tried to elucidate the role of these molecules in the immunosuppressive therapy used to fight GvHD. Vitamin D is currently a focus of interest but publications in the HSCT setting are limited, particularly in GvHD.I designed the study, selecting populations of interest and appropriate timepoints for measuring vitamin D and GvHD biomarkers. Feedback was provided by main supervisors and scientists from the ECP laboratory in Rotherham General Hospital. I

also approached Transplant Centres (and those with ECP units) to invite them to participate in the study. I analysed the samples alongside Charlotte Burton (Research Assistant) and this work was supervised by Dr Nick Matthews (Senior Scientist) in the ECP laboratory. Subsequently, I analysed the data and feedback was provided by my main supervisors. Although sample size did not allow to draw significant conclusions, this project reproduced the existing evidence of the role of GvHD biomarkers as diagnostic and prognosis predictive tools in the context of aGvHD. In addition, these data suggest that vitamin D may act as a prognostic factor, especially in cases of aGvHD that do not respond to steroids. Moreover, a potential cut-off to define vitamin D deficiency was suggested and supplementation accordingly was encouraged, in order to optimise serum levels of vitamin D and foster its function as mediator of immune homeostasis.

The last study chapter focused on a different topic but still in line with immune reactions in HSCT recipients, to deepen the knowledge of DLI indicated for MC and relapsed disease in the context of RIC HSCT. I collected the data retrospectively from the Royal Marsden Hospital database and analysed it. Feedback was provided by main supervisors and Dr Chloe Anthias (Consultant Haematologist). It confirmed that GvHD after DLI is more frequent in patients with female and/or older donors. Moreover, it showed the beneficial effect of achieving T-FDC in disease remission and improved survival (in patients with relapsed disease), as well as having a younger donor and attaining UWB (unfractionated whole blood) FDC (in patients with MC). Recruitment of younger male donors is encouraged, as this can minimise the risk not only of GvHD, but also relapse and mortality. These findings will be presented as a poster presentation in the EBMT conference 2020, and a peer-reviewed manuscript is in preparation.

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Acknowledgments

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A mis padres y hermana, porque todo lo bueno que tengo se lo debo a ell@s.

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Publications and Presentations

Published papers

Ros-Soto J, Anthias C, Madrigal A, Snowden JA. **Vitamin D: Is it important in haematopoietic stem cell transplantation? A review**. *Bone Marrow Transplantation* (published online 6th November 2018).

Ros-Soto J, Snowden JA, Salooja N, Gilleece M, Parker A, Greenfield DM, Anthias C, Alfred A, Harrington A, Peczynski C, Peggs K, Madrigal A, Basak GW, Schoemans H on behalf of the Transplant Complications Working Party of the EBMT. **Current practice in vitamin D management in Allogeneic Haematopoietic Stem Cell Transplantation: a survey by the Transplant Complications Working Party of the EBMT.** *Biology of Bone and Marrow Transplantation* (published 20th June 2019).

Ros-Soto J, Anthias C, Madrigal A, Snowden JA. **Insights into the role of vitamin D as a biomarker in stem cell transplantation**. *Frontiers in Immunology* (published 8th June 2020).

Peer-reviewed presentations

March 2019. EBMT annual meeting, Frankfurt (Germany) – *Poster presentation*
Ros-Soto J, Snowden JA, Salooja N, Gilleece M, Parker A, Greenfield DM, Anthias C, Alfred A, Harrington A, Peczynski C, Peggs K, Madrigal A, Basak GW, Schoemans H on behalf of the Transplant Complications Working Party of the EBMT. **Current practice in vitamin D management in Allogeneic**

Haematopoietic Stem Cell Transplantation: a survey by the Transplant Complications Working Party of the EBMT.

December 2019. ASH annual meeting, Orlando – *Poster presentation*

Ros-Soto J, Zoubek E, Szydlo R, Johnson P, O'Leary A, Cuadrado M, Burlton C, Madrigal A, Anthias C. **Age matters: Younger unrelated PBSC donors experience less G-CSF-related symptoms and have a faster emotional recovery than older donors.**

August 2020. EBMT annual meeting (virtual) – *Poster presentation*

Ros-Soto J, Snowden JA, Szydlo R, Nicholson E, Madrigal A, Easdale S, Ethell M, Potter M, Anthias C. **Donor factors influence outcomes of patients receiving donor lymphocyte infusion for mixed chimerism after RIC allogeneic SCT.**

August 2020. EBMT annual meeting (virtual) – *Poster presentation*

Zoubek E, Ros-Soto J, Szydlo R, Watson A, Burlton C, Johnson P, O'Leary A, Anthias C. **Bone marrow donor characteristics: Influence on harvest yield and donor recovery.**

<u>Invited speaker presentation</u>

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List of Abbreviations

alloHSCT: allogeneic haematopoietic stem cell transplantation

aGvHD: acute graft-versus-host disease

Ag: antigen

APC: antigen presenting cell

BM: bone marrow

CI: cumulative incidence

CMV: cytomegalovirus

CR: complete response

cGvHD: chronic graft-versus-host disease

DAMPs: damage-associated molecular patterns

DCs: dendritic cells

DLI: donor lymphocyte infusion

DFS: disease-free survival

EBV: Epstein-Barr virus

FDC: full donor chimerism

GvT: graft-versus-tumour

GvL: graft-versus-leukaemia

HLA: human leukocyte antigen

HSC: haematopoietic stem cells

IDO: indoleamine 2,3-dioxygenase

IFN-γ: interferon γ

Ig: immunoglobulins

IL: interleukin

IS: immunosuppression

MAC: myeloablative conditioning

MC: mixed chimerism

mHA: minor histocompatibility complex

MHC: major histocompatibility complex

mST2: trans-membrane molecule

MUD: matched unrelated donor

N: number of participants

NK: natural killer cells

NR: no reported

NRM: non-relapse mortality

OS: overall survival

Paed: paediatric

PAMPs: pathogen-associated molecular patterns

PBSC: peripheral blood stem cell

PFS: progression-free survival

PDGF-α: platelet-derived growth factor α

PR: partial remission

RT: replacement therapy (with $1,25(OH)_2D^3$)

TGF-β: transforming growth factor β

RIC: reduced intensity conditioning

RR: relative risk

sST2: soluble ST2

SR GvHD: steroid refractory GvHD

TGF-β: transforming growth factor β

Th1, 2 and 17: T helper lymphocytes 1, 2 and 17

TNF-α: tumour necrosis factor α

Treg: regulatory T lymphocytes.

UBC: umbilical cord blood

UD: unrelated donor

VD: vitamin D

VDD: vitamin D deficiency

VDR: vitamin D receptor

VUD: volunteer unrelated donor

1 Introduction

1.1 Overview of the Human Immune System

The human immune system aims to protect against infections, react against foreign molecules and remove cellular detritus. It is comprised of two different groups of cells; innate and adaptive immunity. Innate immunity acts within the first few hours of infection, recognising and destroying any microorganism encountered (such as bacteria, viruses, parasites or fungi). Neutrophils, macrophages, dendritic cells (DCs), natural killer (NK) cells and mast cells take part in this front-line response against microbes. Adaptive immunity develops long-term immune memory throughout the days or weeks after the infection. It is formed of B and T lymphocytes, with B lymphocytes being the manufacturers of antibodies, molecules with high-affinity target specific antigens and the eradicators the infectious microbes[2].

All the immune cells derive from haematopoietic stem cells (HSC) located in the bone marrow. HSC produce myeloid and lymphoid progenitor cells. Myeloid progenitors differentiate into erythrocytes, megakaryocytes, granulocytes (neutrophils, basophils and eosinophils), DCs, macrophages and mast cells. For its part, lymphoid progenitors differentiate into and B and T lymphocytes and NK cells[3].

1.1.1 The Innate Immune System

The innate immune system consists of a first line of defence that can rapidly identify pathogens and injured cells through different pattern recognition receptors (PRRs), including toll-like receptors. Cells from the innate immunity respond immediately

after encountering microbes or parts of them (pathogen-associated molecular patterns (PAMPs)), as well as products from injured cells (damage-associated molecular patterns (DAMPs))[4]. Following this, inflammation starts off with cytokine release and subsequent recruitment of activated macrophages, that engage other phagocytic cells, such as neutrophils, and NK cells through chemical signalling with chemokines. Dendritic cells also play a fundamental role in this early immune response, mediating with adaptive immunity and activating naïve lymphocytes in the peripheral or secondary lymphoid tissues after presenting captured antigen/s, thus acting as antigen presenting cells (APCs)[2].

1.1.2 The Adaptive Immune System

B and T lymphocytes mediate in the adaptive immunity after maturing in primary lymphoid tissues (either bone marrow or thymus). Then, these mature naïve cells migrate to the secondary lymphoid tissues (lymph nodes or spleen) to be activated after APCs present a specific antigen associated to HLA (human leukocyte antigens) class II. The pool of activated lymphocytes by a specific antigen proliferates and expands during a process called *clonal expansion*, in order to support the fight against a particular pathogen. Following this, activated lymphocytes differentiate into *effector cells*, that attack and destroy microbes, and *memory cells*, that remains as long-term immunity should they encounter this particular threaten again. The effector cells also differentiate into CD4+ and CD8+, and they carry out the *cellular immunity*. CD8+ migrate to the infection sites and recognise infected non-immune cells that express antigens linked to MHC (major histocompatibility complex) class I. CD4+ T helpers recognise antigens associated to MHC class II in APCs and, following activation, they secrete cytokines to support

B cell activation and production of antibodies (Ab), leading to the beginning of the *humoral immunity*.

Lastly, the regulatory T cells (Treg) are a T lymphocyte subset that probably derive from memory CD4+ T cells[5]. They mediate immune homeostasis destroying self-reactive cells and preventing autoimmune disorders[6].

1.2 Overview of Haematopoietic Stem Cell Transplantation

HSCT is now standard of care in many diseases and the only cure in some[7]. Since it was first reported by Professor Thomas in 1957[8], the numbers of transplants have gradually grown. This is mainly due to the use of reduced intensity conditioning (RIC), that benefits older patients and those with comorbidities and the availability of alternative donors such as haploidentical or umbilical cord[9,10].

The biological basis of HSCT consists of eradication of the disease, immunosuppression of the alloreactive immune cells and reconstitution of the healthy haematopoietic system from the donor stem cells[10]. Depending on the type of conditioning, this may vary: When myeloablative conditioning (MAC) is administrated, tumoral cells are eradicated due to the cytotoxic drugs alongside the graft-versus-tumour (GvT) effect caused by the host immunocompetent cells. On the contrary, when RIC is used, cytotoxicity relies solely on the GvT effect performed by the donor cells[11].

Historically, stem cells have been harvested directly from the bone marrow (BM). However, this practice has been gradually switched to peripheral blood stem cell (PBSC) donation, a less invasive technique that requires donor priming with

granulocyte-colony stimulating factor (G-CSF) and entails a faster recovery with fewer side effects[12,13].

There are two main different types of stem cell transplants: autologous, where stem cells are collected from a patient prior to conditioning, and allogeneic, where stem cells are harvested from a related or unrelated donor.

1.2.1 Autologous HSCT

In the 1980's, the development of stem cell collection and cryopreservation fostered the use of autologous HSCT[14]. This is the main type of HSCT for patients with haematological malignancies (lymphoma, myeloma), solid tumours and autoimmune diseases[15]. High-intensity chemotherapy administrated as part of the treatment can cause irreversible stem cell aplasia, thus autologous HSCT aims solely to re-establish the normal haematopoietic function.

1.2.2 Allogeneic HSCT

The indications for this procedure encompass haematological malignancies, bone marrow insufficiency, haemoglobinopathies, metabolic disorders and immune insufficiencies[15]. Depending on the duration and reversibility of the cytopenias induced, the different types of conditioning can be divided into MAC, RIC and non-myeloablative (NMA)[10]. In the malignant setting, the aim of this procedure is to cure the underlying disease as well as re-establishing the normal haematopoiesis. In selected cases, more intensive conditioning regimes are needed to achieve disease control as well as a profound immunosuppression to ensure a successful engraftment. In non-malignant conditions, the graft transplanted solely aims to restore the haematopoiesis, so less intense conditionings are required[10].

For most of the diseases, the first choice would be a related donor, primarily a sibling. When this is not available, the next option is a voluntary unrelated donor (VUD), which can be approached via international bone marrow registries. Otherwise, frozen UCB (umbilical cord blood) can be an alternative stem cell source[16].

HLA determine the compatibility between a donor and a patient. Genes encoding these proteins (MHC) are found in the short arm of chromosome 6. HLA proteins are displayed on the surface of most human cells, and there are two types: class I (HLA-A, HLA-B and HLA-C) and class II (HLA-DR, HLA-DQ and HLA-DP). Both are involved on response against infection and self-tolerance. HLA class I proteins are expressed in all the nucleated cells and platelets and they present non self-antigens, including intracellular bacterial or viral peptides, to activated CD8+ cytotoxic T cells. HLA class II are expressed on APCs, such as DCs, to present extracellular peptides, previously phagocytosed, to CD4+ T helper cells. When activated immune effector cells recognised non self-antigens attached to the correspondent HLA protein, they mount an immune response that can lead to tissue damage and necrosis. Therefore, HLA proteins have a fundamental role in histocompatibility and transplantation. Guidelines from the British Society of Histocompatibility and Immunogenetics (BSHI) recommends 10/10 high-resolution allelic level matched HLA-A, -B, -C, -DRB1 and –DQB1 donor. HLA-DPB1 typing is encouraged for unrelated donors in order to avoid non-permissive mismatches[17].

Alloreactivity can play an advantage role in alloHSCT. Owing to the expression of minor histocompatibility antigens (mHA), polymorphic peptides presented by HLA proteins on the cell surface, alloreactive donor T cells can recognise mHA and attack not only healthy but also tumoral host cells. This is known as GvT or Graft-

versus-Leukaemia (GvL) effect[11]. GvL can be associated with graft-versus-host disease (GvHD), and the benefit of the former can be counterbalanced by the harmful effect of the latter[18], hence the dissociation of both processes is key to improve patients outcomes[18]. This is important in malignancies, where the therapeutic effect of GvL can be added up to the cytotoxicity derived from conditioning drugs. Nevertheless, in non-malignant diseases GvHD should be avoided since the aim of HSCT is solely to establish a normal functioning haematopoiesis[10].

1.2.3 Immune reconstitution post-HSCT

The conditioning regimen and stem cells infusion is followed by the *aplastic phase*. From then, quantitative and qualitative recovery of the different immune cell subsets do not occur parallelly. Firstly, immune cell proliferation with peripheral blood expansion happens followed by regaining the function of donor-derived immune system, which may take from a few weeks (in case of granulocytes or NK cells) up to a few years after HSCT (mainly B lymphocytes)[19,20]. Several factors contribute to immune reconstitution following HSCT, including donor and patient age, stem cell source, conditioning, patient-donor HLA matching, lymphodepletion, GvHD prophylaxis and peri-transplant infections[21].

1.2.3.1 Reconstitution of Innate Immunity

Neutrophils are the first cells subset to recover in the process of *engraftment*. It occurs within the first month post-HSCT and depending on the stem cell source the average time is 14 days in PBSC, 21 days in BM and 1 month in UCB[19]. Interestingly, vitamin D may enhance neutrophil recovery at this stage, as shown in a study where patients with higher serum levels of $25(OH)D^3$ had a higher neutrophil

count following HSCT compared to those with lower levels. However, the stem cell source did not impact on speed of immune recovery[22]. NK cells recover within 1-2 months but transplant type or patient characteristics do not seem to impact on this process[20]. Also, DCs may take up to 3 moths after HSCT[23].

1.2.3.2 Reconstitution of Adaptive Immunity

The quantitative recovery of B cell occurs within 12 moths after HSCT, but it may take more than 2 years to achieve normal function of production and secretion of antibodies[19,24]. Due to the lack of memory B cells and decreased Ig, HSCT recipients are more vulnerable to viral and encapsulated bacterial infections during this period[19]. In addition, GvHD can impair B cell reconstitution[21].

T cell reconstitution depends on its different subsets resulting in the inversion of the CD4+/CD8+ ratio early post-HSCT. Whereas CD8+ repertoire present a sustained recovery within the first months, particularly CD8+ memory cells as this process can be carried out extra-thymic, peripheral expansion and maturation of naïve CD4+ cell can take up to a few years after HSCT. This process is usually impaired in older patients and those with GvHD[19,21,23]. Interestingly, host immune T cells may survive and be capable of protecting against viral infections such as CMV disease following RIC[25]. Alongside them, donor-derived T cells can also play an important role in host immunity, mainly with in vivo lymphodepletion, and delay the onset of CMV disease[26].

1.2.4 Donor Lymphocyte Infusion

The term *chimerism* derives from *chimaera*, coined in 1956 to refer to individuals that possess a heterogenous cell population from different individuals[27]. In the

HSCT landscape, mixed chimerism (MC), wherein recipient cells coexist with donor cells[28] , is typical from recipients of RIC[29–33] whose immune reconstitution post-HSCT occurs slower compared to MAC. Since MC can potentially lead to relapse of the primary disease, different strategies have been sought to prevent this. Currently, infusion of donor lymphocytes (DLI), a type of adoptive immunotherapy has been successfully used for treatment of MC[28] and relapsed disease[34,35].

Donor-derived T cells target recipient residual immunity to establish full donor chimerism (FDC, ≥ 95% donor cells) and prevent impending relapse[36]. Also, in overt relapsed disease donor T cells may recognise specific antigens in patients tumoral cells and attack them to eradicate disease and achieve remission, the aforementioned GvT effect[11,37]. Unfortunately, GvT may occur simultaneously to GvHD or graft failure, with terrible consequences[18,35,38,39], but a better understanding of GvHD pathogenesis has allowed the development of safer approaches such as dose-escalation, where stepwise increased administration of DLI can control disease relapse without aggravating GvHD[18,34,35,40,41]. This topic will be addressed in depth in Chapter 4.

1.3 Complications of HSCT

HSCT aims to cure haematological malignancies but it entails risks derived from toxicity to conditioning regimes, immunosuppression and immune reactivity between donor immunity and recipient tissues, including GvHD, resulting in potential life-threatening organ damage. Alongside this, second malignancies and relapse are among the major complications after HSCT, which warrants thorough follow-up of HSCT recipients, especially in early post-HSCT[42,43].

1.4 Graft-versus-Host Disease

GvHD was firstly coined "wasting syndrome" or “secondary disease” due to the effect caused in mice after stem cell infusion[44]. It is characterised by host tissue damage by immunologically competent donor cells and cytokine dysregulation, and it is the most frequent complication amongst alloHSCT recipients, with a high morbidity and mortality[45,46].

GvHD has been historically classified depending on its onset: acute if it occurred within 100 days post-HSCT or chronic if it started beyond this time point. Nevertheless, the current classification does not consider time but clinical features: acute GvHD (aGvHD) is characterised by strong inflammation in skin, liver and gut, whereas chronic GvHD (cGvHD) displays more autoimmune manifestations with heterogenous organ involvement, resulting in tissue scarring and fibrosis[45–48]. According to the existing literature, the incidence of aGvHD ranges from 10 to 80%[47] and between 30% to 70% in cGvHD[46].

1.4.1 Acute GvHD

Acute GvHD remains a major cause of morbidity and mortality in the early phase post-HSCT, particularly in the most severe stages of the disease[49,50]. Tissue damage encompasses the epidermis, hepatic bile ducts and gut epithelium[45], and this is classified based on the extension of affected skin, serum bilirubin and/or quantity of diarrhoea or abdominal symptoms[51,52]. Risk factors for aGvHD include patient age, disparities in donor/patient HLA, PBSC grafts, unrelated donor, female donor to male recipient and high-intensity conditioning regimens[45,50,53–55]. Moreover, advanced disease prior to HSCT and gut and/or liver aGvHD can impact negatively in its prognosis[54].

Damaged tissues by conditioning release pro-inflammatory cytokines (IL-1 and TNF-α)[56] and DAMPs that, alongside PAMPs from local pathogens that leak through injured epithelial cells in the gut or skin, activate host APCs[73]. DAMPs and PAMPs are expressed by donor and patient APCs through their HLA molecules to be recognised by the T lymphocytes in secondary lymphoid organs. APCs interplay with donor adaptive immunity, particularly T cells, activating them (effector phase of aGvHD)[50] and triggering a cascade of pro-inflammatory cytokines[45,57]. To trigger T cell alloreactivity, 3 different steps are required: activation of T cell receptor (TCR), co-stimulation of T cells and cytokine effect. After this, alloreactive donor T-cell migrate to GvHD target organs guided by specific chemokines[57,58]. Naïve T cells are thought to play a central role in aGvHD targeting major and minor histocompatibility antigens (mHA)[45] whereas memory T cells prolong this process. Innate and adaptive immunity synergised to amplify inflammation[59]. Neutrophils, NK and macrophages are also involved in this process, but CD8+ T cells are enough to trigger GvHD in situations of donor/patient HLA-mismatch[50]. IL-1, IL-6 and TNF-α exacerbate the inflammatory cascade and lead to tissue damage and necrosis[50,60]. Alongside epithelial and intestinal stem cells, Paneth cells are destroyed during gut GvHD resulting in a decreased of α-defensin (an antimicrobial peptide), with a subsequent loose of microbial diversity and a detrimental effect on mortality[61,62]. Conversely, Treg cells attenuate proliferation of peripheral donor T cells and blunt GvHD while preserving GvL effect[63], making them an attractive therapeutical strategy against GvHD.

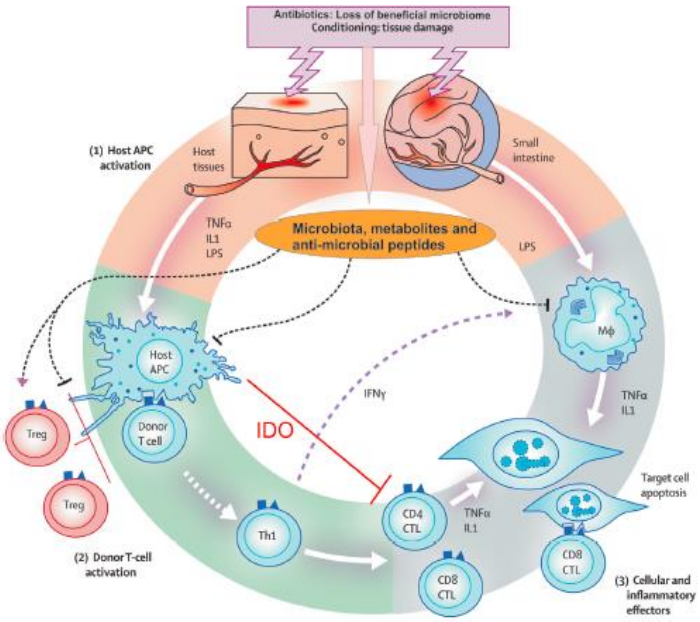


Figure 1-1: Pathophysiology of aGvHD (Ghimire et al, 2017). Open access

1.4.2 Chronic GvHD

cGvHD is the main cause of late non-relapse mortality (NRM) following HSCT[64]. Donor/patient HLA disparity, previous aGvHD, PBSC as stem cell source, female donors for male patients, chronic myeloid leukaemia and older patient or donor age are among the risk factors that can trigger this entity[65,66]. Furthermore, female donors and low platelet count at diagnosis can impact adversely on survival[67].

Pathophysiology of cGvHD differs from aGvHD since the former is a multi-organ disease with a lower inflammatory state but it can potentially lead to scaring and organ disfunction, showing a wide spectrum of presentations that may take up to months or years to appear[64]. It is divided in three phases: inflammation secondary to tissue damage, immune dysregulation with chronic inflammation, and aberrant tissue repair and fibrosis[68].

In the early inflammatory phase, monocytes/macrophages, DCs and B cells act as APCs of DAMPs and PAMPs. After the host immune system has encountered the recipient tissues, tissue injury is perpetuated by innate immunity. Thymic injury secondary to conditioning and calcineurin inhibitors also contribute to cGvHD due to impaired central tolerance and subsequent escape of autoreactive CD4+ T cells during homeostatic expansion[69,70].

Chronic inflammation is characterised by the role of adaptive immunity: B and T cells act as effector cells after recognising antigens presented by APCs to TCR or B cell receptor, respectively. B cell development is disturbed due to the damaged B cell niche, alongside a decrease in B cell destruction that produce allo and auto-antibodies[71,72]. Besides, Th17 lymphocytes that escaped thymic deletion maintain chronic inflammation[73].

In the last phase, activated macrophages release platelet-derived growth factor α (PDGF-α) and transforming growth factor β (TGF-β), resulting in the production of extracellular matrix collagen and biglycan by activated fibroblast, leading to tissue fibrosis and sclerotic lesions, hallmark of cGvHD[74].

Moreover, lymphopenia is common among patients with chronic GvHD, specially T CD4+ and B cells, which makes them more susceptible to infections in the late post-HSCT phase[72].

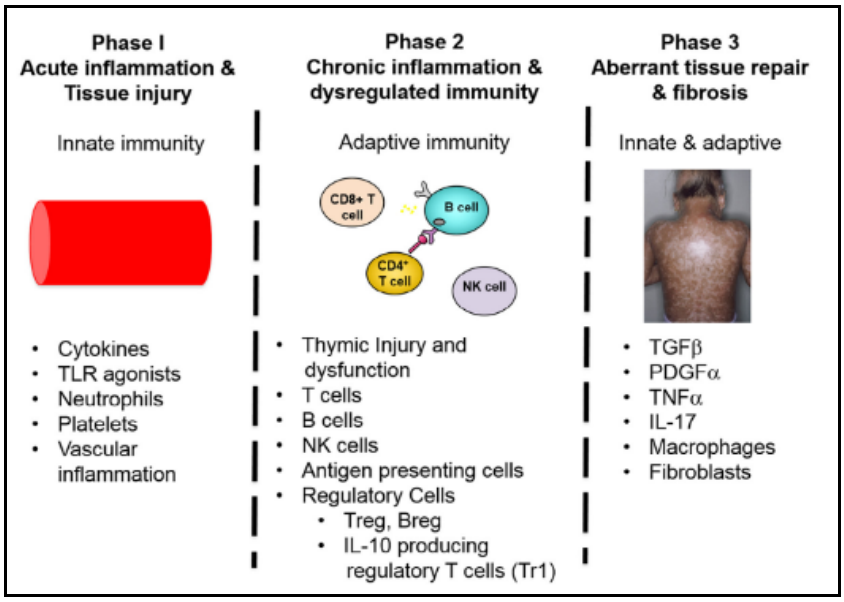


Figure 1-2: Biological phases of cGvHD (Cooke et al, 2017). Open access.

1.4.3 Steroid-Refractory GvHD

Despite many efforts taken to prevent this entity and its harmful consequences, GvHD is still highly prevalent among recipients of alloHSCT[75].

Steroids are the mainstay of first-line therapy in GvHD[47,76,77]. They exert their immunosuppressive effect inhibiting the nuclear factor kappa B (NF-kB), a transcription factor that regulates the secretion of chemokines and expression of HLA class I and II molecules[78,79]. They also hamper leukocyte adhesion to the endothelium of target tissues[80], inhibit macrophage activation, trigger T-cell apoptosis and abrogate cytokine secretion[59]. Low-dose of glucocorticoids is associated with fewer toxicities and has no negative impact on patients outcomes, particularly in the least severe cases[81]. However, a low dose may not provide enough immunosuppressive effect in some patients and further strategies may be required for disease control[82]. This phenomenon is known as *steroid-refractory*

GvHD (SR GvHD) and occurs in more than 50% of patients with this condition[83–86]. Although little is known of its cause/s, it is associated with a poorer prognosis[83,84].

Interestingly, response to steroids has been investigated in a completely different landscape with remarkable findings: In vitro and in vivo studies performed in an asthmatic cohort showed that patients with lower serum levels of $25(OH)D^3$ had a poorer clinical response to steroids than those with higher levels. This was caused by the decreased secretion of IL-10 by CD4+ T cells (as they were less responsive to steroid stimuli) and consequently this hampered recruitment of Treg. However, replacement with $1,25(OH)_2D^3$ overcame this and restored CD4+ IL-10 secretion and consequently, the expansion of peripheral Treg. These patients became more responsive to steroids and improved clinically[87–90]. Nevertheless, the association between vitamin D serostatus and the response to immunosuppressive therapy has not been explored in the context of GvHD, thus we can only hypothesise about the potential applicability of vitamin D as a potent regulator of immune responses in this setting.

SR aGvHD is defined as "disease progression within 3 days or failure to improve over 5-7 days after starting on steroids, or incomplete response after 2 weeks of high dose of steroids"[83,84]. In the **cGvHD** setting, s*teroid refractoriness* is considered when there is no response to steroids after at least 4 weeks of treatment and s*teroid dependency* when inability to wean high dose of steroids after 8 weeks[86]. Surprisingly, there is no evidence of an effective second-line treatment in cases of steroid-refractory GvHD[45,47,76,77], which emphasises the need for validation in further studies and clinical trials. In the meantime, close follow-up in GvHD by a transplant specialist in order to provide best standard-of-care should be sought[91].

1.5 Biomarkers of GvHD

Within the last decade, one focus of research interest lies in specific proteins that act as markers for GvHD in blood[92]. Serum levels of these biomarkers reflect the actual damage caused in target organs by GvHD, rather than the alloreactive reaction of donor cells against host tissues[93,94]. Their serum levels are elevated at the onset of the disease and they rise as the stage of GvHD worsens. Furthermore, higher levels of these biomarkers early after starting on steroids have been associated with treatment failure and poor GvHD-specific prognosis, as response to steroids is a surrogate marker for long-term survival[94–99]. GvHD grading at diagnosis does not correlate with the maximum grade that it will eventually achieve and therefore cannot be used for prognosis purposes[100]. Interestingly, some of the biomarkers achieve higher levels when specific organs are damaged: that is the case of *elafin* in skin aGvHD[101] or *regenerating islet-derived 3α* (REG3α) in gut aGvHD[102]. In specific settings such as cord blood transplantation, s*uppression of tumorigenicity 2* (ST2) has been found to be a good prognosis marker for aGvHD[97].

At the University of Michigan, the *Ann Arbour score* was created to predict outcomes at the onset of aGvHD depending on the disease severity. This new grading algorithm is based on the level of three different biomarkers (TNFR1, ST2, and REG3α) that stratify patients in three risk categories to obtain 1-year NRM from GvHD diagnosis and steroid response as outcomes. As the score increases from 1 to 3, it correlates with a higher NRM (8%, 27% and 46%, respectively, $p<0.001$). Equally, a lower score shows better response rate to treatment than a higher score (86%, 67% and 46%, respectively, $p<0.001$). Therefore, the *Ann Arbour score* reflects accurately the nature of this disease, even in the absence of overt GvHD

symptoms, and predicts treatment failure, allowing early interventions to a more personalised therapeutical strategies[103].

Characteristics	***Clinical application***
Ease of testing	Can potentially avoid invasive biopsies
Widely available technique	
Good reproducibility	
Low cost	
Adequate sensitivity	
High specificity	More accurate diagnosis
Predictive value	Can guide pre-emptive therapy
Correlation with treatment response	Can guide immunosuppressive withdrawal
Involved in pathophysiology	Can be targeted for novel therapy

Table 1-1: Ideal characteristics for a non-invasive blood biomarker for aGvHD (Chen et al*, 2013). Used with permission*

Furthermore, a multicentre study carried out by Hartwell *et al* collected blood samples from 1,287 patients to measure 4 different biomarkers, although only 2 (ST2 and REG3α) enabled the creation of the *Mount Sinai Acute GVHD International Consortium* (MAGIC) algorithm, aiming to predict 6-month NRM. This 2-biomarker model stratifies patients in 2 different categories, depending on the biomarkers levels a week after alloHSCT: 6-month NRM was found 7% and 28% (p<0.001), and severe gut GvHD 8% and 17% (p<0.001) in the low and high-risk cohort, respectively[98]. This proves that, even at the early onset of GvHD, biomarkers can show ongoing tissue damage in target organs and provide information beyond the current clinical manifestations. Similarly, further data was analysed following the *MAGIC score* one week after starting on systemic steroids for aGvHD and it showed that long-term outcomes could be predicted based on the biomarkers concentration

at this particular time point[99]. Another study performed at King's College Hospital generated a panel of serum biomarkers (including hepatocyte growth factor, elafin, soluble interleukin-2 receptor-α, soluble tumour necrosis factor receptor-1 and REG3α) tested in 26 patients following RIC HSCT. Blood samples were drawn at day 0 and +7 post alloHSCT, and on the day of diagnosis of aGvHD. This composite panel was found to increase diagnostic accuracy of aGvHD and predict disease severity before overt clinical manifestations in the context of *in vivo* T-cell depleted alloHSCT[104].

Moreover, in cGvHD a biomarker panel including ST2, *chemokine ligand 9* (CXCL9), *matrix metalloproteinase 3* (MMP3) and *osteopontin* measured 3 months post-alloHSCT could predict the risk of developing cGvHD[105]. Besides, other biomarkers have been identified, including CXCL10 (produced by CD8+ cells), *B cell activation factor* (BAFF, fosters survival and differentiation of activated B cells and Ig production), and immune cells such as CD4+ Th17 and Treg[106–108], but data is still scarce and further studies are warranted in the context on cGvHD.

Serum GvHD biomarkers aim to replace invasive diagnostic tests, monitor response to treatment and identify high-risk patients as they can detect early subclinical disease. They are a reliable tool to tailor the most suitable therapeutical approach for each patient and can potentially identify patients who either need more intensive treatment or limit unnecessary lengthy exposure to immunosuppression, including steroids[94,109]. Nevertheless, most of the studies have focused on the use of biomarkers in the context of aGvHD and at a specific timepoint, particularly within first month after GvHD diagnosis, but little is known of their applicability in the long-term follow-up and in cGvHD[95,105,110].

1.5.1 Elafin

Elafin is an antiprotease secreted by keratinocytes in the inflamed epidermis in response to IL-1 and TNF-alpha. Among its functions, it increases recruitment of inflammatory cells, activates DCs and reduces skin necrosis neutralising proteases released by neutrophils[111–113]. Plasma levels of elafin are higher at the onset of skin aGvHD in patients suffering from this condition compared to those with a drug-related rash. Also, this biomarker gradually increases as the stage of GvHD deteriorates and its plasma levels correlate with the maximum overall grade of GvHD, making elafin a good non-invasive diagnostic and prognostic marker for skin aGvHD[94,95,101].

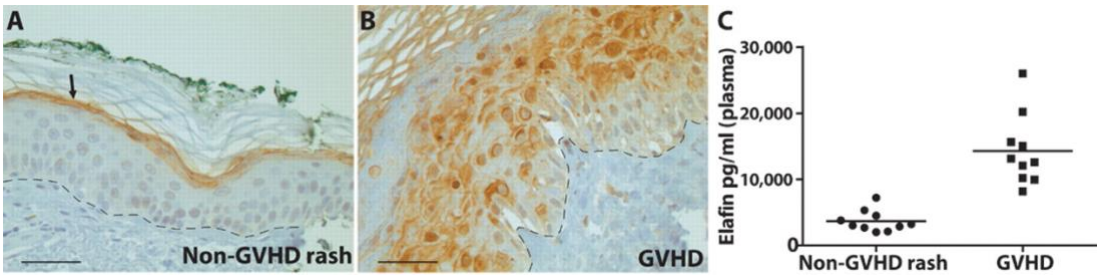


Figure 1-3: Immunohistochemical stain for elafin of skin and serum levels in patients post-HSCT (Paczesny et al, 2010). Used with permission

A study performed by Paczesny *et al* in post-HSCT patients with skin rash showed that patients with higher serum levels of elafin at diagnosis (>6000 pg/ml) were threefold more likely to die from aGvHD (26% *vs* 8%, p=0.02), had higher 1-year NRM (28% *vs* 11%, p=0.06) and lower OS (29% *vs* 53%, p=0.001) compared to patients with lower levels (<6000 pg/ml)[101]. Another study carried out in skin samples from patients with skin acute and cGvHD found a strong association between cutaneous elafin expression and prognosis: Patients with aGvHD and

higher levels of elafin had a significant decreased 2-year overall survival (*p*=0.003), and patients with chronic lichenoid GVHD were more likely to be SR (*p*=0.006) both compared to patients in similar cohorts but with lower levels of cutaneous elafin[95].

1.5.2 Regenerating islet-derived 3-alpha (REG3α)

REG3α is also a novel promising biomarker, highly accurate for lower gut GvHD. It is an antibacterial protein that binds to bacterial peptidoglycans to protect intestinal stem cells in the epithelium of the crypts. REG3α is stored in the mucus but it is found in plasma following reduction of gastrointestinal epithelial barrier and mucosal denudation, the main target of gastrointestinal GvHD[57,102,114]. Interestingly, an in vitro study showed there is an absence of intestinal REG3α during GvHD, and this can foster the intensity of this disease. However, administration of IL-22 enhances local production of REG3α, which abrogates apoptosis of Paneth cells and intestinal stem cells, and helps regenerating gut epithelium[115].

A clinical study assessing a panel of GvHD biomarkers, including elafin and REG3α, predicted higher treatment failure and mortality in patients where makers were raised at the onset of GvHD and 28 days later[94]. Besides, two other studies confirmed that high levels of REG3α in plasma at onset of gut GvHD (>135 ng/mL *vs* ≤135 ng/mL) correlated with lower response to therapy after 4 weeks (44% *vs* 21%, *p*=0.016), higher 1-year NRM (52% *vs* 33%, *p*=0.01) and lower 1-year survival (48% *vs* 27%, *p*=0.001)[102,116].

Moreover, the multicentre *Autologous Stem Cell Transplantation International Crohn's Disease (ASTIC)* trial measured plasma levels of REG3α in patients undergoing autologous HSCT for Crohn's disease to explore the role of this marker in this setting. There was a trend in the difference of REG3α levels in patients with

active endoscopic disease and those in remission (95.4 *vs* 52.4, *p*=0.052). However, REG3α was not found to be a good predictor of disease response as there was no difference between baseline levels from non-responder and responder patients one year after HSCT[117].

1.5.3 Suppression of tumorigenicity 2 (ST2)

ST2 is a member of the interleukin-1 receptor superfamily that binds to IL-33, a pro-inflammatory cytokine. There are 2 different isoforms of ST2: the trans-membrane molecule (mST2) that induces type 1 immune responses and drives T-cell alloreactivity, and the soluble form (sST2), a decoy receptor that inhibits IL-33 and subsequently mitigates inflammation[118,119].

Following conditioning or GvHD, mST2 is upregulated in CD8+ T cells while IL-33 is released by necrotic cells. To foster its pro-inflammatory effect, IL-33 synergises with other cytokines such as IL-12 or Il-18, and after binding mST2 it triggers effector CD8+ T cells that results in an inflammatory status and tissue destruction[120]. Pre-clinical studies have confirmed this, as exogenous of IL-33 can worsen GvHD, situation where sST2 increases to counteract the deleterious effect of this cytokine. Intestinal stromal cells and T cells produce sST2 during GvHD, and blocking it can attenuate GvHD and improve patients outcomes post-HSCT[121]. Conversely, administration of ST2 offsets the harmful effect of IL-33 and abrogates GvHD, confirming its immunoregulatory properties[122]. Interestingly, another immunomodulatory molecule, vitamin D, increased production of ST2 by epithelial cells and lymphocytes and enhanced the production of sST2, which again leads to attenuate IL-33[123], as also seen after administration of anti-TNFα therapy[124]. Nevertheless, under non-inflammatory conditions, IL-33 can promote the

proliferation and recruitment of gut ST2+ Treg, which attenuate macrophagic activation and abrogate lethal GvHD[125]. Interestingly, T9 lymphocytes are activated by IL-33 through mST2 but rather than injure tissues, they mitigate GvHD while simultaneously exerting a strong GvT effect[126].

In a study performed in recipients of MAC HLA-matched related HSCT, increased levels of ST2 on day 28 post alloHSCT was strongly associated with 2-year NRM (17.8% *vs* 5.2%, p=0.008)[109].

Vander Lugt *et al* carried out a study where 381 patients were divided into groups depending on plasma ST2 concentration (<740 pg/mm and ≥740 pg/mm) at day 14 after alloHSCT and GvHD grade at the initiation of therapy. Among patient with grade I or II GvHD, those with lower ST2 had a lower 6-month NRM after therapy than those with higher ST2 (11% *vs* 31%, p=0.001), similarly to patients with grade III or IV GvHD (14% *vs* 67%, p=0.001) compared to patients with similar GvHD grade but higher levels of ST2. Also, among patients with lower gut GvHD, those with lower ST2 and less severe GvHD had a higher 6-month NRM compared to patients with higher ST2 and stage 2-4 GvHD (10% *vs* 71%, p<0.001). In this study, ST2 after HSCT proved to be a better predictor of death risk compared to other risk factors. Anecdotally, ST2 concentration was up to 4-fold higher in patients who underwent MAC than RIC, probably due to a more subdued tissue damage in the latter group[96].

In the UCB setting, a study performed in 113 patients showed that ST2 levels (>33.9 ng/ml and ≤33.9 ng/ml) at day 28 post CBT correlated with the incidence of grade II-IV aGvHD, higher in patients with more severe GvHD (30% vs 13%, p=0.024). The cumulative incidence of grade II-IV acute GvHD at day 180 among patients with high ST2 levels was 66% as compared with 52% of patients with low ST2 levels

(p=0.048). Furthermore, transplant-related mortality (TRM) at day 180 post-UCB was substantially increased in individuals with high ST2 levels at day 28, being its cumulative incidence of 23% *vs* 5% in the high/low ST2 level group (p=0.001)[97]. As it happens with elafin, ST2 is independent of the GvHD grade, and has proved to be particularly useful at predicting risk of lower gut GVHD, especially when combined with REG3α (as previously described)[98].

Besides, high levels of ST2 have also been described in patients with idiopathic pneumonia syndrome[97], engraftment syndrome[118,127], and transplant-associated thrombotic microangiopathy, which could be related to an increased post-HSCT mortality[128].

1.6 Overview of Vitamin D

Vitamin D was discovered in 1919 by Professor Edward Mellanby at Sheffield University, in the United Kingdom, started off studying the effect of rickets in dogs[129,130], and afterwards this knowledge was applied into the paediatric population[131]. Over the last century, research in vitamin D has been gradually growing, allowing us to elucidate its pleiotropic functions, particularly in the field of immunology, where it has been largely studied since the 1980s[132,133].

Vitamin D is fat-soluble steroid hormone that is mainly synthesised in the skin[134], although a small portion is ingested with the diet[135]. To be activated, it needs to be hydroxylated twice, firstly in the liver by 25-hydroxylase (CYP2R1), being subsequently transformed into 25(OH)D^3 or calcidiol, and secondly in the kidney by 1α-hydroxylase (CYP27B1)[136], resulting in 1,25$(OH)_2D^3$ or calcitriol[137]. The latter is

the biologically active metabolite of vitamin D and it binds to a nuclear receptor, the vitamin D receptor (VDR), to trigger gene transcription in target cells[138].

The effect of vitamin D in mineral metabolism and bone homeostasis is well known[33,45]. However, this vitamin is also involved in infectious diseases[139], oncology[140,141], autoimmunity[142–145] and solid organ transplantation[146–148], among others.

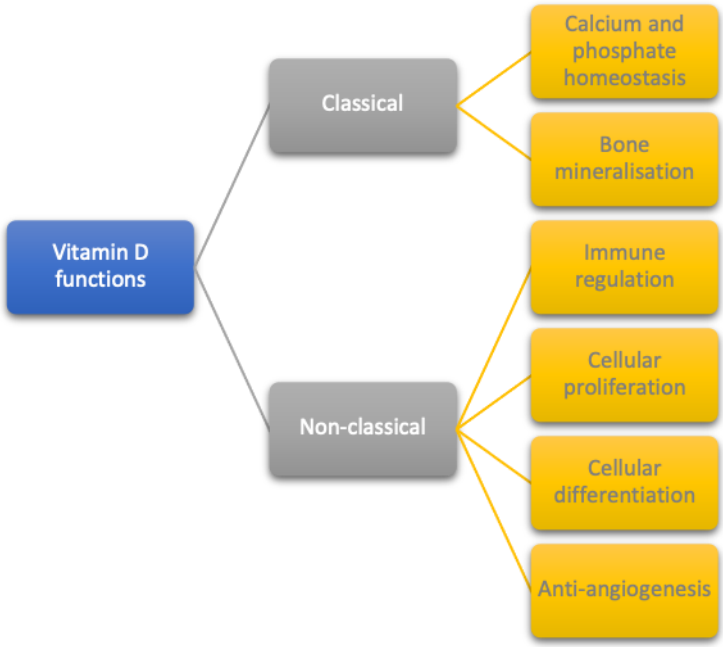


Figure 1-4: Classical and non-classical functions of vitamin D[149](awaiting publication; Open access)

1.6.1 Vitamin D in the Human Immune System

Vitamin D produces opposite effects on both types of immunity. Fostering the innate immunity while blunting its adaptive counterpart it maintains immune homeostasis and a tolerogenic status[136]. Vitamin D exerts its biological function in immune cells as they possess VDR[135,136,143,145,150], including DCs[151–153], monocytes/macrophages[133,154], NK cells[142,155], B[132,151] and T lymphocytes[133,151,156–

[158]. In addition, it can activate 25(OH)D^3 into 1,25$(OH)_2D^3$ because they synthesise 1α-hydroxylase[135,151,159,160]. See Figure 1-5.

1.6.1.1 Vitamin D in Innate Immunity

Monocytes/Macrophages

Maturation of monocytes into macrophages is enabled by 1,25$(OH)_2D^3$, that also reinforces their phagocytic function[133,159]. During infection, substances such as lipopolysaccharides or IFN-γ (interferon gamma), or microorganisms as Mycobacterium tuberculosis, increase the synthesis of 1,25$(OH)_2D^3$ through upregulation of 1α-hydroxylase, which subsequently foster the synthesis of cathelicidin, an antimicrobial peptide[135,159,161]. Furthermore, 1,25$(OH)_2D^3$ reduces the expression of MHC in the macrophages, mitigating their function as APCs and therefore blunting T cell activation[154].

NK cells

Vitamin D abrogates NK cells proliferation and synthesis of TNF-α (tumour necrosis factor alpha) and IFN-γ, mitigating their cytotoxic function[155,162].

Neutrophils

One report showed that 1,25$(OH)_2D^3$ attenuates the synthesis of IL-1b, a pro-inflammatory cytokine, avoiding its harmful effect on target tissues[163]. Also, a clinical study suggested that vitamin D could mediate in immune reconstitution after allogeneic HSCT (alloHSCT) due to the higher neutrophil recovery at day +100 of patients with higher levels of 1,25$(OH)_2D^3$ in serum[22]. However, little is known about the particular effect of 1,25$(OH)_2D^3$ in neutrophils.

Dendritic Cells (DCs)

Vitamin D can hinder antigen presentation by DCs to T-cells (and their subsequent activation) in three different ways: 1) impairing DCs trafficking from damaged tissues to lymph nodes due to the inhibition of the receptor CCR7 and its chemokine CCL2[152,153], 2) downregulating their receptors CD40, CD80 and CD86[164,165], and 3) mitigating IL-12 secretion, which blunts secretion of IFN-γ by CD4+ T-cells since they cannot be fully activated[19,164–167].

The synthesis of $1,25(OH)_2D^3$ by DCs is higher in mature DCs due to the parallel increase in 1α-hydroxylase, but the effect of $1,25(OH)_2D^3$ mainly occurs in the myeloid subset of DCs as they are involved in T cell priming[167]. Moreover, $1,25(OH)_2D^3$ fosters TGF-β secretion (transforming growth factor beta) and IL-10 by DCs[19,27,29,30], enhancing the recruitment of Treg (regulatory T lymphocytes)[88] and contributing to maintain DCs immature (and therefore keeping them in a tolerogenic state), as seen in pre-clinical[152,164,165] and clinical studies in alloHSCT patients[164,28]. Interestingly, an in vitro study showed that IDO (indoleamine 2,3-dioxygenase), an inducer of T-cell apoptosis, is upregulated in immature DCs when $1,25(OH)_2D^3$ was administrated[38], but this could not be reproduced in the clinical setting[164,169].

1.6.1.2 Vitamin D in the Adaptive Immunity

B Lymphocytes

Vitamin D inhibits the production of immunoglobulins (Ig) due to its effect on plasma cells and B lymphocytes[132,170]. These cells possess 24α-hydroxylase (CYP24A1), that enables the inactivation of $1,25(OH)_2D^3$ into calcitroic acid and contributes to eliminate it[151].

T Lymphocytes

The enzyme 1α-hydroxylase increases the local concentration of $1,25(OH)_2D^3$, which mitigates the proliferation of T and B lymphocytes and blunts T cell activation[135,158,171,172]. On the contrary, 24α-hydroxylase can decrease it and avoid toxic levels of vitamin D[159,160]. Furthermore, $1,25(OH)_2D^3$ can also impair T cells homing to gut and skin due to the downregulation of chemokine receptor CCR9 or cutaneous lymphocyte-associated antigen (CLA)[160,173].

Vitamin D inhibits the circulatory pool of **CD4+ helpers Th1 and Th17** and consequently, the production of IFN-γ[87,160,170] and IL-2[172], and IL-17[168,171], respectively. A study performed in alloHSCT recipients on vitamin D supplementation confirmed the inhibitory effect of vitamin D on T cell activation[170]. Conversely, $1,25(OH)_2D^3$ promotes the proliferation of **CD4+ Th2** cells and enhances the secretion of its hallmark cytokines (IL-4, IL-5 and IL-13)[168], shifting from a pro-inflammatory to a tolerogenic status[164]. As shown in *in vitro* and *in vivo* studies performed in alloHSCT patients, $1,25(OH)_2D^3$ supplements inhibits the proliferation of mature **CD8+**[170,173], although this has been contradicted[157]. Moreover, pre-clinical studies showed the favourable effect of $1,25(OH)_2D^3$ in the proliferation of Foxp3+ CD25+ **Treg**[87,88,171], which contribute to control peripheral T immune homeostasis[63].

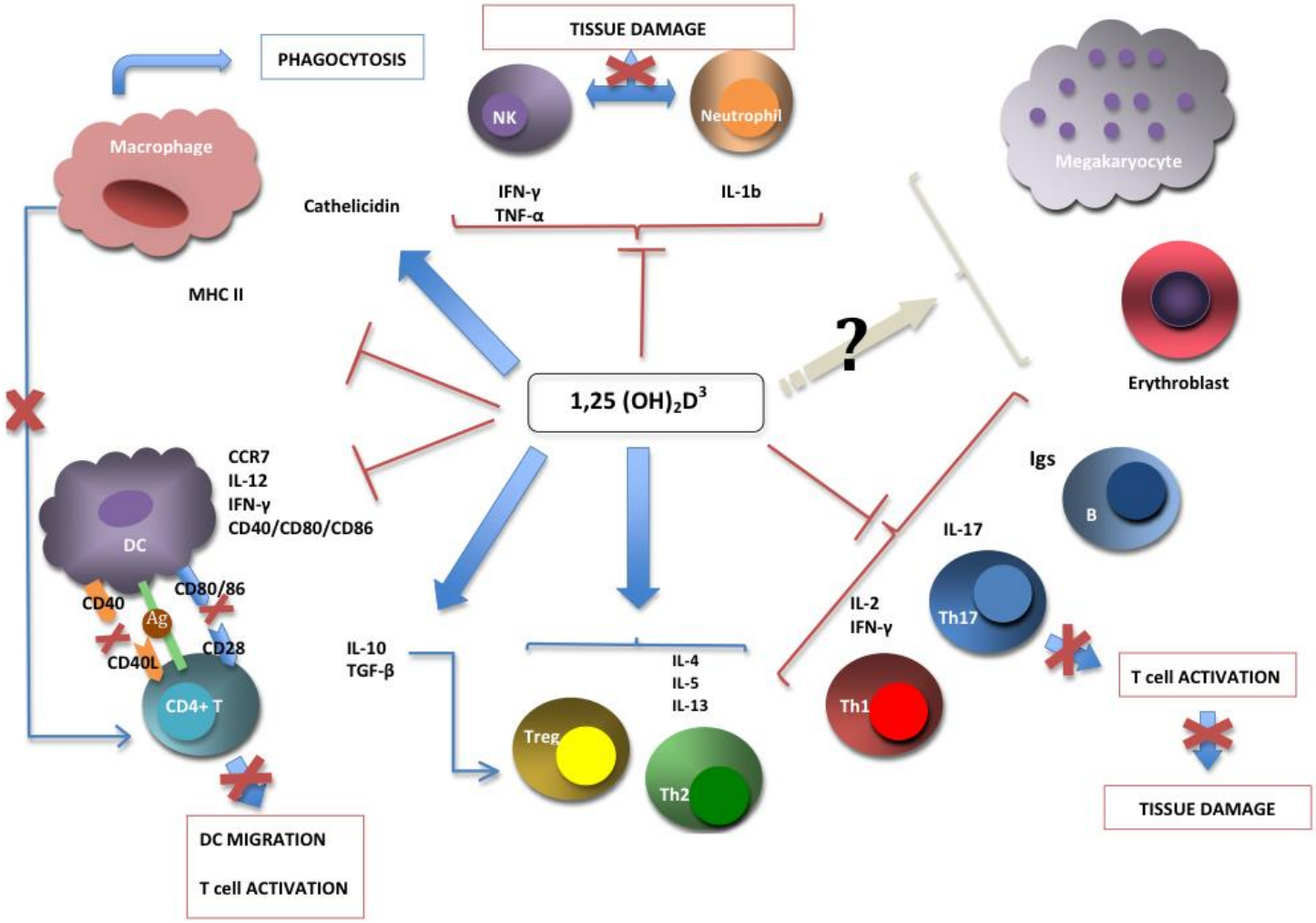


Figure 1-5: Immunomodulatory effect of vitamin D (Ros-Soto, 2018). Used with permission

1.6.2 Vitamin D deficiency

Mucositis, anorexia and low oral intake can potentially lead to nutritional deficiencies of macro and micronutrients. Among the latter, low vitamin D (insufficiency or deficiency) has been reported even before transplantation[22,174,175], which can result in serious complications that can compromise patients health[176,177].

Vitamin D deficiency has currently become a pandemic disease and can affect individuals worldwide[134,178–181]. As seeing in Figure 1-6, many different causes can predispose to it[134,179,180,182,183], but HSCT recipients are exposed to additional factors including any entity involving the gastrointestinal tract[184,185], immunosuppressive therapy[185–188] or injury of vital organs[185,187].

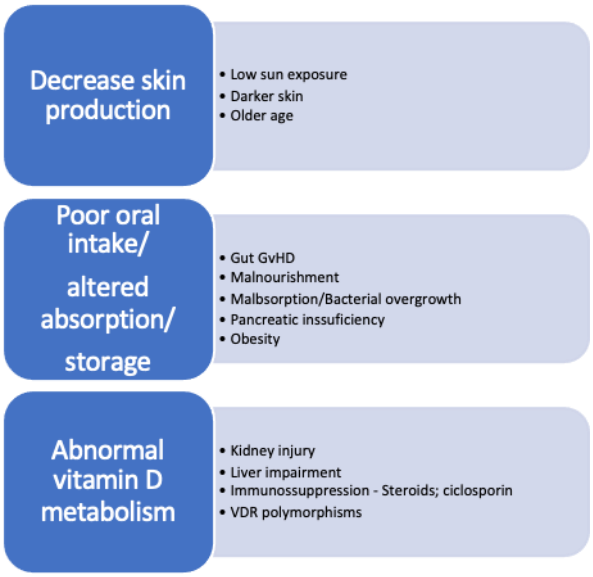


Figure 1-6: Risk factors for vitamin D deficiency

Owing to its half-life of 2-3 weeks, $25(OH)D^3$ is considered the most accurate biomarker of vitamin D metabolism, even better than $1,25(OH)_2D^3$ (the biologically active form) as it only lasts for 2-3 hours hence cannot reflect the actual body stores of vitamin D[189].

The cut-off to differentiate between individual adequacy, insufficiency and deficiency has been established based on the optimal concentration of serum $25(OH)D^3$ to maintain calcium metabolism and preserve bone health, in order to avoid rickets[187]. However, this has been subjected to controversy: in the studies performed in healthy individuals, institutions such as the *Institute of Medicine* defines vitamin D deficiency below 30 nmol/L (12ng/mL)[137] whereas the *Endocrine Society Task Force on Vitamin D* and *NICE* guidelines advocate for a lower threshold, 25 nmol/L (10ng/mL)[186,190], and even a report by Dr Holick, an expert in the field, has established it below 50 nmol/L (20ng/mL)[191].

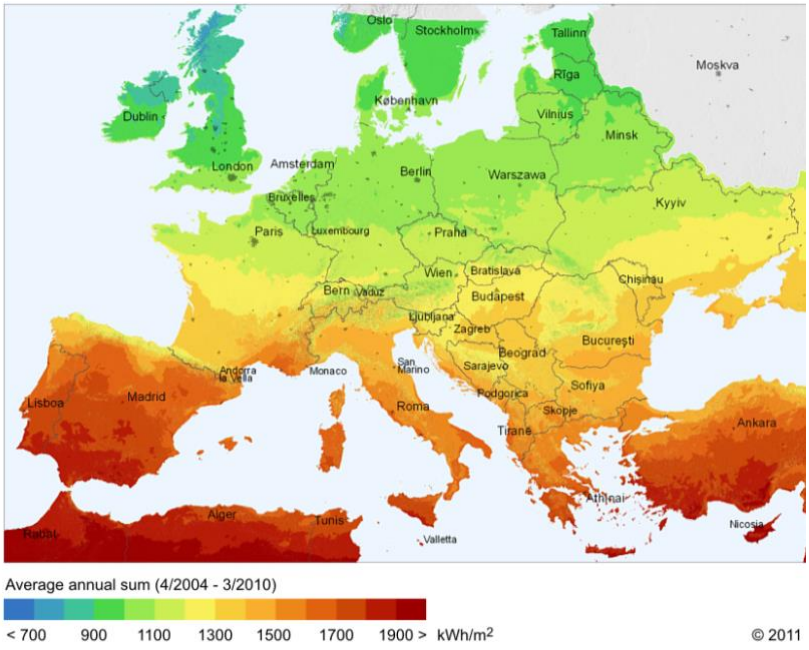


Figure 1-7: Europe average daily solar hours[192]. Used with permission

Similarly, recent publications in the HSCT setting have also shown remarkable discrepancies in this matter, and a cut-off from which vitamin D fosters immunity and consequently overcomes post-HSCT complications[135,136,143] has not been agreed yet[169,175,193,194]. See Table 1-2.

1.7 Vitamin D in Haematopoietic Stem Cell Transplantation

1.7.1 Vitamin D in Immune Reconstitution

A pre-clinical study where adult HSC were treated with $1,25(OH)_2D^3$ showed a 34% higher bone marrow recovery than non-supplemented HSC[195]. Another study reported an increased expansion and proliferation of CD34+ umbilical cells after administration of vitamin D[196]. In a clinical study, African Americans mothers were more prevalent in vitamin D deficiency and this was linked to lower CD34+ count compared to their Caucasian counterparts[197]. Besides, the contribution of donors' VDR genotype in the recovery of the T cell repertoire has been suggested in some reports[167,198], but results could not be reproduced in HSC with VDR absence[199]. Moreover, the immunoregulatory properties of $1,25(OH)_2D^3$ contribute to blunt the production of pro-inflammatory cytokines such as IL-6 (and subsequently hepcidin), leading to stimulation of erythropoiesis[200,201]. Nevertheless, its effect on thrombopoiesis has not been elucidated yet[202].

In the clinical setting, a paediatric study showed that the neutrophil count at the time of engraftment was higher in patients with higher levels of $25(OH)D^3$ than those with lower levels[22], but there has been some controversy on this matter[193,203].

So far, the evidence linking between $1,25(OH)_2D^3$ and immune reconstitution is still very limited to prevent us from drawing any significant conclusion, and further studies are warranted to characterise the role of $1,25(OH)_2D^3$ in immune reconstitution post-HSCT.

1.7.2 Role of Vitamin D in GvHD

Owing to its immunomodulatory effect, vitamin D may play an important role in the pathophysiology of GvHD. Despite the high incidence of vitamin D deficiency in alloHSCT patients[204–206], there is a dearth of clinical studies focusing in the link between the activity of serum $25(OH)D^3$ peri-alloHSCT and GvHD, with discrepancy across the different studies (see Table 1-2):

In aGvHD, Urbain *et al* showed there was a link between patients with low $25(OH)D^3$ serum levels post-HSCT and the likelihood of developing moderate to severe aGvHD[207]. This was also found by Kreutz *et al* in a study where patients with grade III-IV aGvHD were found to have lower levels of $25(OH)D^3$ than those with less severe grades[193]. Critically, other studies contradict the previous results and neither Von Bahr *et al* nor Bhandari *et al*, among others, could find an association between serum $25(OH)D^3$ and aGvHD[68,175,207].

In the chronic setting, Von Bahr *et al* showed that serum $25(OH)D^3$ at transplantation was linked to the development of cGvHD[169], as confirmed by Glotzbecker *et al,* whose study also linked vitamin D with the severity of cGvHD[175]. On the contrary, could not reproduce the previous findings[22].

As shown, there is limited data of GvHD and the potential contribution of vitamin D deficiency in its pathophysiology. Furthermore, the results are contradictory thus currently it is not possible to reach solid conclusions, stressing the need for further research to gain more insight in this topic.

1.7.3 Impact of Vitamin D on Outcomes post allogeneic HSCT

Given the immunoregulatory properties of vitamin D and its effect on diseases such as GvHD, clinical research has been carried out to elucidate its role in the post-alloHSCT outcomes, with mixed results:

Hansson *et al* performed a study in a paediatric population where OS was lower (mainly in the cohort with haematological malignancies) (50% *vs* 87%, p=0.01) and relapse rate higher (4% *vs* 33%, p=0.03) in patients with lower 25(OH)D^3 concentration pre-HSCT compared to those with higher concentration[22], which supports the hypothesis that vitamin D could have a positive impact on the biology of the primary disease. In keeping with this, Beebe *et al* reported paediatric patients with vitamin D deficiency pre-HSCT had lower 1-year OS compared to those with sufficient levels (65% *vs* 93%, p=0.001)[203]. Wallace *et al* also found that paediatric patients with severe 25(OH)D^3 deficiency 100 days after HSCT had a lower OS (p=0.044)[208]. According to the author, this could be due to the immunoregulatory properties of vitamin D and its potential favourably effect on immune reconstitution and infection prevention. Besides, Bandari *et al* correlated pre-HSCT of 25(OH)D^3 with death risk (p=0.01)[68]**.** In the adult context, Von Bahr *et al* showed patients with vitamin D deficiency had a 13% lower 2-year overall survival than sufficient patients[169]. Nevertheless, not all the studies in the HSCT population could reproduce the previous results nor correlate 2-year disease-free survival[169],

progression-free survival[175], relapse rate[170] or OS[175,209] with 25(OH)D^3 status. See Table 1-2.

Interestingly, Hansson *et al* reported a borderline evidence between graft rejection and vitamin D deficiency pre-HSCT (0% *vs* 11%, p=0.06)[203], but this link was not found between 25(OH)D^3 status an another severe condition, veno-occlusive disease (VOD)[68].

1.7.4 Effect of the Vitamin D Receptor in HSCT

Chromosome 12 contains the VDR gene[210]. Specific single nucleotide polymorphism (SNPs) in this gen can downregulate its activity, including *Apa*I AA, which consequently impacts on immune reconstitution of specific lymphocyte subsets post-HSCT. On the other hand, SNPs such as *Apa*I aa and *Fok*I FF enable VDR upregulation[198,211]. Interestingly, VDR SNPs can modulate serum levels of 25(OH)D^3 after supplementation, which can also be contributed by SNPs in CYP2R1 gene[212,213]

Recent publications have reported the link between polymorphisms in the VDR gene and different outcomes after alloHSCT. However, results have been inconclusive[16,142,145–149] thus this merit further research to fully characterise thec contribution of SNPs in VDR gene in the field of alloHSCT, particularly in haploidentical or unrelated donors.

Author/s	Study design	Age	N	VDD cut-off	Incidence VDD	Overall Survival	aGvHD	cGvHD	Comments
Joseph *et al* (2011)[174]	Prospective	Adult	72	<20 ng/mL	At day 0: 70% At day +100: 58%	NR	NR	NR	
Von Bahr *et al* (2015)[169]	Retrospective	Adult	166	< 25 nmol/L	Pre-HSCT: 11% Post-HSCT: NR	Decreased 2-year OS in VDD patients (63%) compared to sufficient VD patients (76%) *(P* = 0.03*)*	No association with $25(OH)D^3$ serostatus	Strong correlation (RR 2.66) with $25(OH)D^3$ serostatus	VDD pre-HSCT was associated with increased CMV disease (*P= 0.005*) No association with 2-year DFS
Glotzbecker *et al* (2013)[175]	Retrospective	Adult	53	<25 ng/mL	Pre-HSCT: 60% Post-HSCT: NR	VD serostatus did not impact OS	No significant differences	2-year CI 63.8% in VDD patients compared to 23.8% in sufficient VD patients (*P* = 0.02) Extensive cGvHD at 2-year was 54.5% in VDD patients compared to 14.3% in sufficient VD patients (*P* = 0.009)	No association with PFS
Hansson *et al* (2014)[22]	Prospective	Paed	123	<50 nmol/L	Pre-HSCT: 69% Post-HSCT: NR	Lower in patients with VDD + malignancies (50%) compared to VD sufficient patients (87%) (*P* = 0.01)	More frequent in patients with sufficient VD (47%) compared to VDD patient 30% (*P* = 0.05)	No significant differences	Relapse rate higher (33%) compared to sufficient VD levels (4%) (*P* = 0.03) No significant association with CMV and EBV reactivation
Kasiani *et al* (2016)[194]	Retrospective	Paed	64	<30 ng/mL	Pre-HSCT: NR Post-HSCT: 73%	NR	NR	NR	
Simmons *et al* (2013)[182]	Prospective	Paed	22	<15 ng/mL	Pre-HSCT: 27% Post-HSCT: NR	NR	NR	NR	

Florenzano *et al* (2016)[214]	Retrospective	Adult	46	<20 ng/mL	Pre-HSCT: 17% Post-HSCT: 85%	NR	NR	NR	53% of patients on VD supplements (but not an interventional study) 36% autologous HSCT and 64% allogeneic HSCT
Wallace *et al* (2015)[208]	Prospective	Paed	135	<20 ng/mL	Pre-HSCT: NR at day +100: 23%	Lower OS in VDD*** (*P* = 0.044)	No significant differences	No significant differences	16% patients on supplements pre HSCT
Sproat (2011)[215]	Retrospective	Adult	58	<20 pg/mL	Pre-HSCT: NR / Post-HSCT: 59%	NR	NR	NR	21% of patients on VD supplements
Kreutz *et al* (2004)[193]	NR	NR	48	<25 nmol/L	Serum levels of $25(OH)D^3$ - pre-HSCT: 36.4+/-2.2nmol/L - Post-HSCT: 27.8+/-1.3 nmol/L	NR	Lower serum levels of $25(OH)D^3$ in grade 3 and 4 (*P* =0.031)***	NR	
Urbain *et al* (2012)[207]	Prospective	Adult	102	<10 ng/mL	Pre-HSCT 23.5% Post-HSCT: NR	NR	Trend of aGvHD if lower levels of $25(OH)D^3$ on day+100 (*P*=0.066)	NR	
Bhandari *et al* (2019)[68]	Retrospective	Paed	136	<20 ng/mL	Pre-HSCT 61%	Every 10ng/mL increase in pre-HSCT VD was associated with 28% decrease in death risk (*P*=0.01)	No significant differences	NR	No significant differences between VDD, insufficient and sufficient group and the incidence of SOS

*** Number patients affected has not reported (NR).

Table 1-2. Observational studies measuring vitamin D serostatus in alloHSCT (modified from Ros-Soto el al, 2018, with permission)[216]

1.8 Conclusion

Among its pleiotropic effects, vitamin D has immunoregulatory properties that contribute to enhance protection against encountered microorganisms while abrogating autoimmunity, thus maintaining immune homeostasis. Multiple factors make HSCT recipients at higher risk of vitamin D deficiency (even prior to stem cell infusion) and this can impact adversely over the course of HSCT, as outlined in this chapter. Owing to the lack of consensus, a cut-off to define vitamin D deficiency has not been established yet and clinical practice in the field of transplantation may change across different centres. To address this, chapter 2 will examine current management of vitamin D deficiency in alloHSCT units.

Acute and chronic GvHD are the main cause of morbimortality post-alloHSCT. Currently, diagnosis can be delayed and a diagnostic test to confirm or rule it out may take up to several days or weeks. GvHD biomarkers are promising diagnostic tools that can speed up this process, predict outcomes in the early post-HSCT phase and monitor response to immunosuppression in order to minimise the detrimental effect of this therapy. For this, chapter 3 will explore the role of vitamin D and GvHD biomarkers measured in subsequent timepoints in patients with *de novo* and SR GvHD, in the context of an observational study.

Lastly, as part of the immunotherapy used to prevent or treat disorders after HSCT, the last chapter will describe the experience of a single centre in treating patients with DLI for MC or relapse disease, examining factors impacting on achievement of FDC, survival and GvHD post-DLI.

2 Current Practice in Vitamin D Management in Allogeneic Haematopoietic Stem Cell Transplantation: A Survey by the Transplant Complications Working Party of the EBMT

2.1 Introduction

As described in Chapter 1, vitamin D exhibits a variety of immunological effects over the course of HSCT[22,169,203,216,217]. In the setting of vitamin D deficiency, dysregulation of immune balance favours a pro-inflammatory status that may lead to auto-immune diseases[144,145]. Many risk factors that cause and contribute to this have been identified, especially low sun exposure, since the skin is the main manufacturer of this vitamin[184,185]. Particularly in HSCT recipients, this can be aggravated due to prolonged hospitalisations and compromised nutritional status[204–206].

Vitamin D deficiency is not only diagnosed among inhabitants of higher latitudes, but also in those living in equatorial regions[134,178–180]. It is a major health concern and research has been carried out to determine its actual prevalence and the potential risks to human health[134,178–181].

Despite the adverse effect of vitamin D deficiency on post-HSCT outcomes[22,169,203], publications providing recommendations for risk assessment of comorbidities before

HSCT do not mention monitoring serum levels of 25(OH)D^3 prior to this procedure[218,219]. Interestingly, clinical guidelines encourage maintaining therapeutic levels of 25(OH)D^3 following HSCT to preserve bone mineralisation and avoid osteoporosis and bone fractures[176,177,206]. However, they do not take into account the role of vitamin D in immune homeostasis and the potential detrimental effect of vitamin D deficiency in HSCT recipients[22,169,175,203,217]. Similarly, a literature review about the optimal nutritional support in patients with lower gut GvHD suggested vitamin D replacement in this population[204]. The reason for this was to counteract bone demineralisation secondary to steroids, but yet again the authors did not comment on the immunoregulatory properties of 1,25$(OH)_2D^3$.

Moreover, we previously commented on studies performed in steroid-refractory asthma and its link with vitamin D serostatus[87–90]. Hypothetically, 1,25$(OH)_2D^3$ could play an important role in the response to immunosuppressive therapy in patients with GvHD, but this has not been investigated yet. Thus, the effect of vitamin D therapy in this setting merits further investigation.

So far, only two previous studies have surveyed the awareness of healthcare professionals in vitamin D deficiency: one in the UK, among primary care physicians and midwifes[220], and another in Belgium, among primary care physicians[221]. Nonetheless, this type of study has never been run across allogeneic HSCT (alloHSCT) units, and therefore the current management of vitamin D deficiency in the alloHSCT community has never been addressed.

2.2 Study hypothesis and objectives

To date, there are no clinical guidelines focusing on vitamin D status and its optimal levels required for prevention of autoimmune diseases post-transplantation and enhancement of immunosuppressive therapy. Consequently, vitamin D deficiency is under-diagnosed and the number of patients who could potentially benefit from supplementation is unknown.

To better define the current understanding of the management of vitamin D deficiency in alloHSCT patients, we conducted an online survey on behalf of the Transplant Complications Working Party across the European Society for Blood and Marrow Transplantation (EBMT) affiliate centres. This survey aimed to describe the current clinical practice in Europe to acknowledge possible discrepancies across the HSCT Programmes, considering their geographical location (as the quantity of sunlight varies across countries) as well as their membership to the European Union.

This study was presented as a poster at the EBMT conference in Frankfurt (2019) and subsequently, it was published in the journal *Biology of Blood and Marrow Transplantation*[222].

2.3 Material and methods

A total 326 EBMT affiliate centres with adult or paediatric alloHSCT programme from 42 countries eligible to take part in this survey were selected from the EBMT registry. AlloHSCT programme directors were invited to complete this survey, or delegate to a healthcare professional member of staff directly involved in patients

care. The study protocol, study questionnaire and letter of invitation were attached to the email. The questionnaire comprised 34 questions divided in different categories, including diagnosis, prescription of vitamin D replacement or follow-up. Each EBMT centre was only allowed to participate in the survey once.

This study was carried out from September to November 2018, and reminders were sent out to centres who had not responded every 3 weeks. Consequently, data was analysed descriptively.

2.4 Results

Demographics

A total of 326 EBMT affiliate centres that perform alloHSCT were invited to participate in this survey. Amongst them, 114 centres from 24 countries returned the questionnaire. Centres characteristics and their location are displayed in Table 2-1 and Figure 2-1, respectively.

Since cutaneous synthesis of vitamin D differs based on the latitude individuals inhabit, centres were classified depending on their location from 50 degrees latitude: above this latitude, inhabitants receive less than 1,800 hours of sunlight per year while below it the number of sunlight hours are greater than 1,800 yearly[223]. Then, we decided to explore the impact of this geographical location in our study: Fifty-two centres (46%) from 11 countries (*northern* countries) are located above and 62 centres (54%) from 13 countries (*southern* countries) below this landmark. Furthermore, 58% (n=66) are adult-only centres, 21% (n=24) paediatric-dedicated centres and 21% (n=24) *mixed* centres, those treating both adult and paediatric

patients. At the time of the survey, 96 centres (84%) were members of the European Union.

Patient group	Response *(%)*
Adult only	58
Paediatric only	21
Adult and paediatric	21
Location	
European	87
Non-European	13
Latitude	
Northern	46
Southern	54
Number of alloHSCT	
<500	35
500-1000	37
1000-1500	13
>1500	15
Centres performing alloHSCT since	
<15 years	8
15-25 years	21
>25 years	71
JACIE accreditation	
Accredited	43
Accreditation in progress	25
No	32
Type of HSCT performed	
Identical Sibling	45
Unrelated (matched and mismatched)	40
Haploidentical	6
Other relative (syngeneic, matched and mismatched)	6
Umbilical cord blood	3
Gross National Income	
High	91
Middle	9

Table 2-1: Characteristics of the participant centres[222]. Used with permission

Only 19% of the centres followed any local or national guidelines. Among them, centres mentioned the *French Paediatric Society Guidelines, Swiss Guidelines* or *Lombardian Regional Statement* (Italy). Furthermore, 18% followed international guidelines such as the *National Institute for health and Care Excellence* (NICE), *UK Osteoporosis, Up-to-date recommendations* or the *Dietary Reference Intake* from the *Institute of Medicine of the National Academy of Sciences*. In most of the mixed centres (67%), the care provided is similar in both adult and paediatric units and therefore they follow the same guidelines for management of vitamin D deficiency.

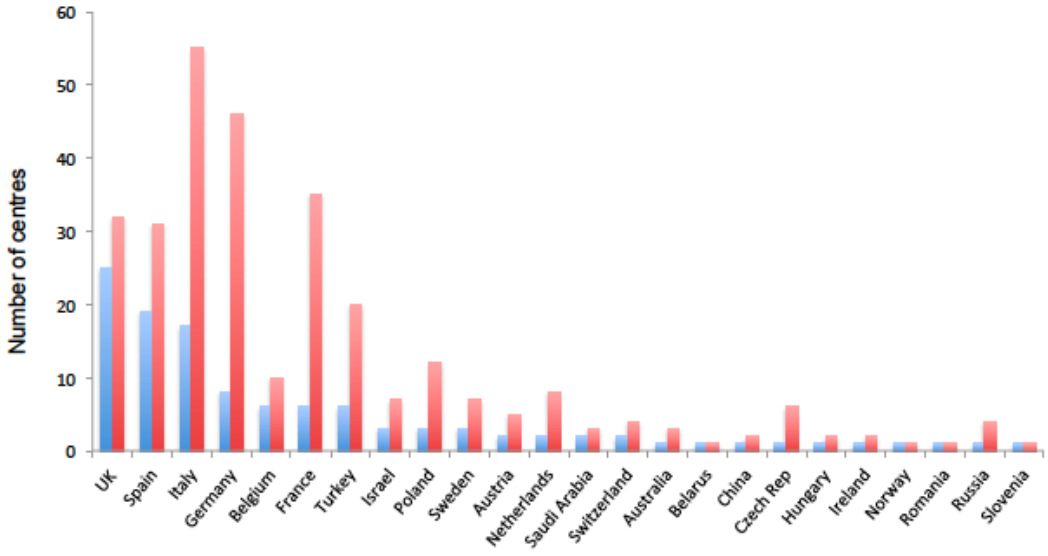


Figure 2-1: Number of centres participating in the survey (blue bar) compared to the total of EBMT centres that performed alloHSCT per country (red bar)[222]*. Used with permission*

Monitoring of vitamin D

Serum 25(OH)D^3 is regularly measured by 47% of the centres before alloHSCT (Figure 2-2). Thirty-seven percent check it in all patients but 10% only in those with risk factors for vitamin D deficiency/insufficiency. Nevertheless, it doubles after alloHSCT and nearly 70% of the centres monitor serum 25(OH)D^3 routinely (Figure 2-3). Fifty-three percent measure it in all patients but 17% only in patients with risk factors for vitamin D deficiency/insufficiency. Following HSCT, serum 25(OH)D^3 is monitored every 3 months (39%), every 6 months (24%), once a year (18%) or at other time-points (19%). The majority of the centres (94%) do not consider seasonality important while monitoring after HSCT.

	Pre-HSCT ***N (%)***	**Post-HSCT** ***N (%)***
Osteopenia/Osteoporosis	108 (94)	100 (86)
Treatment with steroids	86 (75)	78 (68)
Previous fracture	86 (75)	81 (71)
Premature menopause	64 (56)	52 (46)
Established menopause	57 (50)	36 (32)
Total body irradiation	8 (7)	*NR*
Low vitamin D	7 (6)	*NR*
Other*	*NR*	7 (6)

* Risk of avascular necrosis of the femur, breastfeeding, total parenteral nutrition; *NR* = no reported

Table 2-2: Centres responses according to clinical indications to request serum vitamin D in patients undergoing HSCT (several answers were possible) [222]*. Used with permission*

Prescription of vitamin D replacement

Vitamin D replacement was mainly prescribed by transplant physicians (75%), primary care physician (10%), clinical nurse specialists (3%), endocrinologists (3%), other specialist physicians (rheumatologist, gynaecologist and physiatrist) (4%). Some centres (5%) did not prescribe it as it is an over-the-counter drug. Vitamin D was prescribed alone (48%) or in combination with calcium carbonate (52%).

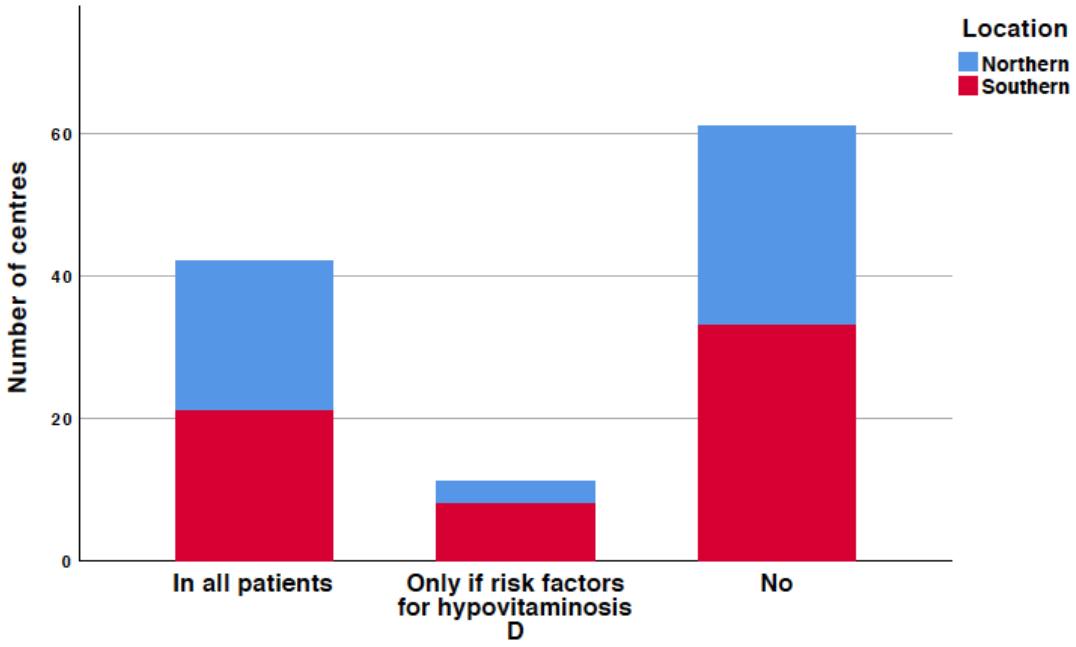


Figure 2-2: Proportion of centres measuring serum vitamin prior to HSCT depending on location[222]. Used with permission

Eighty-three percent of centres prescribed vitamin D replacement based on a specific cut-off for serum 25(OH)D[3]. This varied greatly across centres: ≤ 25 nmol/L (26%), ≤ 30 nmol/L (28%), ≤ 50 nmol/L (37%), ≤ 75 nmol/L (7%) and ≤ 100 nmol/L (2%) (see Figure 2-4).

In northern countries, centres used a median threshold of 50 nmol/L while in those in southern countries the median was 30 nmol/L.

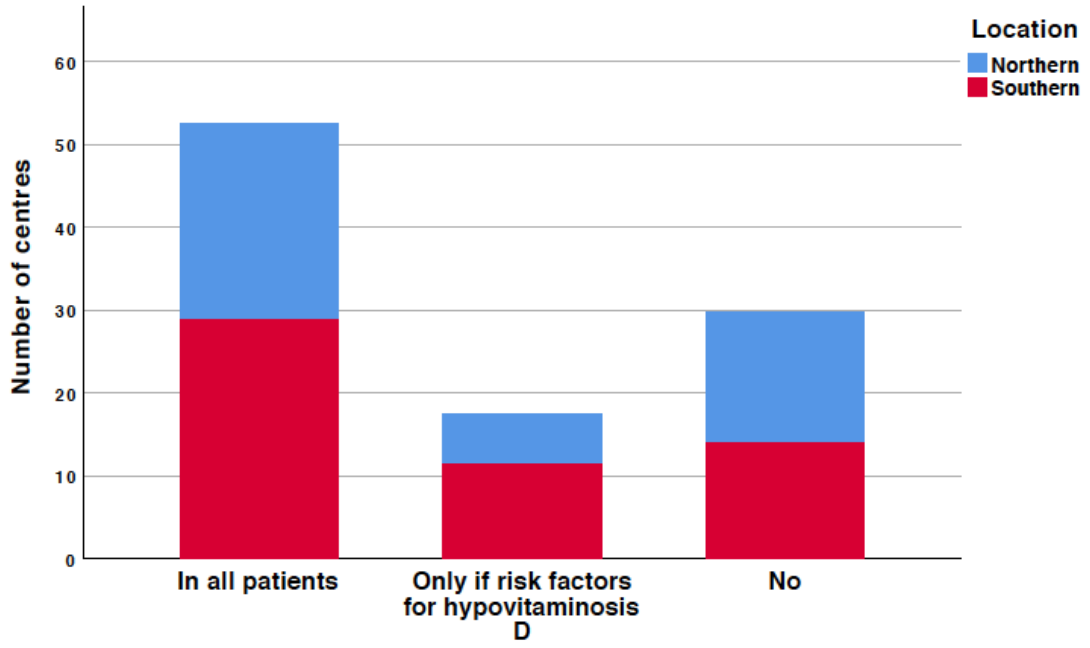


Figure 2-3: Proportion of centres measuring serum vitamin after HSCT depending on location[222]. Used with permission

The main criteria to prescribe vitamin D replacement have been depicted in Figure 2-5.

As part of the treatment course for vitamin D deficiency, only one third of centres (33%) included “loading dose” of vitamin D. Among the responders, 89% provided the loading dose prescribed, being 2,000 IU per day (286 - 20,000) the median loading dose and 6 weeks (1-52) its median duration.

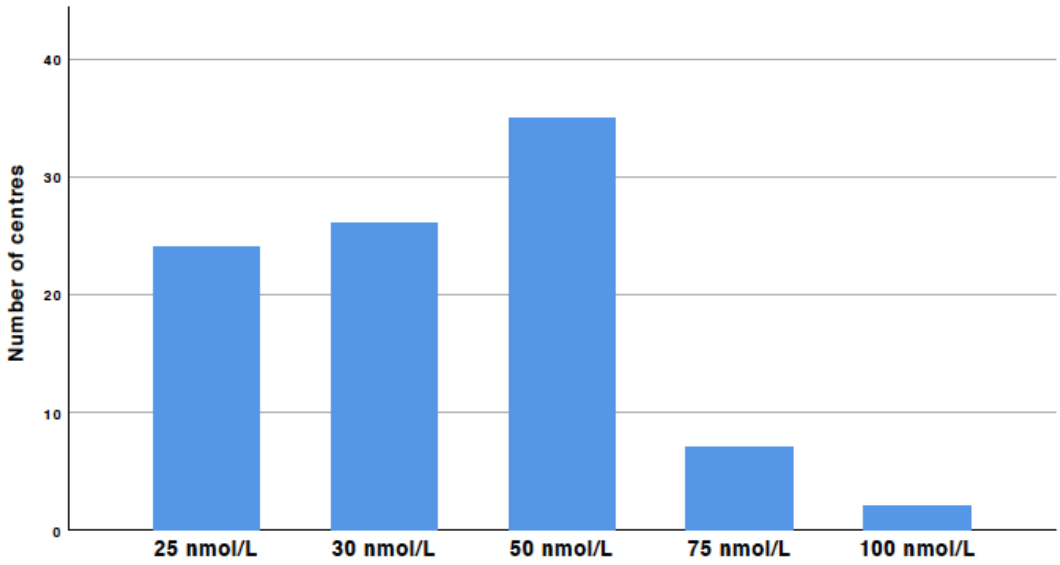


Figure 2-4: Cut-off value for vitamin D deficiency[222]. Used with permission

The majority of centres (98%) prescribed “maintenance” or long-term treatment for vitamin D deficiency. This was provided by 88% of them: 800 UI (67 – 10,000) was the median daily maintenance dose prescribed (see Figure 2-7).

Replacement therapy was discontinued by 69% of the centres based on the following criteria: when serum 25(OH)D^3 reaches therapeutical levels (59%), when DEXA (dual-energy X-ray absorptiometry) scan is reported as normal (12%), when patients report clinical improvement (9%), all of the aforementioned criteria (9%) or other (when growth stops in paediatric patients, after discontinuing immunosuppression or after completing 1 year of treatment) (11%).

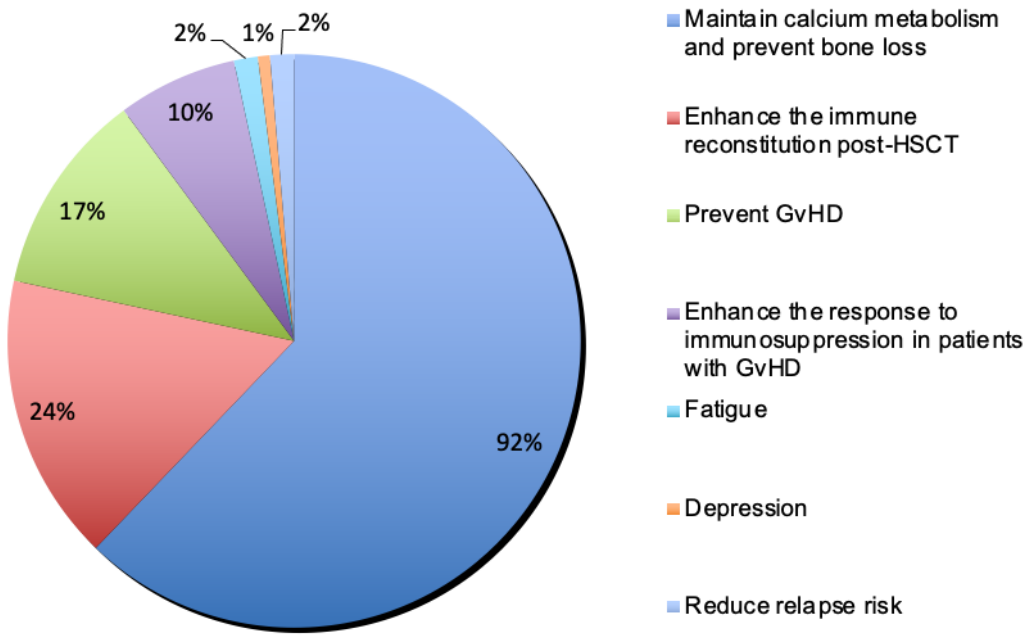


Figure 2-5: Centres responses according to aim for prescribing vitamin D replacement in HSCT patients (several answers were possible)

Follow-up

Most of the patients are followed up at their transplant centre (89%), by the primary care physician (1%) or a mixed model (10%) (See Figure 2-6). Follow-up is usually life-long (57%) its length may vary: between 5 and 10 years (21%), less than 5 years (6%), more than 10 years (4%), until paediatric patients transition to adult team occurs (8%) or other type of follow-up programs (4%).

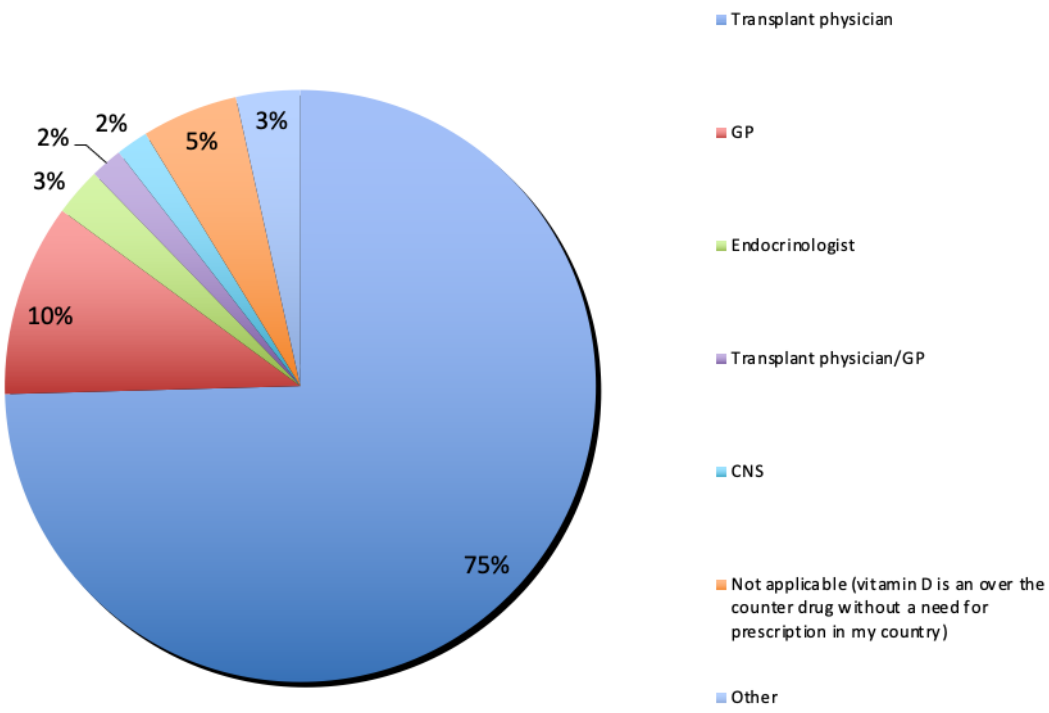


Figure 2-6: Healthcare professionals carrying out follow-up of HSCT recipients

Sixty-nine percent of the transplant centres reported to refer patients routinely to a dedicated osteoporosis service. This occurred in the majority of adult (74%) and mixed centres (79%), but only in half of the paediatric units (48%). Eighty percent of centres monitor bone density as part of the post-HSCT follow-up with a DEXA scan (48% in high-risk patients for osteopenia/osteoporosis and 52% in all of them).

The main criteria to request a DEXA scan are osteoporosis (13%) and osteopenia (87%). If DEXA scan shows an abnormal result, 79% of centres prescribe vitamin D replacement. Most of the centres (78%) repeat DEXA scan after the first test:

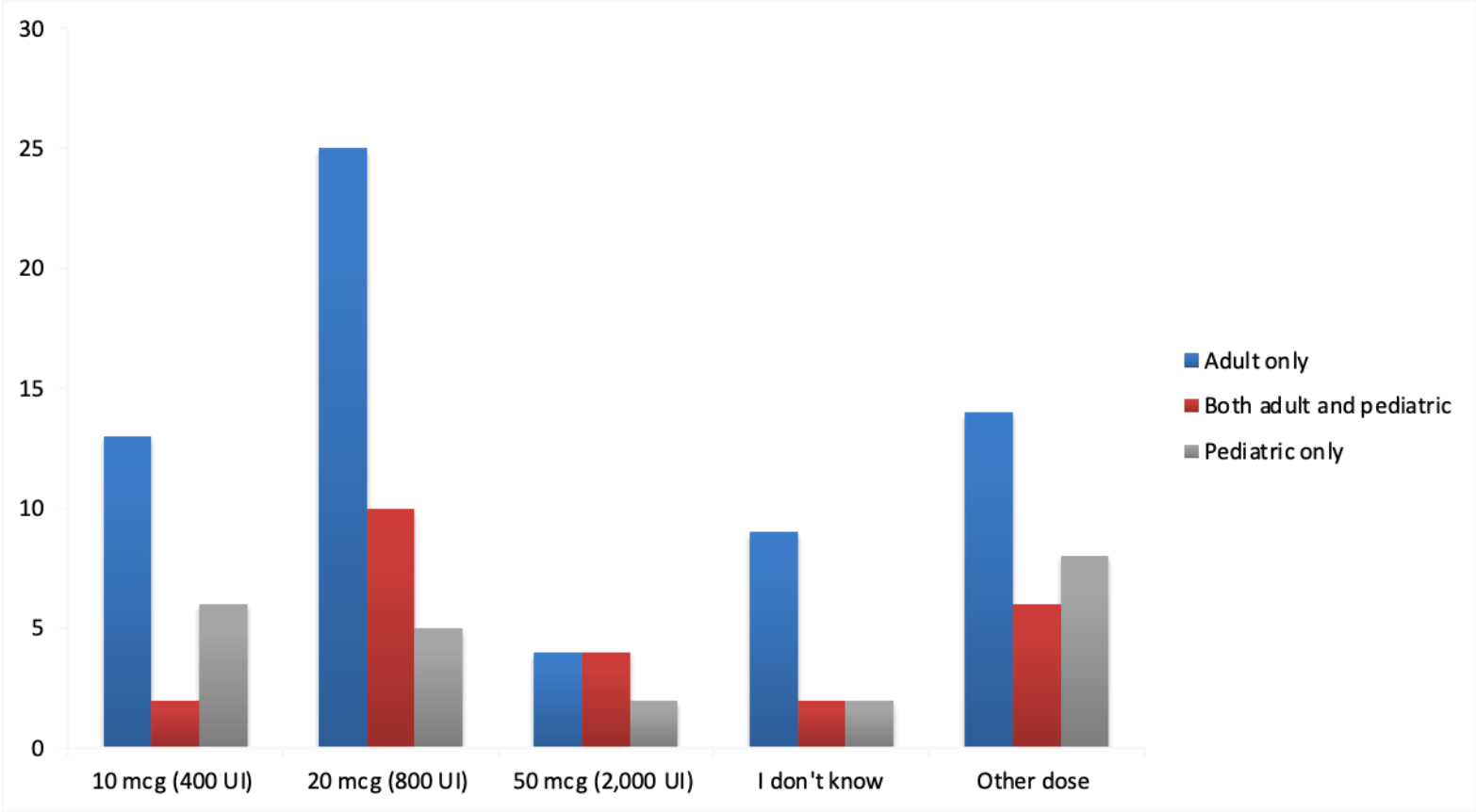


Figure 2-7: Daily dose of vitamin D

Once a year (40%), every 5 years (19%), depending on the results of the last DEXA scan (28%) and the rest used different time-points (13%). DEXA scan is discontinued when it normalised (56%), when it stabilised (33%) or when bone density increased (11%). The health insurance from most countries (92%) cover for this test.

2.5 Discussion

Nearly half of the centres do not request 25(OH)D^3 as part of the pre-HSCT screening, probably because the HSCT clinical guidelines do not encompass any recommendations regarding the measurement of it prior to HSCT[218,219]. Nevertheless, more than 70% of the centres request 25(OH)D^3 following HSCT. This is in keeping with HSCT guidelines, where suggestions include the monitoring of serum 25(OH)D^3 as part of the post-HSCT follow-up in order to prevent bone disease that can lead to bone fractures[176,177,206].

According to the vast majority of the responders (92%), the main clinical indication to start of vitamin D therapy is maintaining bone and mineral metabolism, as recommended in the aforementioned guidelines, in conjunction with calcium and phosphate in serum[176,177,206]. However, they do not mention the frequency or the best time of the year for monitoring it, hence it is not surprising that nearly all the institutions (96%) do not take this into account. Pro-inflammatory cytokines such as IL-1 and TNF-α decrease during the sunnier months due to the increase of the production of vitamin D in the skin, in comparison to the darker months[224]. This is another example of the immunoregulatory effect of vitamin D[135,136,143] and the impact that seasonality has on the concentration of 25(OH)D^3 in serum[134]. Owing to the role of vitamin D in immune homeostasis[22,144,145,169,203,216,217], it is encouraged to monitor

25(OH)D^3 over the course of alloHSCT to treat patients at risk of vitamin D deficiency.

The contribution of vitamin D within acute or chronic GvHD has been a matter of debate[22,68,169,175,193,207]: As previously discussed in Chapter 1, the deficit of vitamin D has been linked to both forms of GvHD in some studies[169,175,193,207] whereas others could not confirm this[22,68,175,207]. Furthermore, little is known of the effect of 1,25$(OH)_2D^3$ on immune reconstitution following HSCT[167,195–199]. Therefore, it is not surprising that only a few centres (17% and 24%, respectively) contemplate vitamin D as a contributor to both phenomenon. Moreover, the effect of vitamin D on the clinical response to immunosuppression has been reported in patients with auto-immune disease such as asthma[87–90], but it has not been explored in the context of HSCT, hence it seems reasonable that only a minority of the responders (10%) considered this as an indication to commence on replacement therapy. The lack of strong evidence in this matter certainly warrants further research to correlate the impact of 1,25$(OH)_2D^3$ with post-HSCT outcomes. This could be achieved with interventional studies using vitamin D treatment, including large sample sizes and serial measurement of 25(OH)D^3 before and after HSCT.

As previously mentioned, the main circulating metabolite of vitamin D metabolism is 25(OH)D^3, as it reflects the overall vitamin D produced in the skin and absorbed with diet[184,185,189]. There is still discrepancy regarding the ideal threshold of serum 25(OH)D^3 to define vitamin D deficiency[137,169,175,186,193,194]. In this study, responses have been heterogenous and depending on the centre, this cut-off varies from 25 to 100 nmol/L. The reason for this is unknown, since less than 20% of centres follow local, national or international guidelines to make their decision. HSCT guidelines that recommend monitoring and replacing vitamin D post-HSCT do not provide

guidance regarding a specific cut-off for vitamin D deficiency[218,219]. Clinicians may have been guided by recent publications in the HSCT setting, where cut-off ranges from 20 to 50 nmol/L[169,175,193,194]. Discrepancy in this matter is also found among institutions as the *NICE* guidelines[186] in the UK or *Institute of Medicine* in the USA[137] (as we previously discussed in Chapter 1). In this survey, most of the centres used 50 nmol/L as the most frequent cut-off for vitamin D deficiency, as has been recently reported in the literature in paediatric[22,217,225] and adult[215,226] alloHSCT recipients. Moreover, the concept of vitamin D *insufficiency* has also been described in some of the aforementioned publications: whereas *NICE* guidelines specify it as the concentration of $25(OH)D^3$ between 26 and 50 nmol/L[186], Dr Holick uses the range between 50 and 75 nmol/L[191].

Anecdotally, we found that some cut-offs were more prevalent depending on the geographical location of the HSCT units: those in southern countries had a trend towards lower thresholds (≤ 30 nmol/L) compared to those in northern countries (≥ 50 nmol/L), despite the fact that it does not always agree with the recent literature[169,170]. Again, the reason for this is unknown although this could be partially explained by recommendations provided by local/national guidelines in those centres that follow them. Current controversy stressed the need for further studies to validate an evidence-based cut-off for vitamin D deficiency in HSCT recipients and treat these patients accordingly.

In healthy individuals, “loading dose” is considered a short-term treatment with high-dose $1,25(OH)_2D^3$ replacement for vitamin D deficiency, while “maintenance dose” is a long-term course of low-dose supplementation prescribed for vitamin D insufficiency[186,190]. In this study, only 33% of centres prescribed loading dose for patients with vitamin D deficiency. In fact, there are no studies performed in

alloHSCT recipients on loading dose of vitamin D, but a report performed in the general population showed promising conclusions[227]. Therefore, future studies are needed to establish the role of the loading dose in the HSCT setting.

Most institutions (98%) supplement patients with maintenance dose of vitamin D, and the median daily dose of vitamin D is 800 IU. Over the last years, prescription of vitamin D supplements has raised worldwide due to an increased awareness of its favourable effects on health and its negligible toxicity[186,228]. In studies performed in healthy individuals, dose varies from 400 to 4,000 IU[185,186,229,230]. In contrast, higher dose is prescribed in HSCT recipients, ranging from daily 1,000 IU to weekly 600,000 IU[170,203,209,225,226,231–233] (see Table 2-3). HSCT patients are at high risk of vitamin D deficiency and potentially to osteopenia and osteoporosis[142,204,206] as a result of the many additional risk factors[186,187,204] that can potentially contribute to lower the concentration of $1,25(OH)_2D^3$ further (including poor compliance to treatment[209]), hence a higher dose of vitamin D replacement therapy is necessary[142,187]. Thus, intensive treatment with vitamin D replacement to fully replenish levels of vitamin D is justified in HSCT patients.

Interventional studies in the alloHSCT setting have followed different strategies depending on the age group investigated: in adults, the dose of vitamin D is usually fixed (following the concept of “one-size-fits-all”)[170,233]. However, in paediatric patients dose is weight-adjusted[170,203,209,217,225,226,231–233], which seems a more accurate approach as otherwise patients actual vitamin D requirements might be underrated (see Table 2-3). Thus, treatment with vitamin D is strongly recommended in HSCT patients diagnosed of vitamin D insufficiency or deficiency, particularly in those with additional risk factors. Ideally, a loading dose pre-HSCT should be administrated, followed by maintenance therapy during the 6-12 months

post-HSCT, although clinical trials to tailor the ideal treatment dose are need, particularly in the adult population.

Interestingly, only a few institutions considered starting on vitamin D therapy to prevent relapse of the primary disease. Reports from pre-clinical studies have shown that $1,25(OH)_2D^3$ inhibits cell proliferation, angiogenesis and triggers apoptosis in tumoral cells[37,60,99–101]. Apart from its effect on solid tumours[136,234–236], *in vitro* studies have shown its anti-tumoral effect has also been studied in multiple myeloma, myelodysplastic syndrome[237] and leukaemia[237,238]. Despite higher serum levels of $1,25(OH)_2D^3$ have a favourable impact on survival[141,236,239,240], results are contradictory in observational studies in the setting of lymphoproliferative disorders[141,241–243]. Therefore, vitamin D therapy is not currently indicated for eradication of the primary disease and prevention of relapse.

In keeping with published guidelines[176,206], the majority of centres have an established pathway for follow-up in osteoporosis services to carry out DEXA scan and prevent osteopenia/osteoporosis in long-term survivors post-HSCT. However, treatment with vitamin D is mainly stopped depending on serum $25(OH)D^3$ rather than DEXA scan, according to most of the responders.

One of the main strength of this survey is that it has been performed by highly experienced HSCT specialists, with a broad experience in assessment and follow-up of HSCT patients. Moreover, this study encompasses many different topics regarding vitamin D deficiency in the context of HSCT (including awareness of this disorder by healthcare professionals, diagnosis, follow-up and current vitamin D therapy).

Nevertheless, one of the limitations of this study is the voluntary nature of reporting without further validation, which can impact on the reliability of the information provided. Also, despite the restorative effect of $1,25(OH)_2D^3$ in immune homeostasis, its role in different aspects of HSCT, such as immune reconstitution or GvHD, has not been fully characterised yet. Thus, this can be the cause of the variations reported across the different HSCT units and may mislead clinicians when implementing the management of vitamin D deficiency in their day-to-day clinical practice. Although this is one of the largest surveys published recently on behalf of the EBMT[244,245], only 1/3 of the centres invited responded to the questionnaire (most of them from high-income countries) which could not reflect accurately the real clinical practice. Since no other surveys have been performed looking into the management of vitamin D deficiency in the HSCT setting, this paves the way for future research in this particular field.

2.6 Conclusion

This study has demonstrated the variations in the management of vitamin D deficiency across international adult and paediatric alloHSCT programmes due to the lack of consensus in the HSCT community. Some recommendations have been provided to standardise criteria and harmonise the management of the aforementioned vitamin. Although there is no agreement in the optimal serum $25(OH)D^3$ level required to foster the immune protective effect of vitamin D to abrogate the detrimental impact of its deficiency on post-HSCT outcome, regular measurement of $25(OH)D^3$ before and after HSCT is strongly recommended, as well as commencing on vitamin D therapy when clinically indicated, as part of the standard of care of HSCT recipients.

Author/s	Age population	N	VDD cut-off	Intervention	Incidence VDD	OS	aGvHD	cGvHD	Comments
Caballero-Velázquez et al (2016)[170]	Adult	150	<50 nmol/L	Control (CG) – no treatment Low dose (LD) – 1,000 IU RT daily High dose (HD) - 5,000 IU RT daily From day -5 to day +100	NR	No significant differences	No significant differences	Lower CI at 1-year of overall and moderate + severe cGvHD after RT in LD (37.5% and 19.5%) and HD (42.4% and 27%) compared to CG (67.5% and 44.7%), respectively ($P < 0.05$)	No significant differences in relapse and non-relapse mortality
Duncan et al (2011)[225]	Paediatric	67	<20 ng/mL	50,000 IU of ergocalciferol weekly for 6 weeks following HSCT	Pre-RT 37.3% Post-RT 36.7%*	NR	NR	NR	*Only 22 patients had VD levels tested after treatment
Wallace et al (2016)[231]	Paediatric	75*	<20 ng/mL	G1: 2,000-8,000 IU/day G2: 15,000-100,000 IU/week From before HSCT to day +30 post-HSCT	Pre-HSCT: G1:51%-G2: 48% Post-HSCT (day +30) G1: 57% G2: 36%	NR	NR	NR	*10 patients underwent autologous HSCT
Robien (2012)[226]	Paediatric + adult	95**	<50 nmol/mL	200-1,000 IU/day (duration NR) in long-term post-HSCT patients	Post HSCT 11%	NR	No significant differences	No significant differences	**Only 59% were on RT and they had higher VD levels (94 nmol/L) compared to those not on RT (65.2 nmol/L) ($P = 0.001$)

Silva et al (2011)[233]	Adult	12	NR	Patients with active cGvHD on ≥ 1st line IS + RT due to bone disease. Compared to CG of 24 patients with cGvHD on 1st line IS but not on RT	NR	NR	NR	50% study cohort stopped IS after 6 months on RT (5 CR, 6 PR, 1 no response) compared to 20% of CG	Retrospective study
Campos et al (2014)[209]	Paediatric	66	<20 ng/mL	All patients received 400 to 800 IU/day of RT during hospitalization, and 39 (59%) after discharge for an average of 140 days	Pre HSCT: 32% At day +180: 51%	No significant differences	No significant differences	No significant differences	Poor treatment compliance justifies higher VDD post HSCT
Wallace et al (2018)[232]	Paediatric	10	<20 ng/mL	Single ultra-high dose based on body weight and VD levels pre-HSCT was administrated prior to day 0 (maximum 600,000 units)	Serum levels of $25(OH)D^3$ pre-HSCT: 28.9 ± 13.1ng/mL Serum levels of $25(OH)D^3$ post-HSCT: 80.4 ± 28.6 ng/mL	NR	NR	NR	All patients achieved therapeutical VD levels
Beebe et al (2017)[203]	Paediatric	72	<20 ng/mL	RT provided to 46 patients	Pre HSCT: 28% Day +100: 27%	Lower 1-year OS in VDD (65%) compared to VD sufficiency (93%) pre-HSCT (P = 0.001)	No significant differences	No significant differences	On day +100 N=62 Higher rate of viral infections in patients with sufficient VD levels (93%) compared to low (65%) (P = 0.001)

*** Number patients affected no reported (NR)

Table 2-3: Interventional studies measuring vitamin D in alloHSCT (modified from Ros-Soto el al, 2018, with permission)[216]

3 Exploratory study of the Impact of Vitamin D in the Response to Immunosuppression in Patients with Graft-versus-Host disease following Allogeneic Haematopoietic Stem Cell Transplantation or Donor Lymphocyte Infusion

3.1 Introduction

Graft-versus-host disease (GvHD) is an unpredictable and potentially debilitating complication of allogeneic haematopoietic stem cell transplantation (alloHSCT)[70]. Acute GvHD (aGvHD) pathophysiology is characterized by strong inflammatory reaction[57] while chronic GvHD (cGvHD) shares features of autoimmunity[50,64]. As we commented in Chapter 1, the relationship between vitamin D and GvHD has received considerable attention in recent years[169,175,193,207]: the detrimental effect of vitamin D deficiency in immune homeostasis can lead to expansion of donor immunocompetent T cells, cytokine dysregulation and subsequent recipient tissue damage[246,247]. Despite a number of studies have been investigated this association, results are still inconclusive[22,68,169,175,193,207].

3.1.1 Steroid resistance

Steroids are the up-front treatment for acute and chronic GvHD (cGvHD) due to their immunosuppressive effect[47,76,248]. However, less than 50% of patients will respond to them, requiring stronger immunosuppression with a subsequent increase

in morbidity and mortality[83,84]. Amongst the different therapies indicated in steroid-refractory GvHD (SR GvHD), ECP is an immunomodulatory treatment that has proved to increase the pool of circulating regulatory T cells (Treg) with a concurrent improvement of GvHD symptoms[249]. However, studies looking into the contribution of vitamin D in the pathophysiology of SR GvHD have not been performed as yet, thus its impact on the response to further immunosuppressive therapy (IST), including ECP, remains unknown.

We have previously discussed that a number of studies performed in patients with asthma confirmed the link between serum $25(OH)D^3$ and clinical response to steroids: As a reminder, Xystrakis *et al* and Nanzer *et al* showed that treatment with vitamin D improved clinical outcomes in steroid-refractory asthmatic patients[87,90]. To be consistent with this, another similar study correlated higher serum levels of $25(OH)D^3$ with an increased number of Treg[89]. The rationale behind this could be that steroids are unable to induce IL-10 secretion by CD4+ T, abrogating subsequent Treg recruitment[168]. Further research in this field has found that Th17, a pro-inflammatory T cell subset, can become refractory to glucocorticoids if they possess the P-glycoprotein MDR1+ (*multi-drug resistance type*), which hampers the inhibition of IL-17, IL-22 or IFN-gamma while on this immunosuppression and perpetuates tissue injury[250]. Supplementation with $1,25(OH)_2D^3$ can restore the secretion of IL-10 by CD4+ T cells, incrementing peripheral Treg and contributing to disease control[88,89].

In the context of SR GvHD[251], the combined effect of vitamin D therapy with steroids can exert an anti-inflammatory synergic effect that mitigate tissue damage after hampering the secretion of inflammatory cytokines[252] (such as TNF-α) by monocytes[253]. Nonetheless, there is a lack of clinical trials and interventional studies

in the GvHD setting, hence further investigation is required to characterise the effect of vitamin D therapy in this field and elucidate whether this can contribute favourably on patients' outcomes.

3.1.2 Extracorporeal Photopheresis

There is no standard second line treatment for patients who fail to respond to steroids[45,47,76,77]. One of the current approaches that has been used for this purpose is ECP[254,255]: this a cell-based immuno-modulatory therapy that separates leukocyte-enriched plasma from peripheral blood and exposes these cells to ultraviolet A (UVA) radiation after administration the photosensitiser agent 8-methoxypsoralen (8-MOP), and then return the cells back into the patient[256]. Indications for ECP encompass patients with erythrodermic cutaneous T-cell lymphoma (CTCL)[256], aGvHD refractory to steroids and calcineurin inhibitors (specially in those with skin aGvHD) and cGvHD with skin, oral or liver involvement[254,255]. In GvHD, its actual mechanism of action is far from being understood, but in CTCL 8-MOP binds to leukocytes DNA resulting in cell apoptosis. After re-infusion, patient's APCs carry out phagocytosis of cell detritus, presenting antigens and subsequently activating immunocompetent cells that will ultimately target the presented molecules[254]. In addition, ECP has proved to expand Treg, fostering its immunomodulatory effect[249].

The rate of response of patients with GvHD to ECP ranges from 20% to 80%, but in order to attain complete response (CR) of GvHD ECP should be performed twice or three times per week[249,255]. ECP has a favourably side effect profile (fever, nausea and headache) without an increased risk of infections in patients with GvHD, alongside an improvement of quality of life[255].

3.1.3 GvHD biomarkers

GvHD biomarkers are non-invasive tests that can predict prognosis and therapeutic response, stratifying patients into risk categories to tailor immunosuppression accordingly[100]. GvHD is diagnosed clinically, ideally supported by biopsy of the involved tissue, although histological confirmation can be challenging[47]. The analysis of these markers allows repetitive measurement with serial blood samples in a short timeframe, simplifying the current GvHD diagnostic pathway[94,109].

The literature review in Chapter 1 showed a number of studies carried out in this field. Moreover, in the SR GvHD setting, Barker *at al* analysed 3 different biomarkers for aGvHD (elafin, ST2 and REG3α) in 20 patients with SR cGvHD prior to starting on ECP. This study revealed that all biomarkers were significantly increased in patients compared to controls. In addition, elafin and ST2 correlated with the NIH skin scores in patients with skin involvement (r=0.7, p=0.0012, and r=0.52, p=0.019, respectively), and supported a relationship between 25(OH)D^3 with ST2[257]. Despite these findings, these molecules have only been applied into the context of aGvHD, and specific biomarkers for cGvHD have not been validated yet in clinical trials because of the inaccuracy of these molecules[258].

As previously discussed, some biomarkers enable the classification of clinical outcomes independently: ST2 can stratify patients' risk of non-relapse mortality (NRM) without considering clinical status of GvHD[258]. Furthermore, combination of biomarkers such as ST2 and REG3α can also create and grading that can predict long-term outcomes in patients with aGvHD[98,103].

There is strong evidence to support the role of elafin, ST2 and REG3α as validated biomarkers for GvHD[98,100,103,257,258]. However, their analysis is laborious and

experience is lacking in many centres in the UK. However, the ECP unit at Rotherham General Hospital has been studying them for a number of years and they were willing to participate actively in this research project.

3.1.4 Response endpoints in GvHD studies

Complete remission (CR) has been defined as the complete resolution of GvHD signs and symptoms[259,260]. A clinical study in patients with newly diagnosed aGvHD reported that day 28 after commencing on IST with steroids can be a valid endpoint to assess early response to treatment[261]. This time point was also found to be an early predictor of survival post-HSCT, including 2-year transplant-related mortality (a better outcome than overall survival (OS) since relapse can impact detrimentally on the latter[100,261]). However, another study in a similar cohort concluded that this time point could not predict accurately long-term outcomes because cGvHD may also occur at some point and this can influence on survival[259]. Moreover, *durability of response* has also been evaluated as a long-term endpoint of interest[260].

Factors associated with successful response to IST at day 28 after initiating treatment include HLA-matched sibling donor and absence of gut or liver GvHD. In addition, higher GvHD grades and older age at transplant can also have a negative impact on response to treatment[259].

Early identification of failure to GvHD therapy is essential to find strategies promptly, tailoring the treatment to decrease therapy-related toxicities and improve outcomes in high-risk patient.

3.2 Gap in the knowledge and study rationale

Vitamin D has a fundamental role in immunity and therefore in stem cell transplantation. Its deficiency can contribute to GvHD but, surprisingly, vitamin D supplementation has not become part of the current standard of care in HSCT recipients yet.

In disorders such as asthma, patients deficient in vitamin D had an improved clinical response to steroids after supplementation with this vitamin[87,90]. This is probably due to the immunoregulatory properties of vitamin D[150], that enhance the immunosuppressive effect of steroids. Nevertheless, studies correlating the serostatus of vitamin D with the response to IST (including steroids or ECP) in patients with GvHD have never been carried out, hence the potential benefit of replacement with vitamin D in this setting remains unknown.

So far, clinical trials performed in patients with aGvHD on steroids have shown that day 28 post-treatment is the best time point to assess the *initial response to therapy*, whereas *durability of response* has been better evaluated later (3 and 6 months post-treatment)[260]. Studies with GvHD biomarkers have also shown that day 28 post-treatment can assess properly the response to therapy in patients with acute GvHD[94,97]. The levels of biomarkers at this endpoint correlated with the activity of the disease (CR or non-CR) and OS[259]. Nevertheless, the applicability of GvHD biomarkers beyond this time point has not been investigated yet.

Most published studies in the field of GvHD biomarkers have involved patients with aGvHD. There is limited data on cGvHD (specially in patients on IST, such as ECP[257]), and whether biomarkers are as relevant as in the acute setting requires

further research. It is therefore important to understand their role in this group of patients. The pathophysiology of GvHD following DLI is similar to post-HSCT[35], hence the interest to explore whether GvHD biomarkers could be used for diagnosis and/or follow-up of GvHD in the context of DLI.

In addition, the relationship between vitamin D and ST2 has been described[123,257] but whether it is associated with other biomarkers remains unknown. This could strengthen the evidence of the contribution of vitamin D in the pathophysiology of GvHD and encourage replacement with vitamin D to improve GvHD outcomes.

3.3 Study overview

I approached centres with Transplant Programme and/or ECP units via email/telephone, and four centres accepted to participate in this study. Study sites were the Royal Marsden Hospital NHS Foundation Trust, the King's College Hospital NHS Foundation Trust, the Royal Hallamshire Hospital (Sheffield Teaching Hospitals NHS Foundation Trust) and the Rotherham General Hospital NHS Foundation Trust. Patients with clinical diagnosis of *de novo* aGvHD, or those with steroid-refractory (SR) acute or chronic GvHD that were deemed candidates to second line treatment for GvHD following alloHSCT or donor lymphocyte infusion (DLI) were identified by the HSCT clinician / CNS during the Transplant Clinic at each site. Patients who fulfilled the inclusion criteria were invited to participate in the study and provided with the Patient Information Sheet. If they accepted, the consent form was signed thereafter. I also attended the Transplant Clinic at the Royal Marsden Hospital and participated actively in this process. There were two control groups: one with healthy staff from the Anthony Nolan charity, and another with patients from the Royal Marsden Hospital on the day +28 following allogeneic HSCT

(named *healthy HSCT controls*). The latter was used to explore whether immune reconstitution could impact on the levels of vitamin D and/or GvHD biomarkers. I emailed all the AN staff to participate in the study (attaching the Patient Information Sheet), and selected them from the pool of volunteers, ensuring a variety of age and race to decrease selection bias. They were consented before the blood sample was drawn. Recruitment of patients on the day +28 post-HSCT occurred as described for patients with GvHD. For both cohorts of controls only one baseline sample was required. Recruitment period encompassed 18 months, from October 2017 to March 2019, with an additional 6-month period to collect remaining follow-up samples, completing the study at the end of September 2019.

All participants deemed eligible by the responsible HSCT physician had a 5 ml clotted blood sample drawn either at diagnosis of aGvHD (*de novo* aGvHD cohort), or prior to start on ECP as second-line treatment (SR acute and cGvHD cohorts), alongside other blood samples requested as part of their standard of care. For each population, consecutive samples were drawn 1, 3 and 6 months after the baseline (See Study flowchart). These were taken and stored by the Research nurses at each study site.

The 5 ml blood sample was drawn in a “yellow top” serum gel tube and labelled with the patient unique study ID (in place of patient name) and date of the sample at the time points previously described. This unique ID was made up with letters and numbers: ‘H’ for centre (H1=Royal Marsden Hospital, H2=Royal Hallamshire Hospital, H3=Rotherham General Hospital, H4=Anthony Nolan and H5=King’s College Hospital) and ‘S’ for each subject (S01, S02, etc). The whole blood study samples were transferred to the local laboratory where they were centrifuged for 7 minutes at 3500 rpm and serum was aliquoted into fresh cryotubes and frozen at

-80^{o}C in the study specific freezer area until the study was completed. Then, study samples were shipped from the study centres inside a thick-walled polystyrene box with dry ice to the Photopheresis Laboratory at Rotherham General Hospital for analysis of 25(OH)D^{3} and GvHD biomarkers (ST2, elafin and REG3α). Laboratory work was performed from November 2019 to February 2020.

The study was conducted in accordance with the study protocol, and standards of good clinical practice and national and local regulatory requirements. Research and development approvals were sought at each centre before patient recruitment.

See appendix 2 for patient information sheet, consent form and notice of research ethics committee approval.

3.3.1 Study sponsorship, insurance and ethical approval

The study was sponsored and insured by University College London (UCL). Ethical approval was granted on the 8th September 2017 by the NHS Health Research Authority (HRA) National Research Ethics Service at all the sites involved in the study (appendix 2).

3.3.2 Study objective and endpoints

Primary Objective:

To determine whether vitamin D contributes to the responsiveness to steroids in *de novo* aGvHD, or to ECP in SR acute and cGvHD in adult recipients of allogeneic HSCT/DLI.

Secondary Objectives:

i) To confirm whether GvHD biomarkers are useful in early diagnosis of aGvHD
ii) To determine whether GvHD biomarkers can be used for monitoring therapy response and long-term follow-up in patients with acute or cGvHD on IST
iii) To determine whether vitamin D and/or GvHD biomarkers impact on survival in patients with acute and/or cGvHD on immunosuppression
iv) To determine whether there is a relationship between serum vitamin D and GvHD biomarkers (in *Supporting data*)

Primary endpoint

Difference in 25(OH)D^3 in *responders* (patients who achieved CR of GvHD-related symptoms at 1 month post-treatment) *vs non-responders*

Secondary endpoints

i) Difference in 25(OH)D^3 and GvHD biomarkers at baseline between patients and controls
ii) Difference in 25(OH)D^3 and GvHD biomarkers at 1, 3 and 6 months compared to baseline (day 0)
iii) Difference in 25(OH)D^3 and biomarkers between survivors and deceased
iv) Correlation between 25(OH)D^3 and GvHD biomarkers at baseline, 1, 3 and 6 months post-treatment (in *Supporting data*)

3.3.3 Study participants

3.3.3.1 Inclusion and exclusion criteria

Inclusion criteria

- Aged 18 and over
- Patients with clinical diagnosis of aGvHD (according to *Modified Seattle Glucksberg criteria*[51]) following alloHSCT/DLI who require treatment with topical, oral or intravenous steroids
- Patients with clinical diagnosis of SR aGvHD (according to *Modified Seattle Glucksberg criteria*[51]) or cGvHD (according to National Institute of Health (NIH) scoring system[262]) following alloHSCT/DLI who are candidates for ECP or other immunosuppression
- Deemed eligible to become part of the study and sign consent form

Exclusion criteria

- Any patient with new diagnosis of cGvHD (including overlap syndrome)
- Any patient in whom steroids or ECP are contraindicated
- Any patient in whom biopsy has ruled out diagnosis of GvHD

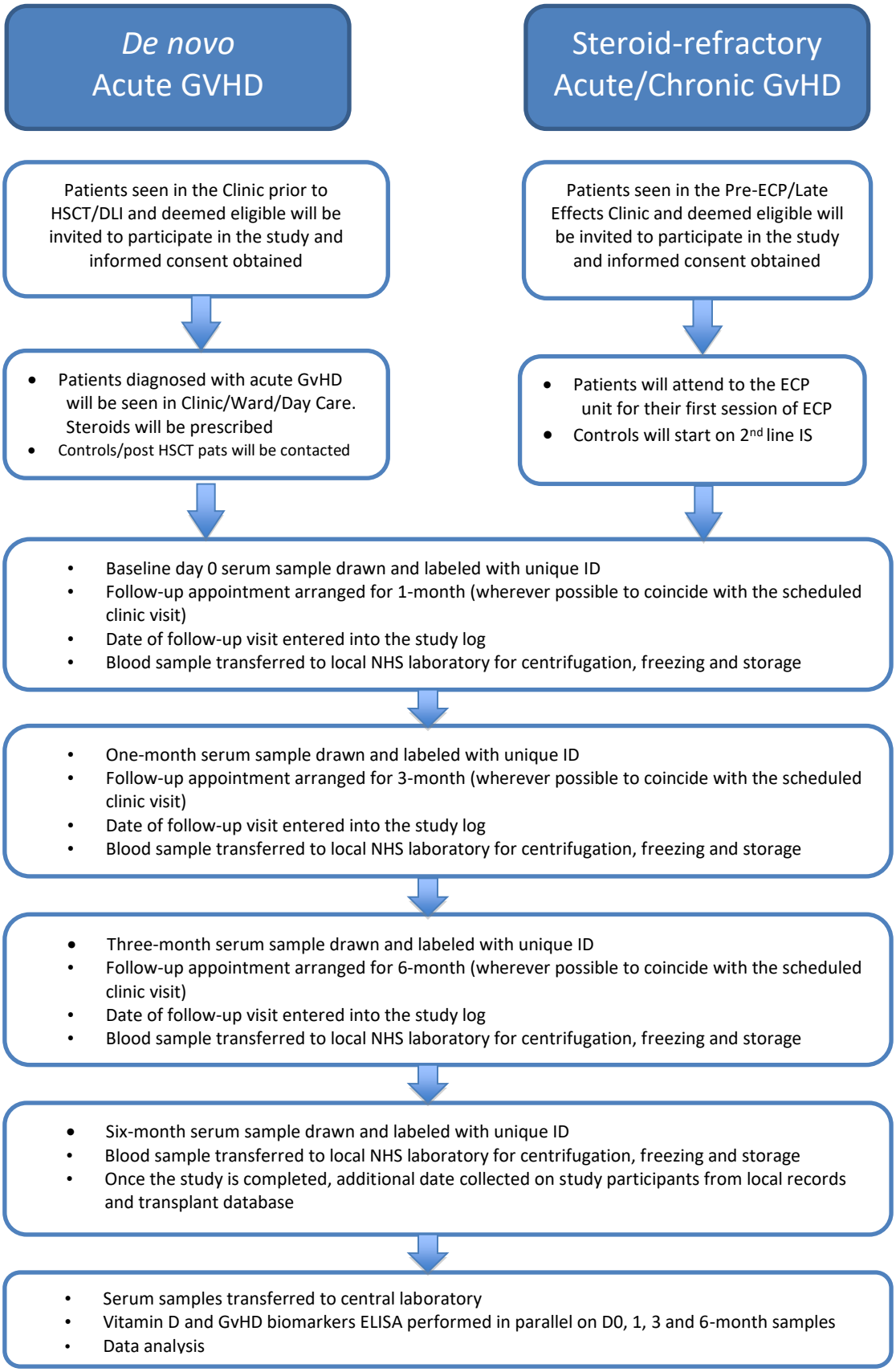
3.3.4 Participant Informed Consent, Registration, Confidentiality and General Ethical Considerations

All patients were provided with the Patient Information Sheet (PIS) and were advised to ask any questions regarding this study. Written informed consent was obtained prior to undergo collection of the first blood sample. Patients were informed that their participation in the study was voluntary, hence they were free to withdraw

at any time without impacting on their medical care. The consent form also recorded whether blood samples and data could be kept and used for future studies. For each patient, the original copy of the signed consent form was retained by the Investigator in the site file. See appendix 2 to find consent form and PIS.

On the study registration log or other documents, patients were identified by their local hospital ID and a study number only. Documents which contain the patient's full name (limited to the consent form) were kept in strict confidence by the Investigators at their centres.

3.4 Study flowchart



3.5 Material and Methods

3.5.1 Solution, reagents and antibodies used for vitamin D analysis

Solution/Reagents	Company	Location
Solid Phase Reagent: mouse monoclonal anti-fluorescein-coated paramagnetic particles in BSA (buffer bovine serum albumin)	Siemens Healthineers	Munich, Germany
Ancillary Well Reagent: Vitamin D-analog conjugated to fluorescein and 1-anilinonaphthalene-8-sulfonic acid in BSA		
VD Calibrator: low or high levels of 25(OH)D^3 in buffered, defibrinated human plasma with BSA		
Ancillary Pack Reagent: releasing agent in buffered saline with sodium azide and stabilizers		
Wash Buffet: Phosphate-buffered saline (PBS) with sodium azide and surfactant		
VD diluent: Phosphate buffer with BSA, cholesterol and sodium azide		

Antibodies	Company	Location
Lite Reagent: Mouse monoclonal anti-VD antibody labeled with acridinium ester in buffer with BSA, mouse IgG and sodium azide	Siemens Healthineers	Munich, Germany

3.5.2 Plastic materials used for ELISAs (Trappin-2/Elafin and ST2)

Plastic material	Company	Location
96-well microplates	R&D systems	Minneapolis, MN
Plate sealers	R&D systems	Minneapolis, MN
50mL tubes conical tubes	Fisher scientific	Massachusetts, MA
Reagent reservoir/solution basin	Starlab	Milton Keynes, UK
1.5mL microcentrifuge tubes (Eppendorf)	Fisher scientific	Massachusetts, MA
Pipettes tips	Starlab	Milton Keynes, UK

3.5.3 Solutions, reagents and antibodies used in preparation and execution of Trappin-2/ Elafin and ST2 ELISAs

Solution/Reagents	Company	Location
Wash Buffer: 0.05% Tween-20 in Phosphate Buffered Saline (PSB)	R&D systems	Minneapolis, MN

Reagent Diluent (1% Bovine Serum Albumin (BSA) in PBS)	R&D systems	Minneapolis, MN
Recombinant Human Trappin-2 Standard		
Colour Reagent A (H_2O)		
Colour Reagent B (tetramethylbenzidine)		
Stop Solution (sulphuric acid)		
Streptavidin-HRP 1:200 (horseradish peroxidase)		
Streptavidin-HRP 1:40 (horseradish peroxidase)		
Recombinant Human ST2 Standard		

Antibodies	Company	Location
Goat Anti-Human Trappin-2 Capture Antibody	R&D systems	Minneapolis, MN
Biotinylated Goat Anti-Human Trappin-2 Detection Antibody		
Mouse Anti-Human ST2 Capture Antibody	R&D systems	Minneapolis, MN
Biotinylated Goat Anti-Human ST2 Detection Antibody		

3.5.4 Plastic materials used in REG-3 alpha assay

Plastic material	Company	Location
96-well microplates	MBL International	Woburn, MA

Plate sealers	R&D systems	Minneapolis, MN
50mL tubes conical tubes	Fisher scientific	Massachusetts, MA
Reagent reservoir/solution basin	Starlab	Milton Keynes, UK
1.5mL microcentrifuge tubes (Eppendorf)	Fisher scientific	Massachusetts, MA
Pipettes tips	Starlab	Milton Keynes, UK

3.5.5 Solutions, reagents and antibodies used in preparation of REG-3alpha assay

Solution/Reagents	Company	Location
Coating Buffer – Carbonate buffer solution (ready-to-use)	MBL International	Woburn, MA
Blocking Agent – BSA and sucrose (ready-to-use)		
Sample Diluent – BSA, Tween 20 and HAMA-blocker		
Wash Concentrate – Tween 20		
Streptavidin-HRP (horseradish peroxidase) Diluent – BSA (ready-to-use)		
Streptavidin conjugated peroxidase		
Substrate Solution – TMB/H_2O_2 (ready-to-use)		
Stop Solution (0.25mol/L sulphuric acid) (ready-to-use)		
Recombinant Human PAP1 (REG3α) Standard		

Antibodies	Company	Location
Mouse Anti-Human PAP1 (REG3α) Capture Antibody	MBL International	Woburn, MA
Biotinylated conjugated rabbit Anti-Human PAP1 (REG3α) polyclonal Detection Antibody		

3.5.6 Enzyme Linked ImmunoSorbent Assays (ELISA)

ELISA is an immunoassay used to quantify the concentration of a specific analyte (usually an antigen). It can be fixed in the microplate after binding to a *capture antibody*. Then, a *detection antibody* binds the antigen and form the antigen-antibody complex. If the detection antibody is attached to an enzyme that changes colour directly after adding a substrate, it is called *direct ELISA*. The colour produced in this reaction will be proportionated to the amount of antigen in the sample[263]. If a second detection antibody with a bound enzyme is needed to produce this reaction, this is known as *indirect ELISA.* When the analyte is covered by the capture and detection antibody forming a “sandwich”, this is coined as *sandwich ELISA*. This is the preferred method for detection of 25(OH)D^3 and biomarkers in this study, as described below.

3.5.6.1 Vitamin D Immunoassay

The diagnostic tool to quantify the total concentration of 25(OH)D^3 in human serum is the ADVIA Centaur XP system, using the Vitamin D (Vit D) Total assay.

3.5.6.2 Principle of the procedure

In this study, the vitamin D assay was performed in the Biochemistry Laboratory at Barnsley Hospital NHS Foundation Trust with the *sandwich* ELISA technique: anti-vitamin D antibodies binds covalently to Acridinium Ester (AE), a chemiluminescent molecule that emits energy in form of light (chemical reaction known as *direct chemiluminescence*). This binding does not alter the ability of the antibody binding an antigen. When these labelled antibodies are added to the sample, they bind specifically the analyte or antigen ($25(OH)D^3$). Moreover, paramagnetic particles or PMP (iron oxide crystals attracted to a magnetic field) coated with specific antibodies for a different epitope on $25(OH)D^3$ are added to the solution and bind to it (already bound to the AE-labelled antibodies) during incubation of the cuvette at 37^{o}C (forming a "sandwich"). They are the Solid Phase of the assay (see Figure 3-1). The cuvette is then exposed to a magnetic field that separates the bound to the unbound material, holding the former and discarding of the latter. An acid is added followed by a base, which triggers the oxidation of AE and subsequent light emission (measured in relative light units). The amount of light produced by the reaction is proportional to the concentration of $25(OH)D^3$ in the sample.

3.5.6.3 Detection capability

The Limit of Quantitation or lowest detectable $25(OH)D^3$ concentration of this assay is 4.2 ng/mL (10.5 nmol/L). When at a concentration below 4.2 ng/ml (10.5 nmol/L) the instrument records all values as <4.2 (or <10.5 nmol/L). The limit of normal concentrations of $25(OH)D^3$ ranges from 4.2 to 150 ng/mL (10.5–375 nmol/L)[264].

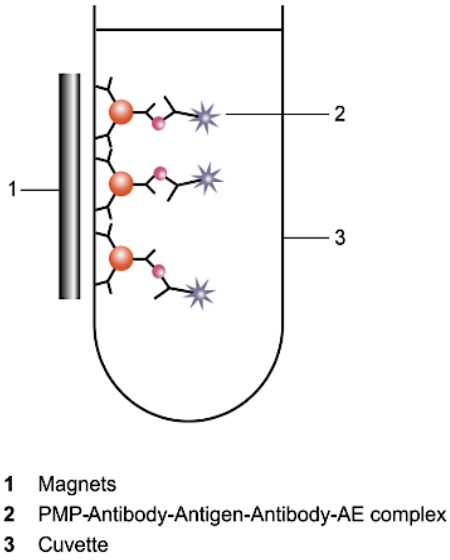


Figure 3-1: Sandwich format assay in cuvette[265]. Used with permission

3.5.6.4 Sample preparation

At least 20µl of sample are required for this assay. Samples should be free of any particle, including fibrin, bubbles and foam. They are placed in the sample rack and loaded at the sample entry queue, in the sample loading area of the ADVIA Centaur XP. Then racks are moved to the in-process queue, where the samples barcode labels are scanned and, subsequently, the sample is aspirated by the sample probe and processed.

Moreover, reagents are ready-to-use liquid solutions after they have been taken out of the fridge. They need to be protected from the light and stored at 2-8ºC.

3.5.6.5 Assay Procedure

The ADVIA Centaur XP carries out this process automatically as follows:

i. 20 µL of sample are distributed into a cuvette and incubated for 15 seconds.

ii. 200 µL of Ancillary Pack Reagent are added to the sample and incubated at 37°C for 4.5 minutes.

iii. 50 µL of Lite Reagent are added to the mixture and incubated at 37°C for 5.5 minutes.

iv. 100 µL of Solid Phase reagent and 50 µL of ancillary well reagent are added and incubated at 37°C for 2.75 minutes.

v. The Solid Phase is separated from the mixture, aspirating the unbound reagent.

vi. Wash 1 is used to wash the cuvette.

vii. Chemiluminescent reaction is started when Acid Reagent and Base Reagent (300 µL each) are added to the sample.

viii. At the end of the processing, the racks reach the sample exit queue, where they are finally removed.

ix. Serum levels of $25(OH)D^3$ are reported in nmol/L.

x. If total $25(OH)D^3$ levels in serum samples are greater than 150 ng/mL (>375 nmol/L) and exceed the linearity of the assay, samples should be diluted (1:2 dilution factor) and retested, setting the dilution point to 150 ng/mL (375 nmol/L). This is done automatically by the system and corrected mathematically for dilution.

3.5.7 GvHD biomarkers Immunoassay

To quantify the concentration of a specific biomarker in a sample, the most common type of immunoassays is *sandwich ELISA*. Capture and detection antibodies bind to different epitopes of the target biomarker, and the subsequent colorimetric signal produced is quantitatively proportionate to the concentration of biomarker present within the sample. Its optical densities are interpreted by interpolating them onto a standard curve generated from a known biomarker concentration (see Figure 3-2).

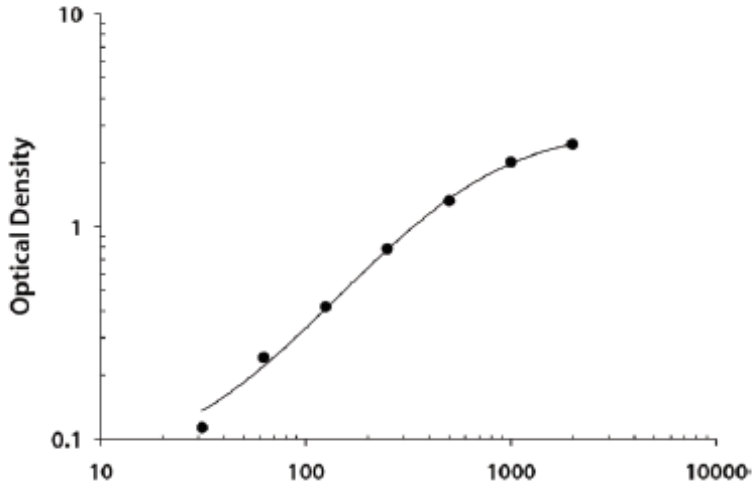


Figure 3-2: Standard curve of Elafin concentration (pg/mL)[266]*. Used with permission*

3.5.7.1 Elafin ELISA method

The Elafin ELISA was performed in accordance with the Standard Operation Procedures (SOP) from the Photopheresis Research Unit at Rotherham General Hospital.

Plate preparation

i. 400ng/ml of capture Antibody (CAb) is prepared in sterile PBS and 100µl is added immediately to each well of the 96-well microplate. CAb and PBS are stored in the fridge (or CAb is quickly defrosted from freezer) and kept sterile by opening in the hood. Plate is sealed with adhesive cover and incubate overnight at room temperature (set at 20°C).

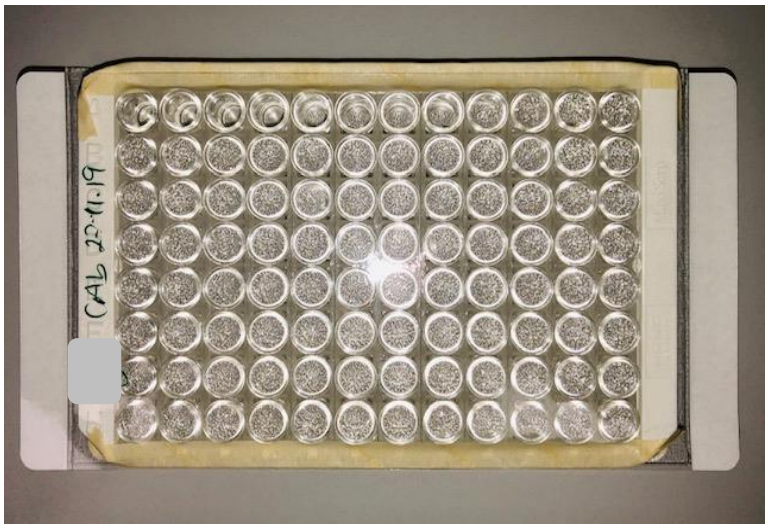


Figure 3-3: Incubation of capture antibody in 96-well microplate

ii. On the following day, Wash Buffer (PBS) is diluted in distilled water (1:25) and washing is performed 3 times by adding 400µL of buffer to each well. After each wash, the buffer is immediately tipped into the waste and blotted against clean paper roll. On the last wash, all bubbles and residual Wash Buffer are aspirated using P100 pipette.

iii. 300µl of Reagent Diluent (RD) (previously diluted in distilled water, 1:10) are added to each well to block non-specific antibody binding. Seal the plate with adhesive cover and incubate at room temperature for 60 minutes.

Assay procedure

iv. Serum sample are removed from the freezer to allow 15 minutes of defrosting before diluting.

v. In the meantime, 48 microcentrifuge tubes are labelled: 8 for the Reconstituted Standard dilutions (2000pg/ml of Elafin with serial 1:2 dilution down to 31.25pg/ml in addition to an RD only for 0 pg/ml) and 40 for the study samples. Aliquots of standard and study samples are prepared as follows:

vi. Standard results are used to create a standard curve to interpolate the sample optical densities onto. They are made from the stock solution straight from the fridge or quickly defrosted from the freezer. Stock solution of the standard is mixed with a pipette before taking required volume (16.7µl) to create the top concentration of 2000pg/ml in RD. Subsequent 1:2 dilutions are prepared in RD until the last one 31.25pg/mL. After each dilution the tip that takes the volume mixes it with the next diluent and a new tip is used to transfer to the next one and mixed and so on. 100µL of standard in RD are added per well of the first two columns and of study sample on the rest of the plate.

vii. Serum samples are prepared in the same way as standards: Stock sample is mixed with pipette before taking 5µL per sample. They are mixed with 995µL of RD (1:200) to make up 1,000µL solution, and duplicate wells are filled with 100µL of this dilution. A new tip is required for each sample to avoid cross-contamination.

viii. The plate is sealed with an adhesive covered and incubated for 2 hours at room temperature. Repeat wash as in step iii.

ix. Detection Antibody (DAb) is taken straight from the fridge and diluted in RD to 10ng/ml100µL is added to each well of the 96-well microplate). Plate is sealed with an adhesive cover and incubated 2 hours at room temperature.

x. Repeat wash as in step iii.

xi. Streptavidin-HRP is diluted in RD (1:200), and 100µL of this dilution is added to each well of the 96-microplate. Plate is covered with an adhesive cover and incubated 20 minutes at room temperature, avoiding direct light.

xii. Repeat wash as in step iii.

xiii. 100µL of Substrate Solution (made up of equal volumes of Colour Reagent A and B) is added to each well of the 96-microplate. Plate is covered with an adhesive strip and incubated 20 minutes at room temperature, avoiding direct light.

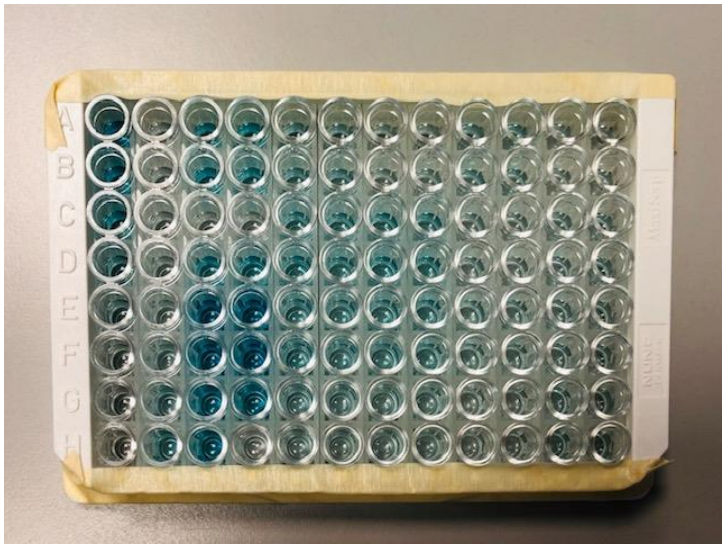


Figure 3-4: Study plate after incubation with Substrate Solution

xiv. To stop colour reaction, 50µL of Stop Solution is added to each well and plate is gently tapped to allow proper mixing prior to analysis.

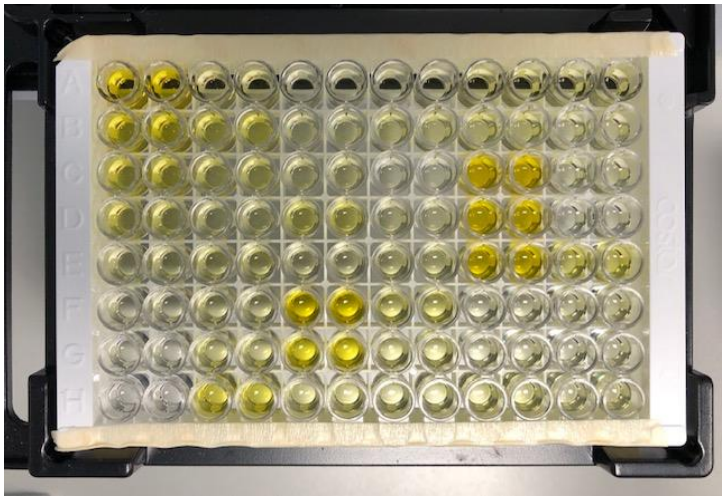


Figure 3-5: Study plate after adding Stop Solution

xv. Plate is placed in loading tray of microplate reader and ScanIT software is started. Wavelength is set to 450nm and 540nm for corrections to ensure a more accurate determination of the optical density of each well.

Figure 3-6: Microplate reader with study plate

3.5.7.2 ST2 ELISA method

The ST2 ELISA was performed in accordance with the Standard Operation Procedures (SOP) from the Photopheresis Research Unit at Rotherham General Hospital.

Plate preparation

i. 1 µg/mL of CAb is prepared in sterile PBS and 100µl is added immediately to each well of the 96-well microplate. CAb and PBS are stored in the fridge (or CAb is quickly defrosted from freezer) and kept sterile by opening in the hood. of CAb is prepared with sterile PBS, Plate is sealed with an adhesive cover and incubated overnight at room temperature.

ii. On the following day, Wash Buffer (PBS) is diluted in distilled water (1:25) and washing is performed 3 times adding 400µL of it to each well. After each wash, the buffer is immediately tipped into the waste and blotted against clean paper roll. On the last wash, all bubbles and residual Wash Buffer are aspirated using P100 pipette.

iii. 300µl of Reagent Diluent (RD) (previously diluted in distilled water, 1:10) are added to each well to block non-specific antibody binding. Seal the plate with adhesive cover and incubate at room temperature for 60 minutes.

Assay procedure

iv. Serum samples are removed from the freezer to allow 15 minutes of defrosting before diluting.

v. In the meantime, 48 microcentrifuge tubes are labelled: 8 for the Reconstituted Standard dilutions (2000pg/ml of ST2 with serial 1:2 dilution down to 31.25pg/ml in addition to an RD only for 0pg/ml) and 40 for the study samples. Aliquots of standard and study samples are prepared as follows:

vi. Standard results are used to create a standard curve to interpolate the sample optical densities onto. They are made from the stock solution straight from the fridge or quickly defrosted from the freezer. Stock solution of the standard is mixed with a pipette before taking required volume (18.1µl) to create the top concentration of 2000pg/ml in 20% FCS. Subsequent 1:2 dilutions are prepared in 20% FCS until the last one 31.25pg/mL. After each dilution the tip that takes the volume mixes it with the next diluent and a new tip is used to transfer to the next one and mixed and so on. 100µL of standard in 20% FCS are added per well of the first two columns and of study sample on the rest of the plate.

vii. Serum samples are prepared in the same way as standards: stock sample is mixed with a pipette before taking 5µL per sample. They are mixed with 995µL of 20% FCS (1:200), and duplicate wells are filled with 100µL of this dilution. A new tip is required for each sample to avoid cross-contamination. The plate is sealed with an adhesive covered and incubated for 2 hours at room temperature.

viii. Repeat wash as in step iii.

ix. Detection Antibody (DAb) is taken straight from the fridge and diluted in RD to 200ng/ml. Following this, 100µL of this dilution are added to each well. Plate is sealed with an adhesive cover and incubated 2 hours at room temperature.

x. Repeat wash as in step iii.

xi. Streptavidin-HRP is diluted in RD (1:40), and 100µL of this dilution is added to each well. Plate is covered with an adhesive cover and incubated 20 minutes at room temperature, avoiding direct light.

xii. Repeat wash as in step iii.

xiii. 100µL of Substrate Solution (made up of equal volumes of Colour Reagent A and B) is added to each well. Plate is covered with an adhesive strip and incubated 20 minutes at room temperature, avoiding direct light.

xiv. To stop the colour reaction, add 50µL of Stop Solution to each well. The plate is gently tapped to allow proper mixing prior to analysis.

xv. Plate is placed in loading tray of microplate reader and ScanIT software is started. Wavelength is set to 450nm and 540nm for correction to ensure a more accurate determination of the optical density of each well.

3.5.7.3 Regeneration islet-derived 3α (REG3α) ELISA method

The REG3α ELISA was performed in accordance with the Standard Operation Procedures (SOP) from the Photopheresis Research Unit at Rotherham General Hospital.

Plate preparation

i. Capture antibody (CAb), directly from the fridge or quickly defrosted from freezer, is diluted in coating buffer (1:200), also directly from the fridge.

ii. Immediately, 100µl of the diluted CAb is added to each well. Plate is sealed with adhesive cover and incubated in the fridge overnight (2-8^{o}C).

iii. On the following day, washing is performed twice by adding 400µL of normal saline 0.9% in each well. After each wash, normal saline is removed by pipette. On the last wash, all bubbles and residual normal saline are aspirated using P100 pipette.

iv. 200µl of blocking agent are added in each well. Seal the plate with adhesive cover and incubate at room temperature for 60 minutes.

v. Content of the wells is removed by pipette (washing not required).

Assay procedure

vi. Serum sample are removed from the freezer to allow 15 minutes of defrosting before diluting.

vii. In the meantime, 48 microcentrifuge tubes are labelled: 8 for the Reconstituted Standard dilutions (100ng/ml of REG3α with serial 1:2 dilution down to 1.56ng/ml in addition to and sample diluent only for 0pg/ml) and 40 for the study samples. Aliquots of standard and study samples are prepared as follows:

viii. Standard results are used to create a standard curve to interpolate the sample optical densities onto. They are made from the stock solution straight from the fridge or quickly defrosted from the freezer. Stock solution of the standard is mixed with a pipette before taking required volume (100µl) to create the top concentration of 100ng/ml in Sample Diluent. Subsequent 1:2 dilutions are prepared in Sample Diluent until the last one 1.56ng/mL. After each dilution the tip that takes the volume mixes it with the next diluent and a new tip is used to transfer to the next one and mixed and so on. 100µL of standard in Sample Diluent are added per well of the first two columns and of study sample on the rest of the plate.

ix. Serum samples are prepared in the same way as standards: Stock sample is mixed with pipette before taking 150µL per sample. They are mixed with 150µL of Sample Diluent (1:2), and each duplicate well is filled with 100µL of this dilution. A new tip is required for each sample to avoid cross-contamination. The plate is sealed with an adhesive covered and incubated for 1 hour at room temperature.

x. Samples are washed off four times with 350µl of wash buffer by pipette and any residual wash buffer is removed at the end.

xi. Detection Antibody (DAb) is taken straight from the fridge and diluted in Sample Diluent (1:101). Following this, 100µL of this dilution are added to

each well. Plate is sealed with an adhesive cover and incubated 1 hour at room temperature.

xii. Repeat wash as in step x.

xiii. Streptavidin-HRP is diluted in SA-HRP diluent (1:101), and 100µL of this dilution is added to each well. Plate is covered with an adhesive cover and incubated 30 minutes at room temperature, avoiding direct light.

xiv. Repeat wash as in step x.

xv. 100µL of Substrate Solution is added to each well. Plate is covered with an adhesive strip and incubated 30 minutes at room temperature, avoiding direct light.

xvi. To stop colour reaction, 100µL of Stop Solution is added to each well and plate is gently tapped to allow proper mixing prior to analysis.

xvii. Plate is placed in loading tray of microplate reader and ScanIT software is started. Wavelength is set to 450nm and 540nm for correction to ensure a more accurate determination of the optical density of each well.

3.5.7.4 Calculation of results

The ScanIT software produces a standard curve after interpolating the mean absorbance for each sample on the y-axis against a known biomarker concentration on the x-axis. The average concentration for each sample duplicate is calculated after plotting it against the standard curve. In some cases, the software cannot provide a specific value as the concentration of the biomarker is over the maximum measured concentration above the standard curve. Subsequent dilutions are then performed (1/2, 1/5 or even 1/10) and analysis is repeated to get results below the standard curve. For diluted samples, the average is multiplied by the dilution factor.

In this study, despite the measures taken, some results were unavailable in the consecutive blood samples, mainly for REG3α.

3.6 Statistical Analysis

Patients were identified following the inclusion criteria at the transplant clinic, haematology ward or ECP unit at the different study sites. Data was collected prospectively and recorded in the data collection forms, and subsequently transferred into a spreadsheet in an encrypted laptop at the end of the study.

SPSS, version 25 (IBM Inc., Chicago, IL, USA) was used for statistical analyses and graphs generation. Descriptive statistics are presented as frequencies with percentages for categorical variables, and medians and interquartile ranges for continuous variables. The Chi-square test and the Mann-Whitney U/exact Kruskal-Wallis test were used to determine categorical and continuous variables, respectively, between groups. Differences in $25(OH)D^3$ and biomarkers serum levels between specific follow-up time points (1, 3 and 6 months) and baseline (day 0) were assessed with Wilcoxon Rank Sum test for continuous values. Correlations were determined by Pearson's/Spearman's rank correlation test (in *Supporting data*). Univariate comparisons to test significant variables associated with treatment response were made using logistic regression. Survival curves were estimated with the Kaplan-Meier method, and the independent impact on risk of significant variables in univariable analysis was assessed using a Cox cause-specific hazard model. A two-tailed p value of <0.05 was considered significant for all statistical tests. Where missing data points these variables were omitted from the analysis, but cases were still available for further testing.

Vitamin D deficiency was defined as serum levels of $25(OH)D^3$ <50 nmol/L and vitamin D adequacy when $25(OH)D^3$ levels ≥50 nmol/L. Complete remission (CR) was defined as the complete resolution of GvHD-derived signs and symptoms.

3.7 Results

Initially, 37 patients and 44 controls took part in the study. Out of this initial number of patients, 8 were removed from the analysis for different reasons (samples taken at wrong time points (n=1), biopsy ruled out GvHD (n=4), patient recruited twice (n=1), samples lost (n=1), and patient recruited for aGvHD arm but developed overlap cGvHD (n=1)). Eventually, the total number of patients participating in this study accounted for 29. Only 3 of them had a biopsy for new diagnosis of aGvHD, all of them confirming gut involvement. As previously described, serial blood samples were taken at specific time points - baseline (day 0), 1, 3 and 6 months later. For controls, only one-off sample was taken: Out of the 44 controls, 28 were Anthony Nolan staff members (AN controls) whereas 16 were patients following alloHSCT at the Royal Marsden Hospital, approximately 1 month post-HSCT. Half of them (n=8) developed clinical GvHD thus they were removed as controls, but the remaining (healthy HSCT controls, n=8) did not report any GvHD-related signs or symptoms at the time of censoring. Patient and control characteristics are shown in Table 3-1.

Unfortunately, not all patients had the 4 serial samples drawn due to different reasons, mainly missing samples at specific time points and early deaths (See Table 5-1in *Supporting data*, Chapter 5).

	Acute GvHD *n*=16	SR Chronic GvHD *n*=5	SR Acute GvHD *n*=8	Healthy HSCT controls *n*=8	AN controls *n*=28
Patient age (years), median (range)	60.9 (22.2-69.7)	38.1 (34.7-70.4)	59.0 (36-68.6)	60.5 (41.5-65.5)	41.4 (25.2-66.2)
Sex, *N*					
male/female	10/6	2/3	4/4	3/5	12/16
CMV status, *N*					
+/-	8/8	3/2	2/3	4/4	-
Unknown	-	-	3	-	
Ethnicity, *N*					
Caucasian	12	3	8	6	26
Afro-Caribbean	2	1	-	-	-
Asian	1	1	-	2	2
Unknown	1	-	-	-	-
BMI, *N*					
Underweight	3	-	-	-	-
Healthy	5	3	2	3	15
Overweight	5	-	1	3	9
Obese	3	2	3	2	4
Unknown	-	-	2	-	-
Disease, *N*					
Myeloid	13	3	8	6	-
Lymphoid	3	2	0	2	
Disease status prior to HSCT, *N*					
CR	10	5	6	6	
Non-CR	4	-	2	2	-
Unknown	2	-	-	-	
Donor sex, *N*					
Male/female	11/5	3/?	4/2	4/4	-
Unknown	-	2	2	-	
Donor age (years), median (range)	26.8 (19.3-64.4)	27.4 (25.3-65.2)	27.0 (21.2-51.9)	35.2 (41.5-65.5)	-
Donor CMV, *N*					
+/-	7/9	3/?	2/4	6/2	-
Unknown	-	2	2	-	
Donor type, *N*					
Sibling	1	3	2	3	
MUD	10	2	5	3	-
Other	5	-	1	2	

Matching, *N* Matched Mismatched	 11 5	 5 -	 7 1	 6 2	 -
Conditioning, *N* MA RIC	 3 13	 2 3	 2 6	 - 8	 -
T depletion, *N* None Alemtuzumab ATG	 2 8 6	 - 4 1	 - 4 4	 - 6 2	 -
GvHD prophylaxis, *N* CsA CsA + other	 14 2	 4 1	 3 5	 8 -	 -
HSCT source, *N* PBSC BM Unknown	 16 - -	 5 - -	 5 1 2	 8 - -	 -
Acute GvHD, grade, *N* I II III IV	 6 4 4 2	 - 	 - - 6 2	 - 	 -
Chronic GvHD, grade, *N* Mild Moderate Severe	 - 	 - 5 -	 - 	 - 	 -
Time from HSCT to 1st sample (days), median (range)	102 (21-397)	418 (153-1159)	160.5 (47-305)	33 (26-38)	 -

N = number of patients

Table 3-1: Patient and control characteristic

3.7.1 *De novo* acute GvHD

Sixteen patients with newly diagnosed aGvHD were recruited into the study. Five patients (31%) were subclassified as *late onset* aGvHD (aGvHD beyond 100 days post-alloHSCT). Three patients (19%) developed aGvHD following DLI.

Baseline characteristics and outcomes of this cohort are summarised in Table 5-13 in *Supporting data*.

Response to steroids

One-month response to treatment was assessed in 12 patients (75%), those who had a second sample taken at this time point. The rate of CR at one month was 50%: Six patients achieved CR to steroids (*responders*) compared to 6 who did not (*non-responders)*. In the responder group, 4 patients (67%) were on topical and 2 (33%) on systemic steroids, while in the non-responder 3 patients (50%) were on topical and 3 (50%) on systemic steroids (p=0.55).

The median levels of 25(OH)D^3, elafin, ST2 and REG3α at day 0 in responders *vs* non-responders at 1 month are displayed in Table 3-2. The only significant difference was the median levels of 25(OH)D^3 at baseline, higher in responders (53.1 nmol/L) than in those who did not respond (32.7 nmol/L) (p=0.037). See Figure 3-7. Then, to take account of the potential prognostic variable of 25(OH)D^3 concentration, logistic regression was performed but it was not found significant (p=0.1).

	Responders	*Non-responders*	*p* value**
25(OH)D^3 (nmol/L)*	53.1 (34.8 - 75.9)	32.7 (29.4 – 49.7)	*p* = 0.037
Elafin (ng/ml)*	28.4 (15.8 - 29.6)	26.8 (13.6 - 503.4)	*p* = 0.63
ST2 (ng/ml)*	47.1 (14.6 - 243.6)	47.3 (23.1 - 164.9)	*p* = 0.75
REG3α (ng/ml)*	44.1 (16.9 - 200.9)	67.7 (4.1 - 382)	*p* = 0.47

**median (range); ** p values derived from Mann-Whitney test*

Table 3-2: Values at baseline in responders vs non-responders after 1 month of treatment with steroids

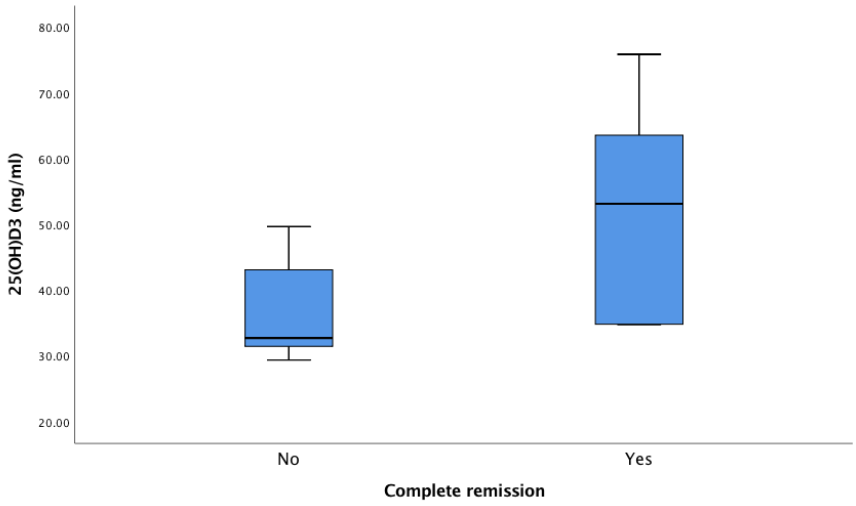


Figure 3-7: : Levels of 25(OH)D3 at baseline and response to steroids at 1 month post-treatment

Interestingly, after dividing patients in 3 categories depending on serum levels of 25(OH)D^3 (<30 nmol/L, 30-50 nmol/L and ≥50 nmol/L), all patients with 25(OH)D^3 ≥50nmol/L responded to steroids, but those encompassed within 30-50 nmol/L

presented mixed responses. The only patient with 25(OH)D^3 levels <30 nmol/L did not respond (p=0.046). See Figure 3-8:

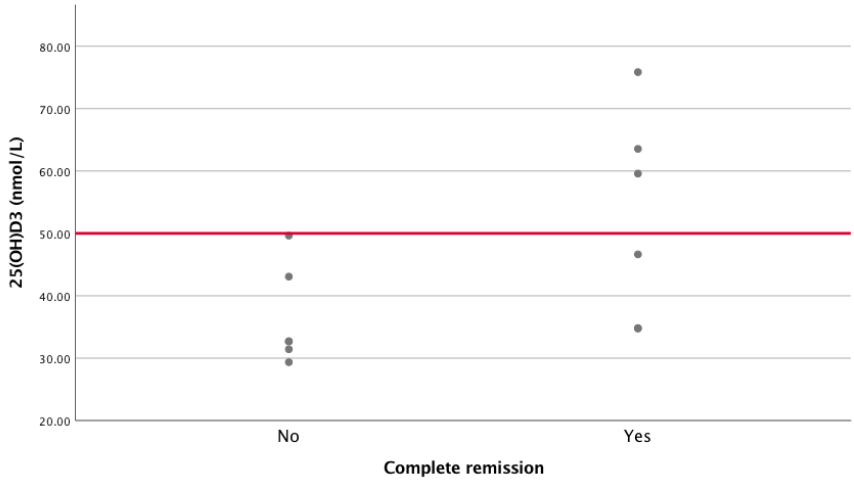


Figure 3-8: Relationship between concentration of 25(OH)D3 and response to steroids after 1 month of treatment

Moreover, 8 and 6 patients had blood samples drawn at 3 and 6 months, respectively. One out of 8 patients (12.5%) achieved CR at 3 months, and 1 out of 6 (16.7%) at 6 months. Only six patients (37.5%) had the 4 consecutive blood samples but all of them reached CR at some point of the study. Comparisons between the median levels of 25(OH)D^3 and GvHD biomarkers could not be made between responders and non-responders neither at 3 nor 6 months because only 1 patient responded at each time point. Moreover, 3 patients relapsed from GvHD, 2 after 3 months and 1 after 6 months of starting on steroids.

Vitamin D and GvHD biomarkers in aGvHD compared to controls

Despite the concentration of 25(OH)D^3 was lower at baseline in patients compared to AN controls, this difference was not significant (38.9 *vs* 50 nmol/L, p=0.21). However, 25(OH)D^3 levels did not differ between patients and healthy HSCT controls (see Table 5-2 in *Supporting data*). Moreover, there was a trend in levels of elafin, higher in patients than in healthy HSCT controls (25 *vs* 15.7 ng/ml, p=0.05). In addition, significant differences were found in the concentration of ST2 (71.1 *vs* 15 ng/ml) and REG3α (55.3 *vs* 10.2 ng/ml), as patients had higher levels than AN controls (both p<0.001). Moreover, there was only a significant difference in elafin levels with healthy HSCT controls at 3 months (30.8 *vs* 15.7 ng/ml, p=0.009). With AN controls, there were significant differences in ST2 and REG3α at 1 (44 *vs* 15, p<0.001; 81.9 *vs* 10.2, p<0.001, respectively) and 3 months (25.3 *vs* 15, p=0.027; 75.3 *vs* 10.2, p<0.001, respectively), and REG3α at 6 months (38.6 *vs* 10.2, p<0.001).

Level of 25(OH)D^3 and biomarkers in aGvHD

At baseline, 12 patients (75%) were 25(OH)D^3 deficient. The median levels of 25(OH)D^3 and GvHD biomarkers at each time point are shown in Table 5-3 (*Supporting data*). Concentrations of all variables were compared between baseline and 1, 3 and 6 months respectively, but none of them were statistically significant (Table 5-4 in *Supporting data*).

Note that 4 patients (25%) had started on IST before the first blood sample was drawn: 3 were on topical (for 3 days) and 1 on oral steroids (for 2 weeks). In order to check whether this could affect the values of the study variables at baseline, both

Mann-Whitney and logistic regression tests were run comparing the group of patients on IST prior to baseline samples *vs* those who were not, but neither of them showed significant differences between those groups in any of the study variables. Likewise, only 3 patients in the study, all of them with *de novo* aGvHD, were on vitamin D supplementation but statistical analysis was not significant.

Relationship between grades of aGvHD and 25(OH)D^3/biomarkers

At diagnosis, there was a trend in levels of ST2: patients with grade III-IV aGvHD had higher levels of this marker compared to those with grade I-II (180.4 *vs* 47.3 ng/ml, *p*=0.051). None of the variables at diagnosis could predict GvHD grade one month later. At one month, patients without GvHD or with grades I-II aGvHD had a higher concentration of 25(OH)D^3 compared to those with grades III-IV (41.6 *vs* 23.3 nmol/L, *p*=0.032). See Figure 5-1, Figure 5-2 and Table 5-5 in *Supporting data*. Comparisons at 3 and 6 months could not be carried out because some grade had solely 1 patient. Further statistical analysis has been described in appropriate section in *Supporting data*.

Relationship between organ involvement and 25(OH)D^3/biomarkers

At diagnosis, the only significant correlation was the levels of elafin with stage of skin aGvHD: elafin at stage 0-I (n=12) was 19.9 ng/ml (7.9 – 32.6) while at stage II-IV (n=4) was 32.2 (28.6 – 503.4) (*p*=0.029). No other significant associations were found between 25(OH)D^3 or any biomarker with the stage of skin, gut or liver, including lower gut aGvHD and REG3α (*p* =0.49) or ST2 (*p*=0.13).

At one month, the previous link between serum levels of elafin and skin GvHD could not be reproduced (*p*=0.83). Other correlations were not found either, possible due

to the low number of patients with stage II-IV aGvHD at this time point (skin, n=2; gut, n=1; and liver n=0). For the same reason, no further analysis was carried out at later time points.

Survival

At the time of censoring, 8 patients had died and 8 were still alive. Survival at 6 and 12 months from baseline blood sample was 91% and 52%, respectively. Time from baseline sample to last follow-up/death was 6.5 months (1 – 24.6). Causes of death were infections (n=4; 25%), infections and GvHD (n=2; 12.5%), relapse (n=1; 6.3%) and gastro-intestinal bleeding (n=1; 6.3%).

Using the Mann-Whitney test, only the levels of REG3α at diagnosis were significantly higher in patients who eventually died compared to those who did not (287.6 *vs* 23.4 ng/ml, p=0.007). See Figure 3-9 (note that patient *number 2*, with stage 2 gut aGvHD, had a very high concentration REG3α). There were no significant differences on elafin (p=0.12), ST2 (p=0.17) or 25(OH)D^3 (p=1.0) at this time point. At 1 month, ST2 (82.9 *vs* 24.3 ng/ml, p=0.019) and REG3α (117.9 *vs* 61 ng/ml, p=0.008) were also higher in deceased patients compared to those alive (See Figure 5-4 and Figure 5-5 in *Supporting data*). Nevertheless, no other significant differences were found at 1, 3 or 6 months follow-up. Cox regression was performed to take account of the potential prognostic variables associated with survival including 25(OH)D^3 and all biomarkers at baseline, but no statistically significant differences were found: 25(OH)D^3 (p=0.47), elafin (p=0.12), ST2 (p=0.34) and REG3α (p=0.25).

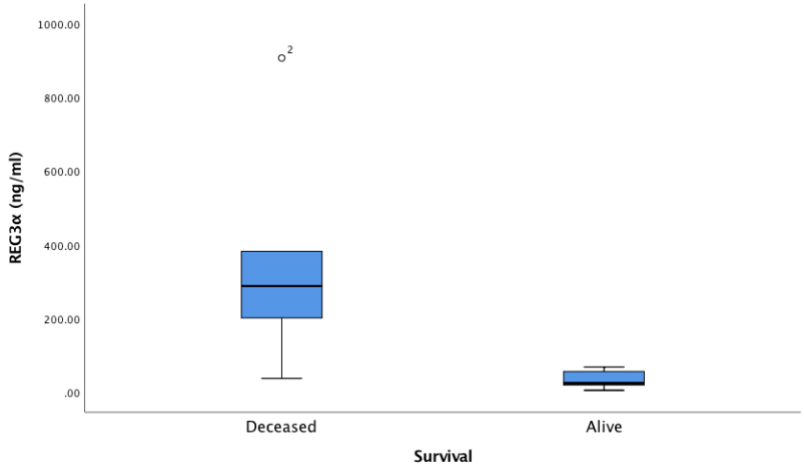


Figure 3-9: Relationship between levels of REG3α at baseline and survival in aGvHD

GvHD Relapse

In total, 3 patients suffered from relapse of GvHD over the 6-month follow-up period: 2 out of 8 (25%) at 3 months, and 1 out of 6 (16.7%) at 6 months. There were no significant differences in baseline variables between patients who did and did not relapse: 25(OH)D^3 (46.6 *vs* 34.8, *p*=0.66), elafin (28.6 *vs* 28.2, *p*=0.88), ST2 (99.4 *vs* 42.7, *p*=0.30) and REG3α (36.3 *vs* 52, *p*=0.66). However, at 1 month there was a trend in higher concentrations of ST2 (73.4 *vs* 24.3, *p*=0.053) and REG3α (98.5 *vs* 61, *p*=0.053) in relapsed patients.

3.7.2 Steroid-refractory chronic GvHD

Five patients with SR cGvHD were recruited for this study. The first blood sample (baseline) was drawn prior to starting on second-line treatment with ECP. All patients where on treatment with steroids and ciclosporin, and one of them (20%) was also on a second calcineurin inhibitor, tacrolimus. At baseline, all patients had

moderate cGvHD. Only one sample was missed at the last endpoint in one of the patients. Characteristics of this population are displayed in Table 5-14 in *Supporting data*.

Response to IST

Response to ECP could not be assessed because none of the patients had achieved CR by the time of censoring.

Vitamin D and GvHD biomarkers in SR cGvHD compared to controls

There were no remarkable differences in 25(OH)D^3, elafin and ST2 between patients with SR cGvHD and controls. Only levels of REG3α were threefold increased in patients at baseline compared to AN controls. See Table 3-3:

	Day 0 ***(N=5)***	**AN controls** ***(N=28)***	**Healthy HSCT controls** ***(N=8)***
25(OH)D^3 (nmol/L)*	46.8 (34.4 – 91.2)	50 (20.4 – 79.2) *p* =0.8	39.9 (22.8 -155.3) *p* =0.56
Elafin (ng/ml)*	32.2 (7.5 – 311.2)	17.3 (6.6 - 2442) *p* =0.27	15.7 (7.9 -32.2) *p* =0.19
ST2 (ng/ml)*	18.7 (9.1 – 285.7)	15 (3.3 -24.3) *p =0.23*	54.7 (18.1 -190.6) *p* =0.14
REG3α (ng/ml)*	33 (13 – 71.8)	10.2 (3.5 – 23.3) *p* =0.002	51.3 (13.4 – 81.1) *p* =0.66

**median (range); p values after comparing the cGvHD populations with each control cohort (Mann-Whitney test)*

Table 3-3: Comparison of 25(OH)D3 and biomarkers between SR cGvHD patients and each study control group

Nevertheless, there were significant differences in SR cGvHD compared to AN controls in levels of ST2 (35 *vs* 15, *p*=0.002) and REG3α (43 *vs* 10.2, *p*=0.002) at one month, in REG3α (30.1 *vs* 10.2, *p*=0.014) 3 months, and in ST2 (48 *vs* 15, *p*=0.001) and REG3α (38.2 *vs* 10.2, *p*=0.004) at 6 months. There were no remarkable differences between these patients and healthy HSCT controls at any follow-up time point.

Level of 25(OH)D^3 and biomarkers in SR cGvHD

At baseline, 4 patients (80%) were deficient in vitamin D. The median levels of 25(OH)D^3 and GvHD biomarkers at each time point are shown in Table 5-6 in *Supporting data*. Concentrations of all variables were compared between baseline and 1, 3 and 6 months, respectively, and solely the difference in concentration of ST2 between baseline and one month was found statistically significant (18.7 *vs* 35, *p*=0.043). See Table 5-7 in *Supporting data*.

Relationship between organ involvement/cGvHD grade and 25(OH)D^3/biomarkers

All patients had moderate cGvHD when the first blood sample was drawn. At 1 month, only 1 patient (20%) improved to mild grade (patient *number 1*). Unfortunately, this patient worsened to moderate grade at 3 months and it remained unaltered throughout the study. At 3 months, patient *number 2* improved clinically to mild grade and continued on this at 6 months. Patient *number 1* had normal 25(OH)D^3 levels as well as patient *number 2* at 6 months, although the latter was vitamin D deficient at 3 months.

Thus, only one patient improved to mild cGvHD grade over the 6-month study period, but comparisons could not be made between this single participant and the

remaining group. A similar scenario occurred with organs affected by GvHD in different stages, as can be seen in Table 5-14.

Survival

Time from baseline sample to last follow-up/death was 6.8 months (5.2 – 17.2). Only 1 patient died nearly 18 months after baseline sample was drawn due to relapse of primary haematological disease, thus survival analysis could not be performed.

3.7.3 Steroid-refractory acute GvHD

Eight patients with SR aGvHD were recruited into the study, and the first blood sample was also drawn before starting on second-line treatment with ECP. At baseline, 6 patients (75%) had grade III and 2 (25%) grade IV aGvHD. At 1 month, 1 patient (20%) had grade II, 2 (40%) grade III and 2 (40%) grade IV. Only 2 patients (25%) had the 3-month blood sample drawn, and they had grade I and III, respectively. Lastly, the only patient (12.5%) who had the 6-month sample drawn had grade I aGvHD at that stage. All patients were on steroids and ciclosporin at recruitment, but one (12.5%) was on ciclosporin and etanercept, and another (12.5%) was solely on steroids. Characteristics of this cohort are displayed in Table 5-15 in *Supporting data*.

Response to IST

Response to ECP could not be assessed because none of the patients achieved CR by the time of censoring.

Vitamin D and GvHD biomarkers in SR aGvHD compared to controls

At baseline, levels of 25(OH)D[3] were significantly lower in patients compared to AN controls. In addition, there was a trend in the concentration of elafin, that was higher in patients than healthy HSCT controls. ST2 and REG3α were also significantly higher in patients compared to both control groups (see Table 3-4).

	Day 0 ***(N=16)***	**AN controls** ***(N=28)***	**Healthy HSCT controls** ***(N=8)***
25(OH)D[3] (nmol/L)*	35.6 (23.9 – 62.6)	50 (20.4 – 79.2) *p* =0.044	39.9 (22.8 -155.3) *p* =0.46
Elafin (ng/ml)*	114.2 (6.9 – 266.5)	17.3 (6.6 - 2442) *p* =0.13	15.7 (7.9 -32.2) *p* =0.059
ST2 (ng/ml)*	231.7 (93.9 – 483.1)	15 (3.3 -24.3) *p* <0.001	54.7 (18.1 -190.6) *p* =0.009
REG3α (ng/ml)*	100.4 (57.1 – 304.1)	10.2 (3.5 – 23.3) *p* <0.001	51.3 (13.4 – 81.1) *p* =0.007

**median (range); p values after comparing the cGvHD populations with each control cohort (Mann-Whitney test)*

Table 3-4: Comparison of 25(OH)D[3] and biomarkers between SR aGvHD patients and each study control group

At one month, levels of ST2 were significantly higher in patients compared to healthy HSCT controls (373.9 *vs* 54.7, *p*=0.019), and to AN controls (373.9 *vs* 15, *p*<0.001). There was also a trend in higher levels of REG3α in patients compared to AN controls (369.7 *vs* 10.2, *p*=0.060). Nevertheless, there were insufficient participants at 3 and 6 months to carry out similar analysis.

Level of 25(OH)D^3 and biomarkers in SR aGvHD

At baseline, 7 patients (87.5%) were deficient in vitamin D. The median levels of 25(OH)D^3 and GvHD biomarkers at each time point are shown in Table 5-8 in *Supporting data*. Concentrations of all variables were compared between baseline, and 1 and 3 months respectively, but none of them were statistically significant (see Table 5-9 in *Supporting data*).

Relationship between organ involvement/aGvHD grade and 25(OH)D^3/biomarkers

Prior to starting on ECP, 6 patients (75%) were grade III and 2 patients (25%) grade IV. At one month, one patient (20%) was grade II, 2 patients (40%) grade III and 2 patients (40%) grade IV. There were neither significant differences between grades and 25(OH)D^3 (p=0.32), elafin (p=0.51), ST2 (p=0.51) and REG3α (p=0.77) at baseline nor after one month of treatment: 25(OH)D^3 (p=0.82), elafin (p=0.22), ST2 (p=0.5) and REG3α (p=0.44).

At baseline, there was a significant difference in concentration of elafin and the skin GvHD grades 0, 2 and 3 (15 *vs* 126.9 *vs* 220 ng/ml, p=0.044). However, no other associations were found between any other biomarkers or 25(OH)D^3 and organs involved. Similarly to other groups, comparisons could not be made at other time points due to the low number of participants.

Survival

Six patients (75%) with SR aGvHD had died at the time of censoring. Six-month overall survival was 33%. Time from baseline sample to last follow-up/death was 1.6

months (1 – 12.4). The causes of death were infections (n=2; 25%), infection and GvHD (n=1; 12.5%), relapse (n=2; 25%) and stroke (n=1; 12.5%).

There was a remarkable difference in the levels of 25(OH)D^3 at baseline between deceased and survivors (30.3 *vs* 51.7, *p*=0.046). See Figure 3-10. However, significant differences could not be achieved with Cox regression (*p*=0.54).

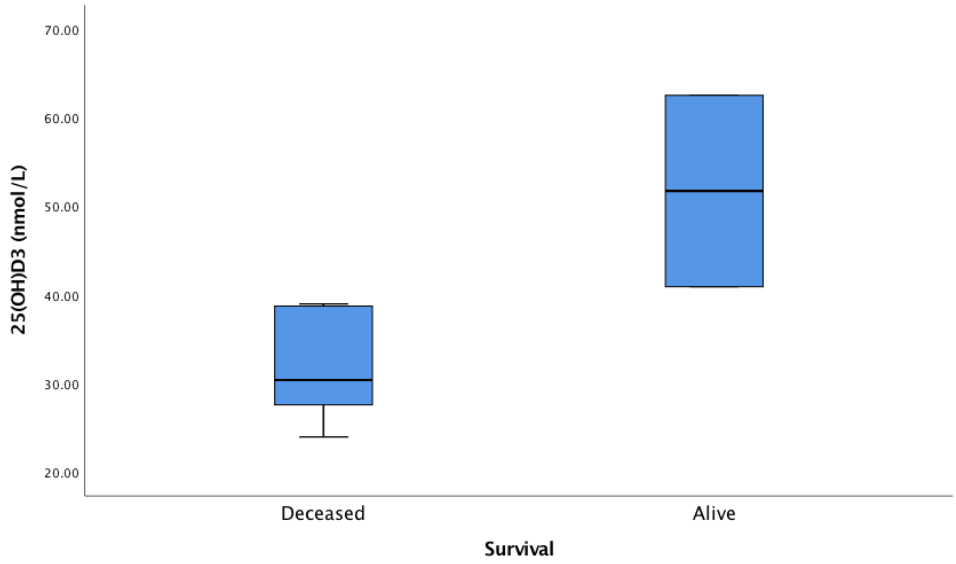


Figure 3-10: Relationship between 25(OH)D^3 prior to second-line treatment and survival

3.8 Discussion

Vitamin D is a potent regulator of immune responses[135,136,143] with a proven implication in the development of GvHD[22,169,203,216,217]. However, the prognosis impact of vitamin D status on the response to IST in GvHD has not been delineated yet. Measurement of 25(OH)D^3 is challenging because it is lipophilic, structurally similar to 25(OH)D^2 and binds strongly to proteins[267,268]. The *gold standard*

technique for detection and quantification of circulating 25(OH)D^3 is *liquid chromatography-tandem mass spectrometry* (LC-MS/MS) assay[268–270]. Nevertheless, its equipment is not available in most of the clinical laboratories due to its high cost, slow throughput and requirement of highly trained techinicians[267,270]. Recent studies have agreed that, in the absence of LC-MS/MS, an immunoassay such as ADVIA Centaur is a more affordable and accurate alternative to assess 25(OH)D^3 in clinical laboratories[270–272].

The prevalence of vitamin D deficiency depends on the threshold applied. In this study, vitamin D deficiency was defined as serum levels of 25(OH)D^3 below 50nmol/L, as seen in studies performed in both general[191] and the HSCT population[22,170,226]. At diagnosis, the majority of patients with *de novo* aGvHD (75%) were vitamin D deficient. This seems reasonable considering that the average time from HSCT until the first sample was drawn was 102 days, and at this early stage post-HSCT patients are strongly recommended to avoid sun exposure[176,177,206]. In addition, 44% of these patients suffered from gut GvHD, and diarrhoea can potentially lead to vitamin D malabsorption[226]. Furthermore, the rate of vitamin D deficiency in SR acute and chronic GvHD was 80% and 87.5%, respectively. These results are in line with previous reports that state the higher risk of vitamin D deficiency in HSCT recipients[142,204], and also with those that have linked vitamin D deficiency with aGvHD[193,207] and cGvHD[169,175].

Response to IST

One month after commencing on IST with steroids is considered the best endpoint to assess early response to treatment in patients diagnosed with aGvHD[50,259,261]. In this cohort, patients who achieved CR at this time point had a significant higher

baseline levels of 25(OH)D^3 (53.1 nmol/L) compared to those who did not respond fully (32.7 nmol/L). In addition, all patients with 25(OH)D^3 above 50nmol/L attained CR but those whose levels were within the “grey area” between 30 and 50 nmol/L presented a mixed range of responses. Although the small sample size refrains from drawing any definitive conclusion, these results could support the hypothesis that vitamin D contributes to the immunosuppressive effect of steroids, as previously reported in the context of asthma[87–90]. Levels of 25(OH)D^3 above 50nmol/L may be required to exert optimally its immunoregulatory properties, as below this cut-off outcomes vary in this study.

All patients in the SR acute and chronic GvHD cohorts received ECP as second-line treatment but none of them attained CR throughout the study, thus response to treatment could not be assessed. A study performed in 219 patients on ECP for SR cGvHD showed that the median duration of treatment was longer than a year, and that long-term ECP treatment was associated with increased disease response[273]. Furthermore, the UK ECP guidelines recommend assessment of the disease status at least 3 months after commencing on this therapy[255]. Thus, long-term studies are recommended in research involving patients on ECP in order to evaluate response to therapy accordingly. Moreover, the high number of fatalities in the SR aGvHD throughout the study did not allow assessment of disease response at later time points.

Many clinical studies have proved the potential of biomarkers in GvHD, particularly in the acute setting: They reflect target organ damage even before clinical manifestations appear[45], and can predict treatment response at early stages of the disease[94,98,99,102,103,116], limiting therapy-derived toxicity in cases of treatment failure[93,103]. However, in this study there was no association between the levels of

biomarkers at diagnosis and response to IST after one month of treatment in *de novo* aGvHD.

Can vitamin D or GvHD biomarkers be used for diagnosis of GvHD?

In *de novo* aGvHD cohort, there were no significant differences found in 25(OH)D^3 concentration at diagnosis between patients and both control groups. Patients are medically advised to avoid direct sunlight[176,177,206], and all controls were UK-based, where vitamin D deficiency is expected to be prevalent due to low number of yearly sun hours[180,192]. In the SR cohorts, GvHD diagnosis had already been made so baseline samples were taken prior to first ECP cycle. Baseline concentration of 25(OH)D^3 was lower in patients with SR aGvHD compared to control groups, following the same rationale as newly diagnosed patients[176,177,206]. Regarding ECP, only two studies have investigated 25(OH)D^3 in this setting: one was performed in a small cohort of patients on ECP for various indication (only 2 of them for cGvHD), trying to elucidate whether UVA radiation emitted during the procedure could impact on 25(OH)D^3, but results were inconclusive[274]. The second aimed to correlate 25(OH)D^3 with GvHD biomarkers, but it could solely link 25(OH)D^3 with ST2[257].

Elafin is a serine protease inhibitor secreted during epithelial damage[275] that as a specific marker of skin GvHD[101,104]. Thus, it is not surprising that in the *de novo* aGvHD cohort its concentration was higher at diagnosis than in controls because most patients (88%) had skin involvement. Moreover, levels of ST2 and REG3α were also raised in this group compared to AN controls. Nearly half of patients (44%) had gut GvHD at diagnosis, and 1/4 of them suffered from severe disease. In patients with SR aGvHD, all biomarkers were higher than both control groups, as most patients displayed grade II-IV skin and/or gut GvHD. ST2 and REG3α have

been associated with lower gut GvHD[94,96,102,109,116], especially when they act combined[98]. REG3α levels are increased during bowel inflammation, as also seen in patients with Crohn's disease[117]. Furthermore, ST2 has an active role in immunity[120,121], and acts as an anti-inflammatory molecule in response to the pro-inflammatory cytokine IL-33 during GvHD[125]. These biomarkers have mainly been investigated in aGvHD and their presence in studies with cGvHD is rare. In this study, only levels of REG3α were increased threefold at baseline in patients with SR cGvHD compared to AN controls. This was also reported by one small study in patients with similar characteristics, who also had higher levels of elafin and ST2 prior to ECP[257]. Another study performed in skin samples showed than patients with chronic lichenoid skin GVHD and higher levels of elafin were more likely to become resistant to steroids compared to a similar cohort with lower levels of this biomarker[95]. Also, ST2 was part of a biomarker panel that predicted cGvHD at three months post-HSCT[105]. Currently, other biomarkers are under investigation in order to provide more accurate information in the context of cGvHD[105,110,258].

Vitamin D and GvHD biomarkers were also compared with organ involvement and GvHD grade: In aGvHD, there was a trending in levels of ST2 at diagnosis, higher in patients with grade III-IV compared to those with grade I-II. No other associations between study variables and the maximum overall grade of GvHD at diagnosis were found. The association of ST2 with aGvHD grade was reported by one study performed in the cord blood recipients where high levels of ST2 at day +28 post-HSCT correlated with the most severe forms of GvHD[97]. Other publications demonstrated the link between aGvHD grading and elafin[94,95,101] or REG3α[94], but these results could not be reproduced in this cohort. In relation to organs affected with aGvHD at diagnosis, there was an association between elafin concentration and staging of skin aGvHD. This was also found in SR aGvHD at baseline, with a

significant difference in increasing concentration of elafin as skin GvHD worsened from stages 0, 2 to 3 (15 *vs* 126.9 *vs* 220 ng/ml). Apart from discriminating GvHD-related from unspecific rash, elafin was found raised in higher stages of skin GvHD (specially between stage 2 and 0-1) in a study carried out in 492 patients post-HSCT[101]. No other significant associations were found linking 25(OH)D^3 or other biomarkers to any specific organ involved at diagnosis. Nevertheless, at 1 month post-treatment, there was a strong association between vitamin D levels and GvHD grades in *de novo* aGvHD cohort, being 25(OH)D^3 concentration nearly double in mild compared to more severe grades (41.6 *vs* 23.3 nmol/L). This has also been reported from two studies where patients with grade II-IV and III-IV aGvHD, respectively, presented lower levels of 25(OH)D^3 than their mild GvHD counterparts[193,207].

Therefore, in this study the three studied biomarkers acted as surrogate markers of aGvHD diagnosis, particularly elafin when skin was involved.

Interestingly, neither 25(OH)D^3 nor GvHD biomarkers were affected by the use of concomitant steroids, which could be owed to the negligible systemic absorption of topical steroids, and the fact that only one subject was on systemic IST. Probably longer treatments course is needed to produce a noticeable impact on their serum levels, especially in vitamin D where treatment with corticosteroids has been reported as a potential cause for its deficiency in HSCT recipients[215,226].

Can vitamin D or GvHD biomarkers be used for follow-up of GvHD?

Clinical trials in aGvHD have evaluated day 28 after commencing on steroids as an endpoint for *initial response to therapy*[259,260]. This has been validated in studies with

biomarkers, where elafin, ST2 and REG3α (among others) correlated with GvHD activity and its response to IST at this endpoint[94,97]. Furthermore, d*urability of response to IST* is another endpoint of interest that has been explored as a tool for long-term assessment in aGvHD (3-6 months after starting on steroids) and re-evaluation of current therapy, to taper IST if disease response or consider other strategies if treatment failure[50,260]. Nonetheless, the applicability of GvHD biomarkers at these long-term time points remains unknown.

At 1 and 3 months, significant differences in levels of ST2 and REG3α between patients with aGvHD and AN controls continued, but at 6 months solely the difference in REG3α remained significant. Across the study, differences were also found at different time points in ST2 and REG3α between patients with acute and chronic SR GvHD and controls. This is in line with the studies previously mentioned, where levels of ST2 and REG3α were higher in patients affected of aGvHD although they were measured at earlier time points[94,96,102,109,116].

Even though patients with aGvHD responded to steroids, concentrations of vitamin D or any of the biomarkers did not vary at any of the consecutive assessments. In the cGvHD cohort, levels of ST2 doubled after one month despite most patients remained on moderate GvHD and only one improved clinically. Therefore, none of the variables could be used to monitor response to treatment in this study.

Furthermore, the small cohort of patients who relapsed from aGvHD at 3 and 6 months did not allow to draw significant conclusions. Larger studies could examine whether GvHD relapse could be linked to vitamin D deficiency, as there is a lack of publications focusing on this particular topic.

Do vitamin D or biomarkers impact on survival?

Acute GvHD is the main cause for early non-relapse mortality (NRM) post-HSCT[49,50], and response to IST 4 weeks after commencing on steroids for aGvHD has been assessed as an endpoint to predict prognosis[261]. However, this early time point may underestimate the actual NRM as this does not take into account further factors that can also impact negatively on post-HSCT outcomes (including cGvHD)[259].

As for GvHD biomarkers, they have shown their predictive value in clinical studies[94–99]: ST2 and REG3α, as part of the MAGIC Algorithm Probability (MAP), predicted NRM in early phase of GvHD[98,100]. These two biomarkers have also taken part of the *Ann Arbour score*, which correlated with a higher NRM as score increments[103]. In cord blood recipients, higher ST2 levels at day 28 post-HSCT were also linked to higher NRM[97]. In addition, raised cutaneous elafin was associated with lower 2-year OS[95]. In this report, REG3α levels at diagnosis and 1 month after commencing on steroids were raised in patients with aGvHD who eventually died compared to survivors. Interestingly, a high concentration of REG3α was seen in one of the survivors (patient *number 2*) probably due to clinical stage 2 gut aGvHD. Moreover, ST2 levels were also increased in deceased participants 1 month after diagnosis. As previously described, this is in line with a number of publications linking REG3α and ST2 with patients outcomes[94,96,102,116]. Furthermore, in the SR aGvHD cohort there was a remarkable difference in levels of 25(OH)D^3 at baseline, lower in deceased patients than survivors (30.3 *vs* 51.7 nmol/L). Von Bahr *et al* reported how vitamin D deficiency peri-HSCT could impact detrimentally on survival[169], supported by a number of publications in HSCT recipients[22,68,203,208]. However, it is unclear whether these results are biased as this particular population may require prolonged

hospitalisations and their performance status may refrain them from spending time outdoors, resulting in a lower exposure to sunlight (among other cause for vitamin D deficiency).

Thus, REG3α and ST2 could be used as prognostic markers early after aGvHD diagnosis. Furthermore, vitamin D may have a prognostic value in those patients with aGvHD who are refractory to first-line IST.

Is there any correlation between vitamin D and GvHD biomarkers?

The only long-lasting significant association between variables was found in the SR cGvHD population between 25(OH)D^3 and elafin, at baseline and at the first month (see Figure 5-6 and Figure 5-7 in *Supporting data*). Two different publications have reported a significant association between 25(OH)D^3 and ST2[123,257], but none of the associations found in this study have been reported yet.

Further analysis

The only significant difference found between the three study populations at baseline was the concentration of ST2, higher in both acute compared to the chronic group (results in *Supporting data*). Acute GvHD is an exacerbated inflammatory disease, and when it becomes resistant to steroids it entails long-lasting alloreactivity with subsequent cumulative tissue damage[45,47]. This hyperactive immune system can explain the significant increment of ST2 to offset the detrimental effect of IL-33 as GvHD worsens[118,119,121]. Although intense, the inflammatory phase in cGvHD can be more subtle than in aGvHD[65,107], supporting the hypothesis that pathophysiology behind each different type of GvHD may justify the discrepancy found in ST2 concentration.

3.8.1 Study strengths and weakness

3.8.1.1 Study design

Sample size could not be calculated beforehand because similar studies with comparable objectives have not been done before. This study comprised a low number of patients for different reasons: Firstly, GvHD has lower prevalence in the UK compared to other countries due to the administration of *in vivo* lymphodepletion with alemtuzumab or ATG as part of the conditioning pre-transplant. Besides, recruitment last only one and a half year as this study was part of a MD project with limited resources (including time and funding) and only four NHS centres accepted to participate in it. Moreover, the volume of patients with SR GvHD treated at Rotherham has considerably decreased over the past three years as other ECP units have opened in its surroundings.

Nonetheless, this study was conducted in three different populations with GvHD, and inclusion criteria were strictly followed. Research and recruitment bias were avoided having a well-trained in-site clinician responsible for patient recruitment, sample collection and transport to local research laboratory for processing and storage. Due to sample size, it would not have been meaningful to correlate clinical transplant data with GvHD biomarkers and vitamin D results, thus it was not performed.

Owing to the small sample size, results should be interpreted cautiously. Although these findings cannot be extrapolated to the entire transplant population, this points towards an active role of vitamin D in the immunosuppressive effect of steroids in patients newly diagnosed with aGvHD. As future measures to ensure higher number of study participants, longer recruitment period with a higher number of study

centres involved (including those with larger ECP units), alongside logistic support and funding from national (*British Society of Bone and Marrow Transplantation and Cellular Therapy*) and international (*European Bone and Marrow Transplant*) organisations with special interest in research in the HSCT field should be sought.

3.8.1.2 Laboratory analysis

This study was highly controlled in order to achieve the least variability possible: all samples were batched up together and sent off to Rotherham General Hospital to be analysed at the same time. Correspondent reagents for elafin, ST2 and REG3α had the same lot number in order to prevent lot-to-lot variation and obtain a homogenous result and were analysed in the same laboratory to avoid between-laboratories biases. Furthermore, vitamin D ELISA was also carried out in the same laboratory with the same reagents on the same day to avoid intra-laboratory bias.

3.9 Conclusion

This is preliminary data and although statistical significance has not been reached in a number of the analysis performed, the results obtained here have potential clinical relevance.

Although our findings do not provide definite evidence of causal relationship between vitamin D and response to IST, vitamin D levels may exert an immunosuppressive effect in the long-term response to steroids. Besides, 50 nmol/L could be a recommendable cut-off to define vitamin D deficiency in HSCT recipients to start on supplementation, and 30 nmol/L on replacement therapy. Furthermore, vitamin D may have predictive prognosis, particularly in patients with aGvHD who

fail to response to steroids. Moreover, monitoring vitamin D post-HSCT and managing its deficiency accordingly is highly recommended.

In patients with aGvHD, elafin, ST2 and REG3α are useful diagnostic tools, especially elafin when skin is involved. Despite none of these markers nor vitamin D proved to be effective for follow up in any of the populations, ST2 may play an important role in this SR aGvHD. Moreover, REG and ST2 may predict outcomes in early stages of aGvHD. Apart from the discrete initial result of raised REG3α, the small number of patients in chronic SR GvHD precluded from definitive conclusions in this cohort. Further research is warranted to evaluate the utility of serial measurements of biomarkers to tailor IST based on their concentration, meet the needs of individual patients and maximise therapeutical benefit.

Although the number of patients included in this study was low, and thus limited in statistical power, these results may support the contribution of vitamin D in the dysregulated immunity underlying GvHD, providing the basis for further studies with larger numbers of patients to better understand the potential interaction with IST in relation to clinical manifestations, prognosis and biomarkers of GvHD.

4 Donor lymphocyte infusion as treatment for mixed chimerism and relapsed disease following reduced intensity conditioning allogeneic haematopoietic stem cell transplantation: a single centre experience.

4.1 Introduction

As discussed in the first three chapters of this thesis, vitamin D has an impact on immune responses in allogeneic HSCT. Its deficiency can lead to immune disorders, including graft-versus-host disease (GvHD). One of the main causes for this is donor lymphocyte infusion (DLI), an adoptive immunotherapy indicated for mixed chimerism (MC) and/or relapsed disease following HSCT. DLI is a common practice in the UK because of the extended use of reduced intensity conditioning (RIC) prior to stem cell infusion[276]. The use of RIC has allowed elderly patients to become candidates for transplantation as it diminishes transplant-associated complications[9,10]. However, it may fail to achieve full donor chimerism (FDC) (≥95% of donor cells), entailing higher risk of graft loss and disease relapse[10,277]. Many conditioning regimes in the UK contain lymphodepleting agents to promote engraftment of donor haematopoietic stem cells[278]. Amongst them, the most commonly used is alemtuzumab (CAMPATH-1H), a monoclonal antibody against

CD52 antigen expressed in lymphocytes for *in vivo* lymphodepletion[276,278]. However, MC is more likely to occur following RIC with alemtuzumab[279] but studies looking into this are scarce and little is known of the factors to attain FDC or disease remission, and those that contribute to improve survival[29–33,277,280]. Thus, this is a good opportunity to study a UK-based population of post-HSCT recipients requiring DLI, to identify patients and donors' factors that can predict achievement of FDC and disease remission, describe potential complications (including GvHD) and their impact on patients' outcomes, increasing the evidence-based knowledge in this field to optimise this therapy accordingly.

4.1.1 Donor lymphocyte infusion

The infusion of DLI has proved to be a feasible and effective strategy in high-risk diseases (prophylactic DLI), MC (where recipient cells coexist with <95% of donor cells)[28], positive minimal residual disease (MRD) (preemptive DLI) and overt disease relapse/progression (therapeutic DLI)[34,35]. Donor T cells are administrated to counteract the remaining immunocompetent host cells and achieve FDC, in cases of MC, or target tumour cells, in relapsed disease[36]. This procedure may entail complications, including GvHD and graft failure[35,38], as described in 4.1.4.

4.1.2 Effect of DLI on chimerism

The most common technique for monitoring chimerism following HSCT is the semi-quantitative fluorescent polymerase chain reaction (QF-PCR) of short tandem repeats (STRs or microsatellites). STRs are highly polymorphic tandem repeats found across the genome that enables differentiation between individuals[281]. Since

this is a semi-quantitative assay, it allows to monitor the dynamics of chimerism, differentiating between progressive or stable MC[28].

When MC is diagnosed, the best approach prior to DLI infusion is the reduction of dosage of immunosuppression[34]. If this does not improve chimerism, DLI is the next step to attain FDC. Clinical studies in the RIC setting have shown that the rate of patients achieving FDC after DLI ranges from 37% to 86%[29–33]. In a clinical trial where 36 patients received DLI at day +60 post-HSCT, 60% of patients with MC attained FDC[29]. In a study where patients with Hodgkin's disease (HD), 86% of those receiving DLI for MC attained FDC[30]. Another study with heavily treated patients with follicular lymphoma (FL), 17/28 (60.7%) patients with MC attained FDC. Also, a study performed in 65 patients with different haematological malignancies, 9/15 (60%) patients achieved FDC after DLI[32]. Lastly, a retrospective study with 27 paediatric patients with non-malignant diseases reported that 37% of them attained FDC 6 weeks after DLI, although this is an early time point to assess response to chimerism[33].

The incidence of MC is lower in peripheral blood stem cell (PBSC) recipients compared to bone marrow (BM), possibly explained by the higher number of stem cells infused in the former and the elimination of the recipient haematopoietic cells by the graft T cells[28]. Non-myeloid malignancies[277] and GvHD post-DLI[280] have also been found to be predictive of FDC following DLI**.** Apart from this, no other factors have been linked to improvement in chimerism[282].

4.1.3 Effect of DLI in disease relapse

Graft-versus-Tumour (GvT) effect (also seen in the literature as Graft-versus-Leukaemia (GvL) effect) consists of an immunologic reaction where antigen-specific T cells target tumour cells[11]. The immune cells involved in this phenomenon are predominantly CD4+ and CD8+ T cells. These cells target minor histocompatibility antigens (mHA), which are polymorphic peptides expressed by HLA molecules in both tumoral and healthy recipient cells[11,283]. The GvT effect facilitates the eradication of the malignant cells, so disease control can be achieved[37]. Nevertheless, GvHD is the major complication associated with DLI and this is linked to GvT effect[18,39]. This was firstly published by Weiden *et al* in the late 70's, where relapse rate was 2.5 times less prevalent in patients who developed GvHD compared to those who did not[16], as confirmed afterwards[18].

In the RIC setting, patients with relapsed disease have a response rate to DLI between 58% to 77%[30,31]: In a study where patients with HD relapsed post-HSCT, 58% achieved CR after DLI[30]. Also, in a similar scenario but where patients had FL, 10/13 (77%) achieved CR after disease recurrence[31]. Similarly, a study were patients received DLI for relapsed disease but most of them (86%) underwent myeloablative conditioning (MAC) HSCT, 54 (34%) were in remission after DLI[284]. Depending on the type of malignancy and tumour size, the dose of DLI may differ[18] but high dose can have a detrimental effect on patients' health[41] thus the best initial dose of CD3+ to exert its optimal effect remains unknown[34]. Some variables can contribute to disease response to DLI, including low tumour burden, relapse beyond 1 year after HSCT and cGvHD[36]. Since patients with high tumour burden can be less responsive to DLI[36,285], cytoreductive therapy prior to it may be required. This approach has not shown to impact on disease status or outcomes post-DLI[36,41,286],

but achieving CR prior to DLI has proved to be a modifiable factor that can influence on DLI efficacy[34]. No other donor or patient factors have been associated with the response to DLI in the context of relapsed disease[36,41].

4.1.4 Complications following DLI

4.1.4.1 Graft-versus-host disease

GvHD is the most common treatment-related complication following DLI. Its presentation and treatment does not differ from after HSCT[287], although it has been reported a later onset when it occurs after DLI[35]. GvHD can be associated with GvT effect, facilitating the eradication of the malignant cells and leading to a lower relapse rate and greater survival[288,289]. However, GvHD can also have a deleterious impact on outcomes, and strategies such as dose-escalation of DLI have shown to dissociate this harmful effect from GvT[18,34,35,41].

Following allogeneic HSCT, acute GvHD (aGvHD) occurs in 10 to 80% of patients[47] and chronic GvHD (cGvHD) between 30% to 70% of them[46]. GvHD rate after DLI is lower, as shown in recent studies in the RIC setting: aGvHD 11% to 37%, cGvHD 18% to 59% and any type of systemic therapy-requiring GvHD 6% to 42%[29–33,280]. Moreover, studies where more than half of the patients underwent MAC HSCT showed that the incidence of GvHD is generally higher: aGvHD 33% to 43%, cGvHD 33% to 46% and any of systemic therapy-requiring GvHD is 8.5% to 55%[36,41,284].

Different risk factors can trigger GvHD after DLI, including chimerism response[280], timing between HSCT and DLI[32,36,41], initial T cell dose $\geq 1\times10^{8}$/kg[41] and donor age:

older donor age has been reported as an independent risk factor for acute and chronic GvHD after HSCT[66,290], and particularly those who are older than 31 years of age have a detrimental effect on patients outcomes[291–293]. Interestingly, one study showed that the incidence and severity of GvHD depended on patient/donor matching (worse and more prevalent among those with unrelated donors)[18], but these results could not be reproduced[285]. No other patient or donor factors were associated with the development of GVHD post-pDLI[36,38,41,280].

4.1.4.2 Graft Failure post-DLI

Apart from attacking tumoral malignant cells and exerting GvT effect, graft T cells can react against the host haematopoietic stem cells due to the expression of mHA in their surface, leading to bone marrow aplasia and graft failure (GF)[294]. This usually occurs within 6-8 weeks after DLI infusion[35]. Early reports estimate the incidence of aplasia post-DLI or GF in 19%-34%[294–296], but recent studies have shown that the current incidence is lower than previously described (10%)[34,36,41]. In one of them, GF post-DLI was only developed by 3/118 patients (2.5%), where 1 of them died of relapse shortly after DLI[36]. Some publications agreed that this phenomenon is more prevalent in patients with MC and predominant host hematopoiesis[294,295], but it has also been described in patients with FDC[296].

4.1.5 Outcomes following DLI

Survival

DLI has a therapeutic benefit in disease control and leads to an improvement on outcomes in the MAC[20] and RIC[9,10] setting. In the latter, the incidence of overall survival (OS) varies depending on the underlying disease[34] and the clinical

indication for DLI: one study showed that the 5-year OS in patients with MC or relapsed/progressive disease was 80% and 40%, respectively[280]. Similarly, another publication reported a 5-year OS of 73% in the MC subgroup and 42% in those who relapsed or progressed[32]. Furthermore, in patients with FL who underwent DLI for either MC or relapse, 1-year and 4-year OS rates of the whole population were 80% and 76%, respectively[31].

Some factors have proved to have a detrimental impact on survival in patients undergoing DLI: male patients[284], patient age ≥60 years[41], high-risk lymphoproliferative disorders[41], active disease prior to DLI[34,284], higher tumour burden at relapse[284], poor-risk cytogenetics[284], shorter timing from HSCT to DLI[32,41] and maintaining MC after DLI[280,282]. Focusing on the latter, different studies in the RIC setting have reported the favourable effect of achieving FDC after DLI on OS: In a study with MDS/AML patients, those who attained sustained FDC or stable MDC after preemptive DLI had a significantly superior 5-year OS compared to those who did not attain it (91% *vs* 62%)[280]. Besides, another study showed that patients who achieved FDC had higher 2-year OS compared to those who remained on MC (95% *vs* 57%)[282]. Moreover, a study performed in patients who underwent MAC HSCT correlated grade II-IV aGvHD with poorer OS[284]. However, a study in the RIC setting did not find any significant association between mortality and aGvHD[29]. Similarly, cGvHD has shown to exert a favourable impact on relapse and survival in the MAC setting[284,289] although this could not be reproduced in the context of RIC[29].

Relapse

Relapsed or progressive disease is one of the main causes of mortality following DLI[21,23], especially in the era of RIC[10,12]: The 5-year relapse/progression rate in

patients who had DLI for relapsed disease was 69%[280]. In patients with MC, the 4-year relapse incidence was 5%[30]. Last but not least, the 1-year and 4-year estimated relapse risk after DLI in a mixed population with MC and relapsed patients were 16% and 26%, respectively[31].

Risk factors contributing to an increased risk of relapse following DLI are timing from HSCT to DLI less than a year[41], patient age greater than 60 years[41], initial T cell dose ≥1×10^7/kg[41] and unchanged MC after DLI[30,31].

GRFS

Graft-versus-host disease-free/relapse-free survival (GRFS) is a composite endpoint that encompasses grade III-IV aGvHD, cGvHD requiring systemic therapy, relapse and death within the first year post-HSCT. It is a more meaningful marker of HSCT success compared to other outcomes such as OS because the latter does not account for ongoing morbidity[297].

Both patients and donors clinical factors have been identified to impact on this: One study where nearly half of them underwent RIC reported a 1-year GRFS of 31%, which was better in paediatric patients, those with BM sibling donors and patients with low-risk disease[297]. Moreover, a publication with patients in the MAC setting stated that PBSC, matched unrelated donor (MUD) and high-risk disease were associated with worst GRFS. One-year GRFS was also 31%[298]. Another study reported that 1-year GRFS was 55% after MAC HSCT. Older patient and donors age, as well as high-risk disease and diagnosis of acute lymphoblastic leukaemia (ALL) were linked to a worse GRFS. Interestingly, this study showed that prophylactic DLI had a favourable impact on GRFS[288].

As previously described, studies published so far have used GRFS as a useful and accurate marker in the recovery following allogeneic HSCT. However, the role of this endpoint after DLI has not been explored yet.

4.2 Gaps in the knowledge and rationale

Different studies have shown the favourable impact of DLI on outcomes of patients with MC and relapsed disease, including higher overall survival and lower relapse rate[30,32,41,285]. In addition, the reported incidence of acute and chronic GvHD is lower than post-HSCT[29–33,280]. Nevertheless, there are a limited number of publications about DLI experience in the context of RIC HSCT, and they come predominantly from UK-based centres[29–33,277,280]. Moreover, there are discrepancies across the studies in the RIC setting in i) heterogeneity in patient population; ii) limited indications for DLI; iii) wide range of conditioning regimes used; iv) diversity of type and dosing schedules of *in vivo* lymphodepleting agents (although most recruited patients received alemtuzumab[276,278,279], ATG (anti-thymocyte globulin) has also been used[32,280]); v) different DLI dosage[31,32,280]; and vi) lack of well-defined endpoints.

Large prospective studies and clinical trials to add robustness to the current evidence-based data in recipients of RIC HSCT are lacking, hence it is difficult to reach solid conclusions about the immunological effects of DLI in this landscape. This study aims to assess the experience of a UK transplant centre and define the role of DLI in MC and recurrent disease following RIC HSCT.

4.3 Study overview

4.3.1 Study hypothesis, objectives and endpoints

I hypothesised that DLI improves outcomes following RIC allogeneic HSCT and it is therefore justified in the management of patients diagnosed with MC or relapsed disease. I also sought to explore factors impacting on survival and response to treatment (achieving FDC in the MC cohort, and disease remission in the relapse cohort), and establish toxicities associated with current dose schedules.

4.3.1.1 Primary objective

To determine survival following DLI for treatment of MC or disease relapse after RIC HSCT

4.3.1.2 Secondary objectives

Following DLI for treatment of MC or disease relapse after RIC HSCT, I aim to determine:

i) Response to DLI

ii) Clinical factors that contribute to disease response

iii) Clinical factors that impact on survival

iv) Outcomes following DLI

v) Impact of dose of DLI and interval between doses on the incidence of GvHD

vi) GRFS in MC cohort

4.3.1.3 Primary endpoints

Overall survival at 1, 2 and 5 years after first dose of DLI for MC or disease relapse

4.3.1.4 Secondary endpoints

i) Overall response rate to therapy: Rate of patients achieving full donor chimerism (MC cohort) or disease remission (relapse cohort) after DLI.

ii) Relapse post-DLI: Cumulative incidence of relapse after DLI

iii) Non-relapse mortality: rate of deceased patients due to other causes than relapse

iv) Disease-free survival (DFS): Rate of patients who have not relapsed after DLI

v) Incidence of acute and chronic GvHD following DLI: Cumulative incidence of acute and chronic GvHD

vi) DLI dose at GvHD onset

vii) Incidence of GF post-DLI: rate of patients with GF

viii) GRFS following DLI in the MC cohort

4.3.1.5 Inclusion and exclusion criteria

4.3.1.5.1 Inclusion criteria

Adult patients who received preemptive or therapeutic DLI after RIC HSCT followed-up at RMH from 1st January 2005 to 31st December 2016.

4.3.1.5.2 Exclusion criteria

i) Adult patients who have received MAC conditioning prior to HSCT

ii) Paediatric patients

4.3.2 Methods and statistical analysis

Transplant patients were identified following the inclusion criteria from the PROMISE database at the Royal Marsden Hospital. Data were collected retrospectively from this database and EPR (Electronic Patient Records), including patients and donors characteristics, that are displayed in Table 4-1. Data was kept on a spreadsheet in an encrypted laptop. For statistical analysis purposes, patients with MRD positive (n=2) were included within the relapse cohort. *Relapsed post-DLI* in the relapsed cohort was defined as subsequent recurrent disease in those patients who entered remission following treatment with DLI.

SPSS, version 25 (IBM Inc., Chicago, IL, USA) was used for statistical analyses and graphs generation. Descriptive statistics are presented as frequencies with percentages for categorical variables, and medians and interquartile ranges for continuous variables. The Chi-square test and the Mann-Whitney U/exact Kruskal-Wallis test were used to determine categorical and continuous variables (patient, disease and transplantation-associated variables), respectively, between groups (*responders* (patients who achieved FDC or remission after relapse) versus *non-responders* to DLI (FDC not achieved or disease progression)). Univariate comparisons to test significant predictors of treatment response were made using logistic regression, and those significant (p<0.05) were entered into a multivariate analysis. Survival curves were estimated with the Kaplan-Meier method. Cumulative incidence of relapse post-DLI was calculated by competing-risk analysis using Fine and Gray method, and non-relapse mortality (NRM) was the competing event. Cumulative incidence of acute and chronic GvHD after DLI were estimated by the same method but treating non-GvHD mortality as a competing risk.

Results are presented as medians with ranges and P values where appropriate. Where missing data points these variables were omitted from the analysis, but cases were still available for further testing.

4.3.3 Schedule for DLI at The Royal Marsden Hospital[299]

During the study period, indications for DLI at this centre were morphological or radiological disease progression, MC and low level of residual disease. This procedure was contraindicated in patients on immunosuppression and those with active GvHD.

For patients with disease relapse, the initial dose of DLI if sibling donor was $1x10^7$ CD3+/kg or $1x10^6$ CD3+/kg if unrelated donor (UD). If subsequent treatment with DLI was required, dose was escalated to $3x10^7$ CD3+/kg if sibling donors or to $3x10^6$ CD3+/kg if UD.

In patients with MC or residual disease, the starting dose was $1x10^6$ CD3+/kg if sibling donors or $3x10^5$ CD3+/kg if UD. If required, DLI dosage was escalated to $3x10^6$ CD3+/kg if sibling donor or $1x10^6$ CD3+/kg if UD.

Characteristics	Total (*N*=100)	MC (*N*=61)	Relapse (*N*=39)
Patients' age (years), median (range)	56.4 (17.6 – 71.4)	58 (19.4-70.8)	52.1 (17.6-71.4)
Patient sex, *N*			
Male/female	67/33	43/18	24/15
Disease, *N*			
AML	44	31	13
ALL	7	3	4
MSD	14	9	5
NHL	16	10	6
HL	10	4	6
MM	3	0	3
MPN	6	4	2
Disease risk			
Early	54	38	16
Intermediate	22	10	12
Late	24	13	11
EBMT score			
1-2	24	15	9
3-4	49	32	17
5-6	26	14	12
Unknown	1	0	1
Patient CMV, *N*			
Positive	47	33	14
Negative	53	28	25
Donor sex, *N*			
Male/female	60/40	34/27	26/13
Donor age (years), *N*			
≤31	25	18	7
>31	75	43	32
Donors' age (years), median (range)	45.3 (17.3-74)	45.1 (17.3-68.9)	44.4 (17.7-74)
Donor CMV, *N*			
Positive	38	27	11
Negative	61	34	27
Unknown	1	0	1
Matching, *N*			
Sibling	55	30	25
MUD	37	26	11
MMUD	8	5	3

Conditioning, *N*			
FMC	77	49	28
Flu/Bu/Campath	10	5	5
BEAM/Campath	6	3	3
Other	7	4	3
HSCT source, *N*			
PBSC	94	59	35
BM	6	2	4
Previous auto HSCT, *N*			
Yes/No	6/94	3/58	3/36
D/R gender, *N*			
Match/mismatch	49/51	30/31	19/20
D/R CMV, *N*			
Match/mismatch	64/35	39/22	25/13
Unknown	1	0	1
Acute GvHD post-HSCT, *N*			
None	68	48	20
Acute grade I-II	30	13	17
Acute grade III-IV	2	0	2
Chronic GvHD post-HSCT, *N*			
None	98	61	37
Mild	2	0	2
Moderate/severe	0	0	0
Time from HSCT to DLI (days), median (range)	221 (83-1699)	221 (88-690)	259 (83-1699)
Donor CD3+% pre-DLI, median (range)	45 (1-87)	26 (4-100)	77.5 (1-100)
Donor CD15+% pre-DLI, median (range)	100 (1-100)	100 (8-100)	100 (1-100)
Unfractionated whole blood donor chimerism%, median (range)	88 (12-100)	86.5 (15-100)	94.5 (12-100)
Number of DLI doses, median (range)	1 (1-3)	1 (1-3)	1 (1-3)
Maximum dose (x10^6/kg), median (range)	1 (0.10-100)	1 (0.10-50)	10 (0.5-100)
Cumulative dose (x10^6/kg), median (range)	1.5 (0.10-160)	1 (0.10-61)	10 (0.5-160)

Table 4-1: Patient and treatment characteristics

4.4 Results

One hundred patients were eligible for inclusion in the study. Sixty-one patients (61%) received DLI due to T-cell MC and 39 (39%) for relapsed disease. Median age at 1st dose of DLI was 56.4 years (range 17.6 – 71.4). The median follow-up time was 36 months (1.4-160.1). Characteristics of patients and donors, stem cell transplantation and DLI therapy are provided in Table 4-1.

4.4.1 Treatment response

4.4.1.1 Mixed chimerism cohort

Pre-emptive DLI was administrated to 61 patients due to **T-cell MC**. In this cohort, the median follow-up time from 1st DLI was 1345 days (55-4803).

The median T and unfractionated whole blood (UWB) chimerism pre-DLI were 27% (4%-87%) and 87% (15%-100%), respectively. Only 13 patients (21.3%) with T-MC had UWB FDC. Following DLI, the median T and UWB chimerism were 98% (0%-100%) and 100% (0%-100%), respectively. T-FDC was achieved in 40 patients (65.6%). When it happened, the median time from 1st DLI to achievement of T-FDC was 158 days (28-4803).

Factors impacting on achievement of T-cell FDC

Patients with female donors (22 (81.5%)) compared to male (18 (52.9%), OR 1.7 (1.4-5.3), *p*=0.004) and those whose donors were CMV (cytomegalovirus) negative (26 (76.5%) compared to CMV positive (14 (52%), OR 2.9 (1.4-5.8), *p*=0.004) contributed to attaining T-FDC following DLI (both variables were found statistically

significant in univariate and multivariate analysis). However, no other donor or patients' characteristics, including patient age (p=0.65), disease risk (p=0.87), HLA matching (p=0.36), CMV serostatus (p=0.27) or donor age (p=0.85) (among others displayed in Table 4-1) were statistically significant.

4.4.1.2 Disease relapse cohort

Therapeutic DLI was administrated to 39 patients: 33/39 (84.6%) due to overt **relapsed disease** and 6/39 (15.4%) to MRD positive. The median follow-up from 1st DLI was 529 days (42-4568). Nineteen patients (48.7%) achieved remission following DLI. The median time from 1st DLI to remission was 121 days (21-218). To decrease tumour burden and improve disease control, 31 patients (79.5%) received chemotherapy prior to DLI. However, this variable was not found significant in the different analysis carried out.

Factors impacting on disease remission

Only achieving FDC in the T lineage (T-FDC) (16 (76.7%)) *vs* T-MC (1 (12.5%), OR 18.7 (1.9-185.4), (p=0.012)) contributed to disease remission. However, there were no patient or donors' factors impacting on disease response (displayed in Table 4-1).

4.4.2 GvHD

Cumulative incidence of aGvHD at day +100 post-DLI was 23% (16-33). Twenty-nine patients (29%) developed **aGvHD after DLI**. Eight patients (27.6%) had grade I, 12 (41.4%) grade II, 6 (20.7%) grade III and 3 (10.3%) grade IV. The median minimum dose of donor CD3+ to trigger aGvHD was higher in patients with matched

related donors (MRD) (n=16) 5 x 10^6/kg (1-100) compared to those with unrelated donors (UD) (n=13), that was 1 x 10^6/kg (0.5-50) (*p*=0.041). The median dose of CD3+ to develop grade I-II aGvHD was 3.5 x 10^6/kg (0.5-100) whereas for grades III/IV was 5 x 10^6/kg (0.5-10) (*p*=0.78). In the **whole population,** only donors above 31 years of age (27 (36%)) *vs* ≤31 years (2 (8%), OR 5.5 (1.3-23), *p*=0.021)) contributed to a higher incidence of aGvHD post-DLI. This result was also reproduced in the **MC** cohort (>31 years: 16 (37.2%) *vs* ≤31years: 2 (11.1%), OR 5.3 (1.1-24.5), *p*=0.033). In the **relapse** cohort, only CMV positive donors (6 (54.5%)) *vs* CMV negative (5 (18.5%), OR 5.3 (1.1-24.5), *p*=0.026)) impacted negatively on aGvHD.

Cumulative incidence of cGvHD at 1-year post DLI was 22% (15.2-31.9). Twenty-four patients (24%) developed **cGvHD following DLI**. Nine patients (37.5%) had mild, 12 (50%) moderate and 3 (12.5%) severe. The median minimum dose of donor CD3+ to trigger cGvHD in patients with MRD (n=15) was 1 x 10^6/kg (1-50), similar to those with UD (n=9), that was 1 x 10^6/kg (0.5-10) (*p*=0.12). The median dose of CD3+ to develop mild cGvHD was 1 x 10^6/kg (1-50) whereas for moderate-severe was 1 x 10^6/kg (0.5-10) (*p*=0.67). In the **whole population**, only achieving T-FDC was a risk factor for developing cGvHD post-DLI (21 (34%)) *vs* T-MC (3 (10.3%), OR 4.4 (1.2-16.4), *p*=0.025). In the **MC** cohort, only female donors (10 (37%)) *vs* male (4 (11.8%), OR 4.4 (1.2-14.2), *p*=0.026)) had a significant impact on cGvHD. In the **relapse** cohort, there were no factors impacting GvHD.

4.4.3 Graft failure post-DLI

GF was diagnosed in 4 patients (4%), all in the MC cohort. Median time from 1st DLI to diagnosis was 121 days (35-233). However, only 2 of the cases (2%) occurred

within 3 months after DLI (35 and 63 days, respectively). One was treated with stem cell boost and another had a second allogeneic HSCT. At the date of censoring, both patients were alive.

4.4.4 Survival

The 1, 2, 5-year OS for the **whole** population was 73% (95% CI 63.7-80.7), 62% (95% CI 52.1-71) and 50% (95% CI 39.7-60.8), respectively. The cumulative incidence of NRM post-DLI at 1, 2 and 5 years was 22% (15.2-31.9), 28% (20.4-38.5) and 31% (22.8-41.9), respectively. At censoring, forty-seven patients (47%) had died, 26 (55%) in the MC and 21 (45%) in the relapse cohort. NRM occurred in 23 of the deceased patients (49%): 10 of these patients died of infection (44%), 1 of haemorrhagic cystitis and renal failure (4%), 1 of relapsed lung cancer (4%), 1 of pancreatic cancer (4%), 2 of GvHD (8%), 1 of liver failure (4%) and in 7 the cause is unknown (32%). Other factors, including dose of DLI or timing between doses were not found statistically significant.

Mixed chimerism cohort

OS at 1, 2 and 5 years was 85% (95% CI 74.4-92), 74% (95% CI 61.6-83.1) and 65% (95% CI 51.6-76.3), respectively. Patients whose donors are ≤31years (17 (94.4%)) compared to those whose donors were >31 years (23 (53.5%), p=0.007), donors who achieved/remained in UWB FDC (36 (76.6%) *vs* those who remained in UWB MC (4 (28.6%), p<0.001) and patients who developed acute GvHD post-HSCT (13 (100%)) *vs* those who did not (27 (56.3%), p=0.01) had a better OS. In the multivariate analysis, donors' age (p=0.013; HR 12.9 (1.7-96.7)) and post-DLI UWB

FDC ((HR 6.3 (2.5-15.9), p<0.001) were statistically significant. Nevertheless, none of the patients' factors were statistically significant (see Table 4-3).

Relapse cohort

OS at 1, 2 and 5 years was 54% (95% CI 38.5-68.4), 44% (95% CI 29.4-59) and 24% (95% CI 10.7-45.5), respectively. In this cohort, there were no patient or donor factors associated with survival. However, achieving either UWB-FDC (11 (45.8%) *vs* 2 (20%)), T-FDC (10 (45.5%) *vs* 1 (12.5%)) or M-FDC (10 (43.5%) *vs* 1 (12.5%)) was significant in the univariate analysis. After adjusting for these three variables, T-FDC (HR 4.1 (1.5-11.3), p=0.007) was the only one statistically significant.

4.4.5 Relapse post-DLI

In the **whole** population, the cumulative incidence of relapse post-DLI at 1, 2 and 5 years was 14% (8.6-22.8), 17% (11.0-26.3) and 24% (16.2-34.6), respectively. the univariate analysis showed that donor/patient gender mismatch (17 (33.3%)) *vs* match (6 (12.2%), p=0.009)), time from HSCT to DLI ≤6 months (13 (34.2%) *vs* >6 months 10 (16.1%), p=0.022) and not developing aGvHD post-HSCT (20 (29.4%) *vs* 3 (9.4%), p=0.028) had a significant impact on relapse. However, in the multivariate analysis only gender mismatch ((OR 4.0, 1.5-10.4), p=0.006) and time from HSCT to DLI (OR 3.0, 1.3-6.9), p=0.010) had a detrimental effect in relapse post-DLI.

Mixed chimerism cohort

Fifteen out of sixty-one patients (24.6%) relapsed following DLI. The cumulative incidence of relapse at 1 year was 14.8% (95% CI 8-27.1), at 2 years 16.4% (95% CI 9.3-29) and at 5 years 24.9 (95% CI 15.6-40). In this population, median donor

age was 45 years old, so this was used as a cut-off to create a categorical variable. Donor age >45 years (11 (36.7%)) *vs* ≤45 years (4 (13%), *p*=0.038), donor/patient gender mismatch (11 (35.5%) *vs* 4 (13.3%), *p*=0.038) and unfractionated whole blood (UWB) MC post-DLI (7 (50%) *vs* UWB FDC 8(17%), *p*<0.001) had significant impact on relapse. However, only donor age ((OR 3.9, (1.2-12.4), *p*=0.024) and unchanged UWB MC post-DLI ((HR 6.8, 2.2-20.6), *p*=0.001) were statistically significant in the MV analysis. NRM at 1, 2 and 5 years was 8.2% (95% CI 3.5-19.1), 14.8% (95% CI 8-27.2) and 16.6% (95% CI 9.4-29.5), respectively.

Relapse cohort

Eight out of nineteen patients (42.1%) relapsed from the primary haematological malignancy after achieving complete remission following DLI. The cumulative incidence of relapse at 1, 2 and 5 years was 12.8% (CI 95% 5.5-29.6), 17.9% (95% CI 9-35.7) and 21.3 (95% CI 11.2-40.3), respectively. In this population, none of the variables had a significant association with relapse post-DLI. NRM at 1, 2 and 5 years was 43.6% (95% CI 30.3-62.7), 48.7% (95% CI 35.1-67.7) and 54.7% (95% CI 39.0-76.8). At censoring, 26 patients (66.7%) had died.

4.4.6 GRFS

Analysis of this composite endpoint was not feasible in the relapse counterpart as this group of patients had already relapsed, and this endpoint only accounts for patients who are disease-free.

The GRFS of the MC cohort at 1-year post-DLI was 71%. Factors contributing favourably to GRFS were patients whose donors were younger than 31 years (15

(83.3%)) *vs* >31 years (15 (35%), *p*=0.008) and those attaining UWB FDC post-DLI (27 (57.4%)(vs UWB MC (3 (21.4%), *p*=0.001) and patients with intermediate/late disease risk (20 (87%)) *vs* early risk prior to HSCT (23 (60.5%), *p*=0.019). However, in the multivariate analysis only younger donors (HR 10.2 (1.2-77.2), *p*=0.025) and UWB FDC after DLI (HR 4.3 (1.7-11.1), *p*=0.002) were significant (see Figure 4-1).

	Total	MC	Relapse
OS			
1-year	73% (95% CI 63.7-80.7)	85% (95% CI 74.4-92)	54% (95% CI 38.5-68.4)
2 “	62% (95% CI 52.1-71)	74% (95% CI 61.6-83.1)	44% (95% CI 29.4-59)
5 “	50% (95% CI 39.7-60.8)	65% (95% CI 51.6-76.3)	24% (95% CI 10.7-45.5)
Relapse post-DLI			
1-year	14% (95% CI 8.6-22.8)	14.8% (95% CI 8-27.1)	12.8% (CI 95% 5.5-29.6)
2 “	17% (95% CI 11.0-26.3)	16.4% (95% CI 9.3-29)	17.9% (95% CI 9-35.7)
5 “	24% (95% CI 16.2-34.6)	24.9 (95% CI 15.6-40)	21.3 (95% CI 11.2-40.3)
NRM			
1-year	22% (95% CI 15.2-31.9)	8.2% (95% CI 3.5-19.1)	43.6% (95% CI 30.3-62.7)
2 “	28% (95% CI 20.4-38.5)	14.8% (95% CI 8-27.2)	48.7% (95% CI 35.1-67.7)
5 “	31% (95% CI 22.8-41.9)	16.6% (95% CI 9.4-29.5)	54.7% (95% CI 39.0-76.8)
DFS			
1-year	64% (95% CI 54.2-72.7)	77% (95% CI 65-85.8)	44% (95% CI 29.4-59)
2 “	55% (95% CI 45.1-64.3)	69% (95% CI 56.4-79)	33% (95% CI 20.4-48.6)
5 “	43% (95% CI 33.3-53.7)	57% (95% CI 43.8-69.2)	21% (95% CI 9.7-38.5)

Table 4-2: Outcomes of the study cohorts

4.5 Discussion

DLI is an immunological modality for cancer therapy that aims to blunt bidirectional tolerance and achieve disease control. Quantification of residual donor haematopoiesis can predict the risk of developing disease relapse or GF[10,277] thus chimerism should be regularly monitored after HSCT, ideally using QF-PCR of STRs[28,281]. In this study nearly 66% of patients with MC transformed into T-FDC

within 6 months after DLI. Female and CMV negative donors were more likely to overcome patients' chimerism and establish FDC successfully, regardless of patients' characteristics. Conversely, one study in the RIC setting didn't find any correlation between chimerism and patient or donor characteristics, stem cell source, immunosuppression or conditioning[282].

In the relapsed cohort, nearly half of patients achieved disease remission within 4 months after DLI. According to the literature, factors such as patient age[41], dose of DLI[41], timing between HSCT and DLI[41] and attaining T-FDC[30,31] contribute to disease control. This study could only confirm the latter, but it did not identify any patient or donors' factors linked to remission. Moreover, a published report where AML/MDS patients were treated with DLI for MC following RIC HSCT showed a 5-year DFS of 65%[280], slightly higher than our result in this cohort (57%). This difference could be explained by the different underlying malignancies studied or the dose of DLI administrated. Interestingly, one study suggested that infusion of high doses of DLI (>1x10^7/kg) could entail co-infusion of regulatory T cells that may hinder GvT effect and lead to increase relapse risk[41]. Nevertheless, I could not prove the link between DLI doses, or timing between doses, neither with relapse nor any other outcome.

Following RIC HSCT, the main cytotoxic effect relies on immunocompetent donor cells (GvT effect). Owing to the reduced antitumor activity of these protocols, adjuvant DLI can enhance GvT and facilitate the eradication of malignant cells with lower toxicity than chemotherapy[11,18]. As previously mentioned, GvT can be inherently linked to GvHD so strategies aiming to mitigate it and limit DLI toxicity have been sought, and dose-escalation has been the most successful[18,34,35,41]. In this study, the cumulative incidence of acute and chronic GvHD post-DLI were

similar (23% and 22%, respectively), and both were in keeping with published data[29–33,280]. Among risk factors for GvHD in this study, donors' factors, including older donor age (>31 years) and CMV positive serostatus contributed to aGvHD. The Nordic Bone Marrow Transplantation Group described the beneficial role of patients and donors' CMV positive serostatus leading to lower incidence of relapse (probably due to GvT effect), without additional increased in the incidence of acute or chronic GvHD[37,300]. Conversely, this data showed that patients with CMV positive donors were more likely to develop aGvHD in the relapse population. This could be a spurious result, but the small cohort size prevents us from drawing any significant conclusion. Furthermore, cGvHD was more prevalent in patients who achieved T-FDC and those with female donors in the MC cohort, as reported in the literature[280,290]. Nevertheless, neither patient/donor HLA mismatch[285] nor timing between HSCT and DLI[32,36,41] could be correlated to cGvHD in this study. Although HLA matching did not influence the incidence of aGvHD, higher doses of DLI were needed in the MRD setting to trigger it compared to the UD counterpart, which may suggest the contribution of not only HLA but mHA in the pathophysiology of this disorder[11,283].

In this study, one-year OS of the whole population was 73%, similar to one published report (80%)[31]. However, the 5-year OS in the MC cohort (65%) and relapse (24%) were lower than in previous publications[32,280]. These results may be underestimated by the retrospective design of this study alongside its heterogenic population, with different underlying haematological malignancies and disease risks. Publications looking into patients' outcomes following DLI in the context of RIC are limited thus further comparisons could not be made.

	T-FDC	Disease Remission	aGvHD	cGvHD	Survival	Relapse	GRFS
Female donor	Increased			Increased			
Donor age >31y			Increased		Decreased		Decreased
CMV – donor	Increased						
CMV + donor			Increased				
Donor/patient gender mismatch						Increased	
Attain T-FDC		Increased		Increased	Increased		
Attain WBC-FDC					Increased	Decreased	Increased
HSCT to DLI <6-12 months						Increased	

Abbreviations: y, years; +, positive; - negative.

Table 4-3: Variables impacting on patients' outcomes

In the univariate analysis, donor age below 31 years, attaining UWB FDC and acute GvHD post-HSCT had a positive impact on OS in patients in the MC cohort. However only donor age and UWC FDC were found to be independent risk factors for survival. Due to the impact of donor age on patients' outcomes following HSCT[288,291,301], unrelated stem cell donors registries are currently focusing on recruitment of younger donors. Attaining FDC after DLI has also been previously reported to improve survival[280,282], although they only focused on the T-cell fraction

of chimerism and did not include UWB. Interestingly, although this study could not confirm an association between GvHD and survival, this has been a matter of debate in the literature, particularly depending on the conditioning pre-HSCT: In the MAC setting, acute[284] and chronic GvHD[284,289] have shown opposite effects on survival following DLI but these results have not been confirmed in RIC[29]. In the relapse cohort, achieving FDC in any fraction of chimerism (UWB, T or M) was significantly associated with OS in the univariate analysis. After adjusting for these three variables, T-FDC was the only variable proved to be an independent risk factor for improved survival, as previously seen in the literature[280,282]. Nearly 80% patients in this cohort received cytoreductive therapy prior to DLI, but it did not impact on response to DLI, suggesting that survival is not corelated with sensitivity to chemotherapy. Since patient factors did not correlate with survival in either cohort, strategies to improve survival after DLI should focus on recruitment of younger donors and achievement of FDC, particularly in the T and UWB fractions.

In the whole population, the 5-year incidence of relapse post-DLI was 24%, similar to a study with patients with MC and relapsed disease[31]. However, these results differ from those published in MC[30] and relapse[280], which could be again due to the heterogeneity of the population included in the analysis alongside the sample size. Donor/patient gender mismatch and timing from HSCT to DLI below 6 months (one study showed that timing below 1 year impacted on relapse[41]) were identified as a risk factor with detrimental impact on relapse. In the MC cohort, ¼ patients (24.6%) relapsed following DLI. Donor age above 45 years and UWB MC post-DLI had significant impact on relapse in this population, confirming what has been already published[30,31]. In the relapse cohort, nearly half of patients (42.1%) had relapsed at the time of censoring, but none of the variables analysed had a significant association with relapse post-DLI. The rest of the results published, including patient

age greater than 60 years or initial T cell dose ≥1×10^7/kg[41] could not be reproduced in this study.

In keeping with current publications[34,36,41], incidence of GF secondary to DLI in this population was low (2%). It is estimated that this phenomenon occurs within 6-8 week after DLI infusion[35], as shown here. Although this is a rare complication following DLI, it can be life-threating and early treatment with stem cell top-up or second allogeneic HSCT may be required.

GRFS is a new composite endpoint that encompasses the most relevant life-threatening complications following allogeneic HSCT[297]. In the study population, 1-year GRFS was higher (71%) than post-HSCT (31%-55%)[288,297,298]. One of the reasons could be the selection of patients included in the study, since they did not suffer from severe co-morbidities associated, including GvHD, and were fit enough to undergo DLI. Furthermore, the incidence of GvHD is lower than after HSCT, including the most severe grades which impair patients' health and lead to a higher mortality rate[46,47]. Among the risk factors to impact favourably on GRFS, younger patient age[288,297], BM sibling donors[297,298] and low-risk disease[288,297,298] have been described in the literature. This study showed that patients whose donors were younger than 31 years and those attaining UWB FDC post-DLI impacted favourably on GRFS, as well as in survival since this is one of the main events included in GRFS[288,297,298].

At censoring, nearly half of the patients had died (47%), 55% in the MC and 45% in the relapse cohort. NRM occurred in 49% of the deceased patients, mainly due to opportunistic infections (44%). GvHD-related death only accounted for 8%, in keeping with published data[30,287]. One prospective trial using pre-emptive low-dose

DLI for patients with underlying haematological malignancies showed 1-year NRM of 14%[29], similar to another publication where patients with FL had DLI for MC or relapse (12%)[31]. Here, the cumulative incidence of NRM post-DLI at 1 year in the whole population was higher, 22%. Again, these differences could be due to the heterogeneity of this population, also considering that the relapse cohort was included in this analysis, which has a remarkably higher NRM (43.6%) compared to the MC (8.2%).

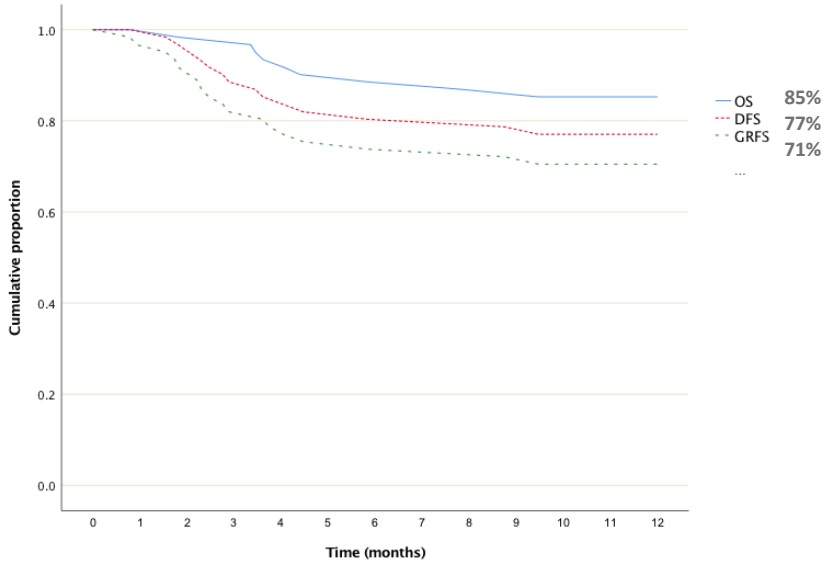


Figure 4-1: Kaplan-Meier estimates for OS, DFS and GRFS at 1-year post DLI

4.6 Conclusions

This study has shown that DLI is a feasible and effective immunotherapy for patients with MC and relapsed disease following RIC HSCT. Factors related to to attain achievement of T-FDC were donor sex and CMV status. However, they did not impact on OS in patients with MC, and female donors entailed a higher risk of

cGvHD. On the contrary, attaining T-FDC impacted favourably on remission and improved survival in patients undergoing DLI for relapsed disease, as previously reported[30,31]. Patients with younger donors and those who achieved UWB FDC after DLI had a better GRFS and OS, the gold-standard endpoint to demonstrate clinical benefit of DLI. Monitoring chimerism, including UWB fraction, should therefore be encouraged following HSCT and DLI. Moreover, GvHD after DLI was less frequent and severe than reported post-HSCT[29–33,46,47,280]. The beneficial actions of DLI through GvT effect outweighs its potential toxicity[11], and selection of younger male donors can minimise the risk not only of GvHD, but also relapse and mortality. The limitations of this study are primarily inherent to its retrospective nature, sample size and the heterogenic population.

This study supports the recruitment of younger male donors due to its favourable impact on outcomes and lower incidence of complications following allogeneic HSCT and DLI.

5 Conclusions

5.1 Introduction

I have presented in this thesis an in-depth investigation of the role of vitamin D in alloHSCT and response to IST in patients with GvHD, the current clinical approach for managing vitamin D deficiency and, the factors that potentially impact on outcomes of patients who undergo DLI following RIC HSCT:

In Chapter 2, an online survey across international adult and paediatric alloHSCT programmes showed the variations in the management of vitamin D deficiency. Since data is scarce in this population, recommendations were provided when evidence was available, including regular monitoring of $25(OH)D^3$ peri-HSCT and starting on vitamin D treatment following clinical indications.

Chapter 3 showed a unique exploratory study performed in GvHD patients. Despite the small sample size, a cut-off of $25(OH)D^3$ serum levels to commence on vitamin D therapy between 30-50 nmol/L was suggested. Interestingly, serum $25(OH)D^3$ may also predict outcomes in SR aGvHD patients. Furthermore, it confirmed the role of GvHD biomarkers as a diagnostic tool of aGvHD, particularly elafin when skin is involved. Nonetheless, the poor recruitment prevents from drawing significant conclusions and requires robust prospective studies to validate the previous results.

Chapter 4 moved away from vitamin D, but still focusing on the immune response of DLI in the context of RIC HSCT. DLI broadens the applications of donor

immunocompetent cells in order to achieve disease control after allogeneic HSCT although it may also induce GvHD. However, escalating DLI dose regimen is an effective intervention with relatively low incidence/severity of GvHD. Furthermore, it showed the importance of achieving T-FDC in patients with relapsed disease, as well as having a younger donor and attaining UWB FDC in those with MC.

5.2 Key findings

Numerous publications have consistently proven the immunomodulatory effect of vitamin D in the field of HSCT[22,169,203,216,217]. Current HSCT guidelines recommend monitoring serum 25(OH)D^3 (the best biomarker for vitamin D metabolism[189]) following HSCT, aiming to maintain mineral homeostasis and bone health[176,177,206]. However, they do not include the assessment of 25(OH)D^3 before HSCT nor the ideal serum cut-off to promote its immunoregulatory properties[176,177,206,218,219]. Despite the controversy raised in this topic, there is growing evidence that vitamin D deficiency can lead to post-HSCT complications such as GvHD[169,175,193,207], and its replacement can enhance the response to IST, as seen in asthmatic patients[87–90].

The survey in Chapter 2 showed the heterogenic clinical practice across the different EBMT HSCT units, as most of them do not follow any guidelines for management of vitamin D deficiency, resulting in a variety of cut-offs for vitamin D deficiency, monitoring, indications for replacement and dosage. The current cut-off is based on the optimal serum levels of 25(OH)D^3 to avoid rickets, but there is a lack of consensus in the concentration required to foster immune homeostasis[169,175,187,193,194]. Finding the ideal cut-off for vitamin D in immunoregulation would make a difference in the management of this deficiency, particularly in a vulnerable population as the recipients of allogeneic HSCT[142,204,206].

For this reason, the observational study carried out in acute and chronic GvHD patients described in Chapter 3 attempted to elucidate it. Although a specific threshold could not be found, results suggested that this may range between 30-50 nmol/L, in line with a number of publications[22,137,215,217,225,226].

Moreover, there is no agreement in the most appropriate treatment of this deficiency[170,185,186,203,209,225,226,229–233] as reflected in the survey where clinical indications and dosage of vitamin D therapy varied between centres, predominantly in the adult units. Vitamin D fosters the anti-inflammatory immune cells while abrogates the pro-inflammatory counterpart[136], hence replacement therapy could potentially reduce the incidence of immune disorders post-HSCT as GvHD[170]. In Chapter 3, higher levels of vitamin D at diagnosis in patients with aGvHD responding to IST at 1 month post-treatment supports its immunomodulatory effect. At baseline, serum levels of vitamin D were lower in deceased patients with SR aGvHD compared to alive patients at the end of the study. This result remains questionable in the light of the poor prognosis described in these patients, but it paves the way for further research in this field.

RIC has broadened the indications of allogeneic HSCT to patients unfit for MAC[9,10], but it also entails residual host immunocompetent cells resulting in mixed chimerism and recurrent of the primary haematological disease[10,277]. DLI can overcome this and establish full donor immunity due to GvT effect that can fight residual tumour cells, but this can also lead to GvHD[288,289]. However, there is a limited number of publications reporting this practice, mostly from the UK where RIC regimens with in vivo lymphodepletion are widespread[276]. Thus, Chapter 4 described the experience of a single UK centre, to establish the nature and severity of GvHD following DLI and explore factors that could contribute to the success of this therapy. As

previously shown[29–33,280], this study confirmed that the incidence of GvHD is lower than post-HSCT and when it occurs, it is less severe. Among other results, it showed that female and older donors could impact detrimentally in patients' outcomes, increasing the rate of acute and chronic GvHD, as seen following HSCT[280,290]. These findings reinforce the evidence that young male donors should be prioritised as stem cell donors when available and encourage their recruitment by unrelated stem cell donor registries. Focusing on this, the deleterious immune response produced by DLI could be mitigated, resulting in a lower (and less severe) rate of GvHD. Moreover, this study showed the favourable effect on survival of attaining FDC in unfractionated whole blood or T-cell lineage in the mixed chimerism and relapsed cohort, respectively.

Early diagnosis of GvHD and prompt start on IST are essential to improve patients' survival[47,76]. Elafin, ST2 and REG3α are GvHD biomarkers used as a diagnostic and prognostic tool that could confirm diagnosis at an early stage and monitor its response to IST[97,101,102]. Chapter 3 examined the use of these proteins, alongside vitamin D, in acute and chronic GvHD. In patients with aGvHD the three markers were raised at diagnosis (compared to non-GvHD controls), especially elafin when skin was involved. Although none of them could prove its role in following-up GvHD or monitoring IST, REG and ST2 were higher at early stages of aGvHD in patients who eventually died compared to those alive at censoring. This showed their role predicting outcomes, in line with recent publications[94–99]. Currently, these biomarkers are not part of the HSCT guidelines nor have been implemented in clinical practice yet, and their use is restricted only to aGvHD setting[95,105,110].

To conclude, strategies such as selection of stem cell (and DLI) donors and evidence-based management of vitamin D deficiency and early diagnosis and

tailoring of IST for GvHD could decrease complications derived from this condition and therapy, impacting favourably on patients' outcomes and improving their quality of life.

5.3 Challenges and limitations

For the observational study described in Chapter 3, the main challenge was patient recruitment. GvHD has low prevalence in the UK owing to the preference for *in vivo* lymphodepletion prior to HSCT[276]. In view of this, a number of actions were taken: i) recruitment period was prolonged to one and a half years; ii) further centres were invited to participate in the study; iii) a new cohort of patients with SR aGvHD was recruited, alongside the initial *de novo* aGvHD and SR cGvHD, to increase the sample size. Moreover, the initial rate of healthcare staff responding to the survey in Chapter 2 was low, so transplant physicians had to be emailed separately to encourage them to participate. Lastly, for DLI study in Chapter 4, retrospective data collection from an electronic database tends to be insufficient, so outstanding data had to be requested to data managers and stem cell lab staff. Moreover, GvHD staging may reported inaccurately, so thorough interpretation of the available data and reports was done to ensure credibility. Although most of the studies performed in the context of DLI are retrospective, data should ideally be recorded prospectively using a study questionnaire.

As limitations, the observational study in Chapter 3 has a small sample size that refrains from drawing any definitive conclusion, although it paves the way for larger clinical studies to power future results. In order to increase sample size, patients with GvHD either post-HSCT or DLI were included, as GvHD pathophysiology seems to be similar following both therapies[35]. Patients who underwent DLI may

have a better performance status following HSCT and suffered from fewer comorbidities than those with GvHD post-HSCT, and this could have affected their levels of vitamin D or GvHD biomarkers (although there is no evidence to support this in the current literature).

Furthermore, experience in the analysis of GvHD biomarkers is limited and only a few centres in the UK could perform it, Rotherham General Hospital being one of them. Assays were undertaken at the end of the study when all the study samples were collected, also using the same lot number for each reagent to avoid lot-to-lot variability, the same laboratory and staff to avoid inter-laboratories variation, and samples were blinded for analysis to eliminate bias.

Moreover, vitamin D samples very sensitive to sunlight, so they have to be safely protected covered in tinfoil material while transported. Vitamin D ELISA was also carried out in the same clinical laboratory with the same reagents on the same day to avoid intra-laboratory variation.

There may have been some degree of selection bias while recruiting controls from the Anthony Nolan cohort since all of them volunteered to participate. However, a broad range of age and race was selected in order to minimise it.

5.4 Dissemination of findings

Findings from Chapter 2 were displayed as poster presentation at the EBMT annual conference in Frankfurt, and a peer-reviewed manuscript was published in the journal *Biology of Bone and Marrow Transplantation*.

Results in Chapter 3 will be submitted as an abstract for the next EBMT annual conference and will be followed by preparation of the manuscript.

Findings from Chapter 4 were recently accepted for poster presentation at the next 2020 EBMT annual conference congress in Madrid, and a peer-reviewed manuscript is in preparation.

5.5 Future projects

Prospective studies with larger patient samples are warranted to define the threshold of vitamin D deficiency and better understand the role of vitamin D in the response to IST in GvHD patients. In order to recruit a larger number of patients to power the study for statistical analysis, these could be carried out as multicentric study with logistic support from BSBMT or EBMT.

Furthermore, prospective clinical trials including vitamin D replacement on HSCT recipients should shed a light in the optimal dosage required in the adult population.

Last but not least, guidelines focusing on the management of vitamin D deficiency in the HSCT community should be written to standardise criteria and be disseminated across the healthcare staff in alloHSCT units to become part of the standard of care of HSCT recipients (i.e. BSBMT, BCSH or EBMT guidelines).

5.6 Conclusion

In conclusion, vitamin D has proven immunoregulatory properties over the course of HSCT, and its deficiency could have a detrimental impact on outcomes post-HSCT.

Further research is necessary to confirm the ideal cut-off of serum vitamin D to justify replacement in the HSCT setting. In the meantime, regular monitoring and replacement therapy if clinically indicated should be encouraged. Furthermore, DLI is a safe and effective type of immunotherapy, with a favourable side effect profile (particularly in patients with male young donors) and its immune response can contribute to favourable patients' outcomes.

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Appendix 1 – "Current practice in vitamin D management in Allogeneic Haematopoietic Stem Cell Transplantation" survey

1) What is your EBMT centre identification code?

PLEASE COMPLETE QUESTIONS 2-5 REGARDING YOUR OWN EXPERTISE & PRACTICE

2) Who is completing this survey?
   - Bone Marrow Transplant Programme Director
   - Transplant Consultant
   - Clinical Nurse Specialist in Bone Marrow Transplant
   - Registrar/Fellow
   - Other (please describe)

3) Are you allowed to prescribe vitamin D?
   - Yes
   - No
   - Not applicable (vitamin D is an over the counter drug without a need for prescription in my country)

4) Are you involved in the care of patients who undergo an allogeneic HSCT?
   - Yes
   - No

5) If *yes*, at which level are you involved in these patients care? Select as many options as required
   - Pre-transplant
   - During the transplant (ward admission)
   - Early post-transplant (< 1 year)
   - Late post-transplant (> 1 year)

PLEASE COMPLETE QUESTIONS 6-30 REGARDING YOUR CENTER POLICY

6) Does your HSCT protocol include routine measurements of vitamin D in serum <u>prior</u> to allogeneic HSCT?
- Yes, in all patients (skip to Q8)
  - Yes, but only in those patients with risk factors for hypovitaminosis D
  - No (skip to Q8)

7) If *yes*, for which of the following patients do you request vitamin D prior to allogeneic HSCT? Select as many options as required
- Patients with known osteopenia/osteoporosis
  - Patients with premature menopause
- Patients with menopause
  - Patients who have received corticosteroids
  - Patients who have had a fracture
  - Other (please specify _____)

8) Do you routinely check vitamin D levels in serum <u>after</u> an allogeneic HSCT?
   - Yes, in all patients (skip to Q10)
     - Yes, but only in those patients with risk factors for hypovitaminosis D
   - No (skip to Q12)

9) If *only for selected patients*, for which of the following patients do you request vitamin D after to allogeneic HSCT? Select as many options as required
- Patients with known osteopenia/osteoporosis
- Patients with premature menopause
- Patients with menopause
- Patients who have received corticosteroids
- Patients who have had a fracture
- Other (please specify _____)

10) If *yes*, at what time point(s) after an allogeneic HSCT do you check it?
- Every 3 months
- Every 6 months
- Once a year
- Other (please specify _____)

11) If *yes*, do you check it during a particular season? Select as many options as required
- Yes, in winter
- Yes, in autumn
- Yes, in summer
- Yes, in spring
- Season is not taken into account

12) Where does the long-term follow-up occur?
- In your HSCT centre
- In a secondary centre (patient's local hospital)
- With the primary care physician
- Other (please specify_____)

13) For how many years post allogeneic HSCT are patients routinely followed up?

14) What is the cut-off value of vitamin D in serum for commencing on replacement in your centre?
- ≤ 25 nmol/L (10 ng/ml)
- ≤ 30 nmol/L (12 ng/ml)
- ≤ 50 nmol/L (20 ng/ml)
- If other, please specify _____
- I do not use cut-off values

15) Do you have any local guidelines or SOP (S*tandard Operation Procedures*) for the management of vitamin D deficiency?
- Yes (please specify which guidelines _____)
- No

16) Do you follow any national or international guidelines for the management of vitamin D deficiency?
- Yes (please specify which guidelines _____)
- No

17) For which of the following reason(s) do you measure vitamin D? (*Click as many options as required)*
- To maintain calcium metabolism and prevent bone loss
- To enhance the immune reconstitution post-HSCT
- To prevent GvHD
- To enhance the response to immunosuppression in patients with GvHD
- Other (please specify)

- I don't measure vitamin D at my centre

18) For which of the following reason(s) do you prescribe vitamin D? (*Click as many options as required)*
    - To maintain calcium metabolism and prevent bone loss
    - To enhance the immune reconstitution post-HSCT
    - To prevent GvHD
    - To enhance the response to immunosuppression in patients with GvHD
    - Other: please specify
    - I don't prescribe vitamin D at my centre

19) If indicated, who prescribes the vitamin D supplements?
    - Transplant physician
    - GP
    - Other (please, specify _____)
    - Not applicable (vitamin D is an over the counter drug without a need for prescription in my country)

20) How is vitamin D mainly prescribed?
    - Alone
    - Combined with calcium (*calcium carbonate*)
    - Within a multivitamin complex
    - I never prescribe vitamin D (skip to Q25)

21) Do patients start on a "loading dose" of vitamin D supplements?
    - Yes
    - No (skip to Q22)

22) If *yes*, which daily "loading dose" do you use?
    - 20 mcg (800 UI) per day
    - 50 mcg (2,000 UI) per day
    - 100 mcg (4,000 UI) per day
    - Other (please specify _____)

23) On average, how long are patients on the "loading dose" **for**?
    ____ weeks
    ____ months

24) What is the daily "maintenance dose" of vitamin D contained in the supplements you/GP prescribe/s?
    - 10 mcg (400 UI) per day
    - 20 mcg (800 UI) per day
    - 50 mcg (2,000 UI) per day
    - Other (please specify _____)
    - I don't know

25) Are vitamin D supplements discontinued eventually?
    - Yes
    - No (skip to Q25)

26) If *yes*, when?
    - If symptoms improve and patients feel better
    - If therapeutic levels of vitamin D are reached
    - If DEXA scan returns to normal
    - Other (please, specify _____)

27) Is there a dedicated osteoporosis service at your centre?
    - Yes
    - No

28) Do you routinely request DEXA scan after allogeneic HSCT?
    - Yes, for all patients
    - Yes, but only for patients with high risk of osteoporosis/osteopenia
    - No (skip to Q32)

29) Which DEXA scan result would trigger the prescription of Vit D?
    - Osteopenia
    - Osteoporosis

30) At which interval post allogeneic HSCT do you repeat DEXA scans?
    - Never
    - Every year
    - Every five years
    - Other (please specify _____)

31) When do you discontinue DEXA scans?
    - When results have normalized
    - When results have stabilised
    - When results have improved

32) Are DEXA scans covered by health insurance in your country?
    - Yes
    - No
    - Under certain circumstances (please describe_____)

33) Does your centre perform adult and/or paediatric allogeneic HSCT?
    - Adult only
    - Paediatric only
    - Both adult and paediatric

34) If you perform both adult and paediatric allogeneic HSCT, does your vit D management policy differ for adults and children?
    - Yes (please describe how it differs)
    - No

Appendix 2 – Notice of Ethical Approval, Amendments, Patient Information Sheets and Consent Form for Chapter 3

NHS
Health Research Authority

Professor Alejandro Madrigal

Anthony Nolan Research Institute The Royal Free Hospital
Pond Street
NW3 2QU

Email: hra.approval@nhs.net

8 September 2017
Dear Professor Madrigal,

Study title:

IRAS project ID: REC reference: Sponsor:

Letter of HRA Approval

Vitamin D and immune responses in haematopoietic stem cell transplantation
225121
17/WM/0325

University College London

I am pleased to confirm that HRA Approval has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications noted in this letter.

Participation of NHS Organisations in England

The sponsor should now provide a copy of this letter to all participating NHS organisations in England.

Appendix B provides important information for sponsors and participating NHS organisations in England for arranging and confirming capacity and capability. Please read Appendix B carefully, in particular the following sections:

- Participating NHS organisations in England – this clarifies the types of participating organisations in the study and whether or not all organisations will be undertaking the same activities
- Confirmation of capacity and capability - this confirms whether or not each type of participating NHS organisation in England is expected to give formal confirmation of capacity and capability. Where formal confirmation is not expected, the section also provides details on the time limit given to participating organisations to opt out of the study, or request additional time, before their participation is assumed.
- Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria) - this provides detail on the form of agreement to be used in the study to confirm capacity and capability, where applicable.

  Further information on funding, HR processes, and compliance with HRA criteria and standards is also provided.

It is critical that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details and further information about working with the research management function for each organisation can be accessed from www.hra.nhs.uk/hra-approval.

Appendices

The HRA Approval letter contains the following appendices:

- • □A – List of documents reviewed during HRA assessment
- • □B – Summary of HRA assessment After HRA Approval

The document “After Ethical Review – guidance for sponsors and investigators”, issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:

- • □Registration of research
- • □Notifying amendments
- • □Notifying the end of the study

The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

In addition to the guidance in the above, please note the following:

- • □HRA Approval applies for the duration of your REC favourable opinion, unless otherwise notified in writing by the HRA.
- • □Substantial amendments should be submitted directly to the Research Ethics Committee, as detailed in the After Ethical Review document. Non-substantial amendments should be submitted for review by the HRA using the form provided on the HRA website, and emailed to hra.amendments@nhs.net.
- • □The HRA will categorise amendments (substantial and non-substantial) and issue confirmation of continued HRA Approval. Further details can be found on the HRA website.

Scope

HRA Approval provides an approval for research involving patients or staff in NHS organisations in England.

If your study involves NHS organisations in other countries in the UK, please contact the relevant national coordinating functions for support and advice. Further information can be found at http://www.hra.nhs.uk/resources/applying-for-reviews/nhs-hsc-rd-review/.

If there are participating non-NHS organisations, local agreement should be obtained in accordance with the procedures of the local participating non-NHS organisation.

IRAS project ID	225121

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/.

HRA Training

We are pleased to welcome researchers and research management staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

Your IRAS project ID is 225121. Please quote this on all correspondence. Yours sincerely,

Emma Stoica Senior Assessor

Email: hra.approval@nhs.net

Copy to:

Ms Misha Ladva [sponsor contact]
Ms Julie Curtis [lead NHS R&D contact]

IRAS project ID	225121

Appendix A - List of Documents

The final document set assessed and approved by HRA Approval is listed below.

Document	Version	Date
Contract/Study Agreement template [material transfer agreement]	template	
Contract/Study Agreement template [data transfer agreement]		
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [UCL Insurance Confirmation Letter]	1	27 July 2017
GP/consultant information sheets or letters [Amended GP letter]	1.2	29 August 2017
HRA Schedule of Events [All Site Activities]	1	
HRA Schedule of Events [Blood Sample Analysis]	1	
HRA Statement of Activities [All Site Activities]	2	
HRA Statement of Activities [Blood Sample Analysis]	2	
IRAS Application Form [IRAS_Form_01082017]		01 August 2017
Letter from funder [Funding letter]		21 June 2017
Letter from sponsor [UCL Sponsorship Letter]	1	27 July 2017
Other [UCL Clinical Trial Policy]	1	08 May 2017
Participant consent form	1.3	08 September 2017
Participant information sheet (PIS) [Acute]	1.3	08 September 2017
Participant information sheet (PIS) [Chronic]	1.3	08 September 2017

Referee's report or other scientific critique report [Peer review]	1	21 June 2017
Research protocol or project proposal [Authorised Protocol]	1.1	15 June 2017
Summary CV for Chief Investigator (CI) [CV PROF ALEJANDRO MADRIGAL]		
Summary CV for student [CV JOSE ROS SOTO]	1.0	06 June 2017
Summary CV for supervisor (student research) [CV PROF JOHN SNOWDEN]		03 July 2017

IRAS project ID	225121

Appendix B - Summary of HRA Assessment

This appendix provides assurance to you, the sponsor and the NHS in England that the study, as reviewed for HRA Approval, is compliant with relevant standards. It also provides information and clarification, where appropriate, to participating NHS organisations in England to assist in assessing and arranging capacity and capability.

For information on how the sponsor should be working with participating NHS organisations in England, please refer to the, participating NHS organisations, capacity and capability and Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria) sections in this appendix.

The following person is the sponsor contact for the purpose of addressing participating organisation questions relating to the study:

Ms Misha Ladva, Joint Research Office
E-mail randd@uclh.nhs.uk; Telephone 02034475199

HRA assessment criteria

Section	HRA Assessment Criteria	Compliant with Standards	Comments
1.1	IRAS application completed correctly	Yes	No comments
2.1	Participant information/consent documents and consent process	Yes	Very minor, non-substantial revisions were made to both Participant Information Sheets and the Consent form following REC review, in order to make them consistent with the protocol.
3.1	Protocol assessment	Yes	No comments
4.1	Allocation of responsibilities and rights are agreed and documented	Yes	A Statement of activities for each site type of participating organisations (All Site Activities and Blood Samples Analysis) has been provided by the sponsor to help set up at the participating NHS organisations. The Statements will act as agreements of the NHS organisations to participate. A Material Transfer Agreement will also be in place to cover the transfer of

IRAS project ID 225121

Section	HRA Assessment Criteria	Compliant with Standards	Comments
			samples.
4.2	Insurance/indemnity arrangements assessed	Yes	The sponsor confirmed that the study is insured under UCL's policy and will be automatically rolled over into subsequent insurance period(s) until the study terminates. Where applicable, independent contractors (e.g. General Practitioners) should ensure that the professional indemnity provided by their medical defence organisation covers the activities expected of them for this research study.
4.3	Financial arrangements assessed	Yes	The sponsor will not provide funding to the NHS organisations (All Site Activities or Blood Samples Analysis) for participating, as detailed in the statements of activities.
5.1	Compliance with the Data Protection Act and data security issues assessed	Yes	No comments
5.2	CTIMPS – Arrangements for compliance with the Clinical Trials Regulations assessed	Not Applicable	No comments
5.3	Compliance with any applicable laws or regulations	Yes	No comments
6.1	NHS Research Ethics Committee favourable opinion received for applicable studies	Yes	No comments
6.2	CTIMPS – Clinical Trials Authorisation letter received	Not Applicable	No comments
6.3	Devices – MHRA notice of no objection received	Not Applicable	No comments
6.4	Other regulatory approvals and authorisations received	Not Applicable	No comments

IRAS project ID 225121

Participating NHS Organisations in England

This provides detail on the types of participating NHS organisations in the study and a statement as to whether the activities at all organisations are the same or different.
There are two site types of participating organisations in the study: • • □All Site Activities (NHS organisations): these sites will recruit and consent participants, and undertake all research activities. The NHS research sites are listed in Part C of the IRAS form. • • □Blood Samples Analysis Centre: Rotherham NHS Foundation Trust will undertake the laboratory analysis of all samples collected in the study. The activities to be undertaken at each site type are detailed in the respective statements of activities and schedules of events.

We note that the heathy controls will be recruited and undertake research activities at Anthony Nolan Research Institute (non-NHS site); as such, these activities are outside the remit of the HRA approval.

The Chief Investigator or sponsor should share relevant study documents with participating NHS organisations in England in order to put arrangements in place to deliver the study. The documents should be sent to both the local study team, where applicable, and the office providing the research management function at the participating organisation. For NIHR CRN Portfolio studies, the Local LCRN contact should also be copied into this correspondence. For further guidance on working with participating NHS organisations please see the HRA website.

If chief investigators, sponsors or principal investigators are asked to complete site level forms for participating NHS organisations in England which are not provided in IRAS or on the HRA website, the chief investigator, sponsor or principal investigator should notify the HRA immediately at hra.approval@nhs.net. The HRA will work with these organisations to achieve a consistent approach to information provision.

Confirmation of Capacity and Capability

This describes whether formal confirmation of capacity and capability is expected from participating NHS organisations in England.
Both the All Site Activities (NHS organisations) and the Blood Samples Analysis Centre participating in England will be expected to formally confirm their capacity and capability to host this research. • • Following issue of this letter, participating NHS organisations in England may now confirm to the sponsor their capacity and capability to host this research, when ready to do so. How capacity and capacity will be confirmed is detailed in the Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria) section of this appendix. • • The Assessing, Arranging, and Confirming document on the HRA website provides further information for the sponsor and NHS organisations on assessing, arranging and confirming capacity and capability.

Principal Investigator Suitability

This confirms whether the sponsor position on whether a PI, LC or neither should be in place is correct for each type of participating NHS organisation in England and the minimum expectations for education, training and experience that PIs should meet (where applicable).
Local Principal Investigators are required at the All Site Activities (Research Sites), and have been identified, as listed in the IRAS form Part C and the respective Statement of Activities. No specific training will be required for any of the team members at these sites. The HRA does not expect that a Principal Investigator or Local Collaborator is required at the Blood Sample Analysis centre. A responsible person has already been contacted and agreed to take part in the study at this centre. No specific training is required of the laboratory staff. GCP training is not a generic training expectation, in line with the HRA statement on training expectations.

HR Good Practice Resource Pack Expectations

This confirms the HR Good Practice Resource Pack expectations for the study and the pre-engagement checks that should and should not be undertaken
The activities at the participating NHS organizations will mainly be undertaken by staff who have adequate contractual relationship with the host organizations, therefore no additional arrangements (honorary research contracts or letters of access) are expected for this study. The MD student undertaking research activities at the NHS organisations would be expected to obtain Letters of Access on the basis of a Research Passport (if University employed) or an NHS to NHS confirmation of pre-engagement checks letter (if NHS employed). These should confirm standard DBS checks and occupational health clearance.

Other Information to Aid Study Set-up

This details any other information that may be helpful to sponsors and participating NHS organisations in England to aid study set-up.
• The applicant has indicated that they intend to apply for inclusion on the NIHR CRN Portfolio.

Health Research Authority

West Midlands - Edgbaston Research Ethics Committee

The Old Chapel Royal Standard Place Nottingham NG1 6FS

Please note: This is the favourable opinion of the REC only and does not allow the amendment to be implemented at NHS sites in England until the outcome of the HRA assessment has been confirmed.

14 May 2018

Dr Jose Ros Soto
Anthony Nolan Research Institute Royal Free Hospital
Pond street
NW3 2QG

Dear Dr Ros Soto,

The above amendment was reviewed on 14 May 2018 by the Sub-Committee in correspondence.

Ethical opinion

Study title:	Vitamin D and immune responses in haematopoietic stem cell transplantation
REC reference:	17/WM/0325
Amendment number:	1
Amendment date:	26 May 2018
IRAS project ID:	225121

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Decision: No ethical issues.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Notice of Substantial Amendment (non-CTIMP) [Substancial_amend_final.pdf]	1	26 May 2018
Participant consent form [Consent Form post HSCT .doc]	1.2	29 August 2017
Participant information sheet (PIS) [Post HSCT volunteers Info Sheet.doc]	1.1	07 March 2018
Research protocol or project proposal [PROTOCOL UCL1.2.doc]	1.2	25 April 2018
Sample diary card/patient card [DCF PATIENT VOLUNTEER.doc]	1.3	15 September 2017

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our Research Ethics Committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

Yours sincerely

Pp

Mr Paul Hamilton Chair

E-mail: NRESCommittee.WestMidlands-Edgbaston@nhs.net

Enclosures: List of names and professions of members who took part in the review

17/WM/0325: Please quote this number on all correspondence

[redacted]

West Midlands - Edgbaston Research Ethics Committee Attendance at Sub-Committee of the REC meeting on 14 May 2018

Committee Members:

Also in attendance:

Name	Profession	Present
Dr Hora Ejtehadi	Senior Academic Lecturer	Yes
Mr Paul Hamilton (Chair)	Parish Administrator	Yes

Name	Position (or reason for attending)
Ellena Stansbury	REC Assistant (Minutes)

Health Research Authority

06 August 2018

Dr Jose Ros Soto
Anthony Nolan Research Institute Royal Free Hospital
Pond Street
NW3 2QG

Dear Dr Ros Soto,

West Midlands - Edgbaston Research Ethics Committee

The Old Chapel Royal Standard Place Nottingham NG1 6FS

Please note: This is the favourable opinion of the REC only and does not allow the amendment to be implemented at NHS sites in England until the outcome of the HRA assessment has been confirmed.

Study title:	Vitamin D and immune responses in haematopoietic stem cell transplantation
REC reference:	17/WM/0325
Amendment number:	2
Amendment date:	22 June 2018
IRAS project ID:	225121

The above amendment was reviewed on 06 August 2018 by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Decision: No ethical issues.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
GP/consultant information sheets or letters [DCF SR acute 1.0.doc]	1.0	22 June 2018
GP/consultant information sheets or letters [GP letter - SR acute 1.0.doc]	1.0	22 June 2018
Notice of Substantial Amendment (non-CTIMP) [2nd AmendmentForm.pdf]	2	22 June 2018
Participant consent form [Consent Form SR acute 1.0.doc]	1.0	22 June 2018
Participant information sheet (PIS) [PIS - SR Acute 1.0.doc]	1.0	22 June 2018
Research protocol or project proposal [PROTOCOL UCL1.3.doc]	1.2	25 April 2018

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our Research Ethics Committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

Yours sincerely

Dr Sarahjane Jones Chair

E-mail: NRESCommittee.WestMidlands-Edgbaston@nhs.net

Enclosures: List of names and professions of members who took part in the review

17/WM/0325: Please quote this number on all correspondence

West Midlands - Edgbaston Research Ethics Committee Attendance at Sub-Committee of the REC meeting on 06 August 2018

Committee Members:

Also in attendance:

Name	Profession	Present
Dr Sarahjane Jones (Chair)	Senior Research Fellow	Yes
Dr Nigel Langford	Consultant Clinical Pharmacologist & General Physician	Yes

Name	Position (or reason for attending)
Ellena Stansbury	REC Assistant (Minutes)

PATIENT INFORMATION SHEET – Acute GvHD

Title: Vitamin D and immune responses in haematopoietic stem cell transplantation

Invitation to participate in the study

Before you decide whether to take part, it is important for you to understand why the research is being done and what it will involve.

Please take time to read the following information carefully. Discuss it with relatives and friends if you wish.

You are free to decide whether or not to take part in this study. If you choose not to take part, this will not affect the care you get from your doctors.

Ask us if there is anything that is not clear, or if you would like more information.

Study summary

This research will be conducted between October 2017 and October 2019. We hope to recruit approximately 40 patients to the study. It will be carried out as part of a MD project.

The aim of the study is to determine whether there is a relationship between vitamin D and the response to steroids, an immunosuppressive medication used for treating graft versus host disease (GvHD).

GvHD happens when cells from your donor ('graft') attack your own cells ('host'). This is usually when your donor is not related to you, although other factors may be involved. It affects nearly half of patients after an **allogeneic stem cell transplant (procedure for which bone marrow cells from a volunteer donor are given to a patient affected by a haematological disease)** and donor lymphocyte infusion (DLI). Depending on when it happens after the transplant, GvHD is classed as 'acute' (weeks later) or 'chronic' (months later). It can cause a wide range of symptoms from mild to severe, which can affect different parts of your body – mainly skin, gut and liver, although any organ can be damaged.

Recent studies have proved that vitamin D produces beneficial effects in the immune system, so it affects the stem cell transplant. Steroids are the first medication recommended for GvHD – but unfortunately less than 50% of patients respond to them and require stronger immunosuppression, with significant risk of side effects.

Specific proteins have been found in the blood of patients with GvHD, known as the GvHD biomarkers. They are called *elafin*, *REG3-alpha* and *ST2*. Biomarker levels are raised at the beginning of acute GvHD (even before it causes any symptoms) and can gradually increase if GvHD doesn't respond to medication.

This is a non-invasive test, which means that you don't need complicated procedures such as an endoscopy (to put a camera inside your bowels and take a small sample of it) or skin biopsy (small sample of your skin). Only a small volume of your blood will be required.

Vitamin D is not part of the routine blood test requested in the Post-Transplant Clinic at The Royal Marsden Hospital/Royal Hallamshire Hospital – so vitamin D deficiency may be missed. Vitamin D tablets are easy to swallow and have minor side effects, so they can be

taken safely if required. This treatment may have a remarkable impact on your 'new' immune system and enhance the way your body responds to steroids, avoiding further medication and its side effects.

So far research has only investigated these biomarkers within a month after the diagnosis of acute GvHD. This study plans to go beyond that, to check the biomarkers regularly for six months to find out whether they can predict the response to steroids in patients with acute GvHD. Vitamin D will be also regularly checked.

We want to build on this study with future studies to discover the best way to monitor the course of acute GvHD and to optimise its treatment.

Do I have to take part in the study?

No, you don't have to take part if you do not wish to.

Why have I been asked to participate?

You have been asked to take part because you are due to receive an allogeneic stem cell transplant or donor lymphocyte infusion, and therefore have a 50% chance of developing acute GvHD.

What would taking part in the study involve?

- We will take four extra blood samples over a period of six months, so we can monitor your vitamin D and GvHD biomarkers in case you develop acute GvHD.
- If you develop acute GvHD, we will take the first extra blood sample – either on the day of diagnosis (before you start treatment with steroids) or within the following 24 hours. We will take a second blood sample four weeks afterwards. The third and fourth blood samples will be taken three and six months after the first, respectively. Each blood sample will be approximately 5ml (equivalent to 1 teaspoon).
- We'll try to take the blood samples at times when you would be visiting the hospital anyway. If this isn't possible, we may need to ask you to come to the hospital specifically for the blood test. Unfortunately we cannot offer any payment to cover travel costs.

What will happen if I don't take part?

You will continue to receive all your normal care at The Royal Marsden Hospital/Royal Hallamshire Hospital.

What are the possible benefits of taking part?

By taking part you may help improve the care of patients with acute GvHD after allogeneic stem cell transplantation or DLI.

What are the possible disadvantages of taking part?

- We will ask you for four additional blood samples. If possible, we will take these samples at the same time as other samples we would be taking anyway.

- We may need to ask you to visit the hospital specifically to give one or more of the blood samples.
- Also, as possible adverse effects of venepuncture (taking blood from one of your veins) you may have pain at the site, bruising or bleeding. The pain and potential bleeding should stop after a few seconds or minutes, and the local bruising may take, as maximum, a few days to resolve.

What will happen if I don't want to carry on with the study?

You can withdraw from the research study at any time, and this decision will not affect your ongoing medical care. All data collected as part of the study will be destroyed, and blood samples collected as part of the study will be disposed of.

What information will be collected, and will it be kept confidential?

In addition to taking blood samples, we will use your medical records to find out other important information about you. This will include details such as your date of birth, the date of your stem cell transplant and any medication you are currently receiving.

All the information that is collected about you during the course of the research will be kept strictly confidential and will comply with data protection regulation. All information will be stored securely at The Royal Marsden Hospital/Royal Hallamshire Hospital and the Anthony Nolan Research Institute.

Any information about you will have your name and address removed so that you cannot be identified. Your data will be stored under a code and not under your name. Only the study team will have access to the code key.

To monitor the implementation of the study, it may be necessary to give access to regulatory authorities and the trust's sponsor representatives. Your research data will be stored for 20 years. If you agree, it will be used for future studies to pursue a better understanding of this disease and its treatment.

What will happen to the sample I give?

The blood samples will be stored under a code and not under your name. Only the study team will have access to the code key.

The samples provided will be stored in the laboratory at The Royal Marsden Hospital/Royal Hallamshire Hospital. Frozen samples will be transferred to Rotherham General Hospital, where laboratory analysis will be performed. The blood samples will be tested for vitamin D and GvHD biomarkers (*elafin*, *REG3-alpha* and *ST2*).

Your anonymised blood samples will be stored until the end of the period of laboratory analysis. If you agree to use the samples for future studies, they will be stored at Rotherham General Hospital. Otherwise, the will be destroyed by June 2020.

Involvement of other healthcare professionals

Your transplant doctor will be informed. If you give us permission, we will also write to your GP about the study.

Who has reviewed the study?

To protect your interests, all NHS research is looked at by an independent Research Ethics Committee. This study has been reviewed and given a favourable opinion by the **Edgbaston Research Ethics Committee**.

What will happen to the results of the study?

The results of this study may be published or presented at scientific meetings. The results may be used for further research, but your data would be presented in an anonymised format. If you would like a summary of the results from the study, please let the clinic doctor know.

Unfortunately we will not be able to provide individual patients with the results of their blood tests.

Who is organising and funding the study?

The Chief Investigator for the study is Professor Alejandro Madrigal, Consultant Haematologist and Scientific Director of the Anthony Nolan Research Institute.

The study is organised by Dr Jose Ros Soto, who is undertaking a clinical research degree (MD Res) at the Anthony Nolan Research Institute and the University College London Cancer Research Institute. The study is being funded by the Anthony Nolan Research Institute, and the study is sponsored by University College London.

What if something goes wrong?

If you have any concerns or wish to complain about any aspects of how you have been approached or treated by staff members during your participation in the research, National Health Service and UCL complaints mechanisms are available for you. The Patient Advice and Liaison Service (PALS) offers confidential advice, support and information on health-related matters. They also provide information about the NHS complaint procedure. You can ask for details of your nearest PALS at your GP surgery, hospital or even phone NHS 111. Please ask your research doctor if you would like more information on this.

In the unlikely event that you are harmed by taking part in this study, compensation may be available.

If you suspect that the harm is the result of the Sponsor's (University College London) or the hospital's negligence, then you may be able to claim compensation. After discussing with your research doctor, please make the claim in writing to Professor Alejandro Madrigal, the Chief Investigator for the research, at the Anthony Nolan Research Institute, Royal Free Hospital, Pond Street, NW3 2QG. The Chief Investigator will then pass the claim to the Sponsor's Insurers, via the Sponsor's office. You may have to bear the costs of the legal action initially, and you should consult a lawyer about it.

Thank you for taking time to read this patient information sheet.

Further Information and Contact Details

For further information please contact the Chief Investigator, Lead Researcher or Principle Investigator at your study site.

Chief Investigator

Professor Alejandro Madrigal

Anthony Nolan Research Institute

Student Researcher

Dr Jose Ros Soto

Anthony Nolan Research Institute

Principal Investigator – Royal Marsden Hospital

Dr Chloe Anthias

Royal Marsden Hospital

Principal Investigator – Royal Hallamshire Hospital

Professor John Snowden

Royal Hallamshire Hospital

PATIENT INFORMATION SHEET – Chronic GvHD

Tittle: Vitamin D and immune responses in haematopoietic stem cell transplantation

Invitation to participate in the study
Before you decide whether to take part, it is important for you to understand why the research is being done and what it will involve.
Please take time to read the following information carefully. Discuss it with relatives and friends if you wish.
You are free to decide whether or not to take part in this study. If you choose not to take part, this will not affect the care you get from your doctors.
Ask us if there is anything that is not clear or if you would like more information.

Study summary
This research will be conducted between October 2017 and October 2019. We hope to recruit approximately 30 patients to the study. It will be carried out as part of a MD project.

The aim of the study is to determine whether there is a relationship between vitamin D and the response to immunosuppressive treatment in patients with chronic graft versus host disease (GvHD) who haven't responded to steroids (also called *steroid-refractory chronic GvHD*) are going to start on Extracorporeal Photopheresis (ECP). We will compare this to patients with similar characteristics but are not candidates for ECP.

GvHD happens when cells from your donor ('graft') attack your own cells ('host'). This is usually when your donor is not related to you, although other factors may be involved. It affects nearly half of patients after an **allogeneic stem cell transplant (procedure for which bone marrow cells from a volunteer donor are given to a patient affected by a haematological disease)** and donor lymphocyte infusion (DLI). Depending on when that happens after the transplant, GvHD is classified as 'acute' (weeks later) or 'chronic' (months later). It can cause a wide range of symptoms from mild to severe, affecting different parts of your body – mainly skin, gut and liver, although any organ can be impaired.
Recent studies have proved that vitamin D produces beneficial effects in the immune system, so it affects the stem cell transplant. Steroids are the first medication recommended for GvHD – but unfortunately less than 50% of patients respond to them so they require stronger immunosuppression, with significant risk of side effects. ECP, a technique where part of your white cells are removed from your blood to decrease the number of donor cells attacking your body, has proved to be effective in steroid-refractory chronic GvHD, decreasing the symptoms and improving the quality of life.

Moreover, specific proteins have been found in the blood of patients with GvHD, known as the GvHD biomarkers. They are called *elafin*, *REG3-alpha* and *ST2*. Biomarker levels are raised at the beginning of the GvHD (even before it causes any symptoms) and can gradually increase if GvHD doesn't respond to medication.

This is a non-invasive test, which means that you don't need complicated procedures such as an endoscopy (to put a camera inside your bowels and take a small sample of it) or skin biopsy (small sample of your skin). Only a small volume of your blood will be required.

Vitamin D is not currently part of the routine blood test requested in the Post-Transplant Clinic, so its deficiency may be missed. Vitamin D tablets are easy to swallow and have minor side effects, so they can be taken safely if required. This treatment may have a remarkable impact on your 'new' immune system and enhance the way your body responds to steroids, avoiding further medication and its side effects.

Most of the research into these biomarkers has focused on patients with acute GvHD, and little is known about those with chronic GvHD – so this study plans to find out whether these biomarkers can be used for diagnosis and follow-up of patients with steroid-refractory chronic GvHD. It will also try to discover whether the level of vitamin D has any influence on the way patients respond to immunosuppressive therapy other than steroids.

We want to use and build on this study to improve the treatment of patients with chronic GvHD where first-line treatment (steroids) has not worked.

Do I have to take part in the study?
No, you don't have to take part if you do not wish to.

Why have I been asked to participate?
You have been asked to take part because you have received an allogeneic stem cell transplant or donor lymphocyte infusion, and you have developed steroid-refractory chronic GvHD.

What would taking part in the study involve?

- **As steroids failed to work for you, your transplant doctor will decide which is the most convenient immunosuppressive therapy for you**. We will take four extra blood samples over a period of six months, so we can monitor your vitamin D and GvHD biomarkers.
- We will take one extra blood sample just before you start on this new treatment (either further medication or ECP). We will take the second blood sample four weeks later. The third and fourth blood samples will be taken three and six months after the first one, respectively. Each blood sample will be approximately 5ml (equivalent to 1 teaspoon) in volume.
- We'll try to take the blood samples at a time when you would be visiting the hospital for your next appointment or ECP session anyway. If this isn't possible we may need to ask you to come to the hospital specifically for the blood test. Unfortunately we cannot offer any payment to cover travel costs.

What will happen if I don't take part?
You will continue to receive all your normal care at The Royal Marsden Hospital/ Royal Hallamshire Hospital/Rotherham General Hospital.

What are the possible benefits of taking part?
You may contribute to improve the care of patients with steroid refractory chronic GvHD after allogeneic stem cell transplantation or DLI.

What are the possible disadvantages of taking part?

- We will ask you for four additional blood samples. If possible, we will take these at the same time as other blood samples we would be taking anyway.
- We may need to ask you to visit the hospital specifically to give one or more of the blood samples.
- Also, as possible adverse effects of venepuncture (taking blood from one of your veins) you may have pain at the site, bruising and bleeding. The pain and potential bleeding should stop after a few seconds or minutes, and the local bruising may take, as maximum, a few days to resolve.

What will happen if I don't want to carry on with the study?
You can withdraw from the research study at any time, and this decision will not affect your ongoing medical care. All data collected as part of the study will be destroyed and blood samples collected as part of the study will be disposed of.

What information will be collected, and will it be kept confidential?
In addition to taking blood samples, we will use your medical records to find out other important information about you. This will include details such as your date of birth, the date of your stem cell transplant, the medication you are currently receiving and whether you have previously experienced acute GvHD.

All the information collected about you during the course of the research will be kept strictly confidential and will comply with data protection registration. All information will be stored securely at The Royal Marsden Hospital/Royal Hallamshire Hospital/Rotherham General Hospital and the Anthony Nolan Research Institute.

Any information about you will have your name and address removed so that you cannot be recognised. Your data will be stored under a code and not under your name. Only the study team will have access to the code key.

To monitor the implementation of the study, it may be necessary to give access to regulatory authorities and the trust's sponsor representatives. Your research data will be stored for 20 years. If you agree, it will be used for future studies to pursue a better understanding of this disease and its treatment.

What will happen to the sample I give?
The blood samples will be stored under a code and not under your name. Only the study team will have access to the code key.

The samples provided will be processed in the laboratory at The Royal Marsden Hospital/Royal Hallamshire Hospital/Rotherham General Hospital. Frozen samples will be transferred to Rotherham General Hospital, where laboratory analysis will be performed. The blood samples will be tested for vitamin D and GvHD biomarkers (*elafin*, *REG3-alpha* and *ST2*).

Your anonymised blood samples will be stored until the end of the period of laboratory analysis. If you agree to use the samples for future studies, they will be stored at Rotherham General Hospital. Otherwise, the will be destroyed by June 2020.

Involvement of other healthcare professionals
Your transplant doctor will be informed. If you give us permission, we will also write to your GP about the study.

W**ho has reviewed the study?**
To protect your interests, all NHS research is looked at by an independent Research Ethics Committee. This study has been reviewed and given a favourable opinion by the **Edgbaston Research Ethics Committee**.

What will happen to the results of the study?
The results of this study may be published or presented at scientific meetings and may be used for further research, but your data will be presented in an anonymised format. If you would like a summary of the results from the study, please let the clinic doctor know.

Unfortunately we will not be able to provide individual patients with results of their blood tests.

Who is organising and funding the study?
The Chief Investigator for the study is Professor Alejandro Madrigal, Consultant Haematologist and Scientific Director of the Anthony Nolan Research Institute.

The study is organised by Dr Jose Ros Soto, who is undertaking a clinical research degree (MD Res) at the Anthony Nolan research Institute and the University College London Cancer Research Institute. The study is being funded by the Anthony Nolan Research Institute, and the study is sponsored by University College London.

What if something goes wrong?
If you have any concerns or wish to complain about any aspects of how you have been approached or treated by staff members during your participation in the research, National Health Service and UCL complaints mechanisms are available for you. The Patient Advice and Liaison Service (PALS) offers confidential advice, support and information on health-related matters. They also provide information about the NHS complaint procedure. You can ask for details of your nearest PALS at your GP surgery, hospital or even phone NHS 111. Please ask your research doctor if you would like more information on this.

In the unlikely event that you are harmed by taking part in this study, compensation may be available.

If you suspect that the harm is the result of the Sponsor's (University College London) or the hospital's negligence, then you may be able to claim compensation. After discussing with your research doctor, please make the claim in writing to Professor Alejandro Madrigal, the Chief Investigator for the research, at the Anthony Nolan Research Institute, Royal Free Hospital, Pond Street, NW3 2QG. The Chief Investigator will then pass the claim to the Sponsor's Insurers, via the Sponsor's office. You may have to bear the costs of the legal action initially, and you should consult a lawyer about it.

Thank you for taking the time to read this patient information sheet.

Further Information and Contact Details

For further information please contact the Chief Investigator, Lead Researcher or Principle Investigator at your study site.

PATIENT INFORMATION SHEET – Steroid-refractory acute GvHD

Tittle: Vitamin D and immune responses in haematopoietic stem cell transplantation

Invitation to participate in the study
Before you decide whether to take part, it is important for you to understand why the research is being done and what it will involve.

Please take time to read the following information carefully. Discuss it with relatives and friends if you wish.

You are free to decide whether or not to take part in this study. If you choose not to take part, this will not affect the care you get from your doctors.

Ask us if there is anything that is not clear or if you would like more information.

Study summary
This research will be conducted between October 2017 and October 2019. We hope to recruit approximately 30 patients to the study. It will be carried out as part of a MD project.

The aim of the study is to determine whether there is a relationship between vitamin D and the response to immunosuppressive treatment in patients with acute graft versus host disease (GvHD) who haven't responded to steroids (also called *steroid-refractory* acute *GvHD*) are going to start on Extracorporeal Photopheresis (ECP). We will compare this to patients with similar characteristics but are not candidates for ECP.

GvHD happens when cells from your donor ('graft') attack your own cells ('host'). This is usually when your donor is not related to you, although other factors may be involved. It affects nearly half of patients after an allogeneic stem cell transplant (procedure for which bone marrow cells from a volunteer donor are given to a patient affected by a haematological disease) and donor lymphocyte infusion (DLI). Depending on when that happens after the transplant, GvHD is classified as 'acute' (weeks later) or 'chronic' (months later). It can cause a wide range of symptoms from mild to severe, affecting different parts of your body – mainly skin, gut and liver, although any organ can be impaired.
Recent studies have proved that vitamin D produces beneficial effects in the immune system, so it affects the stem cell transplant. Steroids are the first medication recommended for GvHD – but unfortunately less than 50% of patients respond to them so they require stronger immunosuppression, with significant risk of side effects. ECP, a technique where part of your white cells are removed from your blood to decrease the number of donor cells attacking your body, has proved to be effective in steroid-refractory acute GvHD, decreasing the symptoms and improving the quality of life.

Moreover, specific proteins have been found in the blood of patients with GvHD, known as the GvHD biomarkers. They are called *elafin*, *REG3-alpha* and *ST2*. Biomarker levels are raised at the beginning of the GvHD (even before it causes any symptoms) and can gradually increase if GvHD doesn't respond to medication.

This is a non-invasive test, which means that you don't need complicated procedures such as an endoscopy (to put a camera inside your bowels and take a small sample of it) or skin biopsy (small sample of your skin). Only a small volume of your blood will be required.

Vitamin D is not currently part of the routine blood test requested in the Post-Transplant Clinic, so its deficiency may be missed. Vitamin D tablets are easy to swallow and have minor side effects, so they can be taken safely if required. This treatment may have a remarkable impact on your 'new' immune system and enhance the way your body responds to steroids, avoiding further medication and its side effects.

This study plans to find out whether these biomarkers can be used for diagnosis and follow-up of patients with steroid-refractory acute GvHD. It will also try to discover whether the level of vitamin D has any influence on the way patients respond to immunosuppressive therapy other than steroids.

We want to use and build on this study to improve the treatment of patients with acute GvHD where first-line treatment (steroids) has not worked.

Do I have to take part in the study?
No, you don't have to take part if you do not wish to.

Why have I been asked to participate?
You have been asked to take part because you have received an allogeneic stem cell transplant or donor lymphocyte infusion, and you have developed steroid-refractory acute GvHD.

What would taking part in the study involve?

- As steroids failed to work for you, your transplant doctor will decide which is the most convenient immunosuppressive therapy for you. We will take four extra blood samples over a period of six months, so we can monitor your vitamin D and GvHD biomarkers.

- We will take one extra blood sample just before you start on this new treatment (either further medication or ECP). We will take the second blood sample four weeks later. The third and fourth blood samples will be taken three and six months after the first one, respectively. Each blood sample will be approximately 5ml (equivalent to 1 teaspoon) in volume.

- We'll try to take the blood samples at a time when you would be visiting the hospital for your next appointment or ECP session anyway. If this isn't possible we may need to ask you to come to the hospital specifically for the blood test. Unfortunately we cannot offer any payment to cover travel costs.

What will happen if I don't take part?
You will continue to receive all your normal care at The Royal Marsden Hospital/King's College Hospital/Royal Hallamshire Hospital/Rotherham General Hospital.

What are the possible benefits of taking part?
You may contribute to improve the care of patients with steroid refractory acute GvHD after allogeneic stem cell transplantation or DLI.

What are the possible disadvantages of taking part?

- We will ask you for four additional blood samples. If possible, we will take these at the same time as other blood samples we would be taking anyway.

- We may need to ask you to visit the hospital specifically to give one or more of the blood samples.

- Also, as possible adverse effects of venepuncture (taking blood from one of your veins) you may have pain at the site, bruising and bleeding. The pain and potential bleeding should stop after a few seconds or minutes, and the local bruising may take, as maximum, a few days to resolve.

What will happen if I don't want to carry on with the study?
You can withdraw from the research study at any time, and this decision will not affect your ongoing medical care. All data collected as part of the study will be destroyed and blood samples collected as part of the study will be disposed of.

What information will be collected, and will it be kept confidential?
In addition to taking blood samples, we will use your medical records to find out other important information about you. This will include details such as your date of birth, the date of your stem cell transplant, the medication you are currently receiving and whether you have previously experienced acute GvHD.

All the information collected about you during the course of the research will be kept strictly confidential and will comply with data protection registration. All information will be stored securely at The Royal Marsden Hospital/ King's College Hospital/Royal Hallamshire Hospital/Rotherham General Hospital and the Anthony Nolan Research Institute.

Any information about you will have your name and address removed so that you cannot be recognised. Your data will be stored under a code and not under your name. Only the study team will have access to the code key.

To monitor the implementation of the study, it may be necessary to give access to regulatory authorities and the trust's sponsor representatives. Your research data will be stored for 20 years. If you agree, it will be used for future studies to pursue a better understanding of this disease and its treatment.

What will happen to the sample I give?
The blood samples will be stored under a code and not under your name. Only the study team will have access to the code key.

The samples provided will be processed in the laboratory at The Royal Marsden Hospital/ King's College Hospital/Royal Hallamshire Hospital/Rotherham General Hospital. Frozen samples will be transferred to Rotherham General Hospital, where laboratory analysis will be performed. The blood samples will be tested for vitamin D and GvHD biomarkers (*elafin*, *REG3-alpha* and *ST2*).

Your anonymised blood samples will be stored until the end of the period of laboratory analysis. If you agree to use the samples for future studies, they will be stored at Rotherham General Hospital. Otherwise, the will be destroyed by June 2020.

Involvement of other healthcare professionals
Your transplant doctor will be informed. If you give us permission, we will also write to your GP about the study.

W**ho has reviewed the study?**

To protect your interests, all NHS research is looked at by an independent Research Ethics Committee. This study has been reviewed and given a favourable opinion by the **Edgbaston Research Ethics Committee**.

What will happen to the results of the study?
The results of this study may be published or presented at scientific meetings and may be used for further research, but your data will be presented in an anonymised format. If you would like a summary of the results from the study, please let the clinic doctor know.

Unfortunately we will not be able to provide individual patients with results of their blood tests.

Who is organising and funding the study?
The Chief Investigator for the study is Professor Alejandro Madrigal, Consultant Haematologist and Scientific Director of the Anthony Nolan Research Institute.

The study is organised by Dr Jose Ros Soto, who is undertaking a clinical research degree (MD Res) at the Anthony Nolan research Institute and the University College London Cancer Research Institute. The study is being funded by the Anthony Nolan Research Institute, and the study is sponsored by University College London.

What if something goes wrong?
If you have any concerns or wish to complain about any aspects of how you have been approached or treated by staff members during your participation in the research, National Health Service and UCL complaints mechanisms are available for you. The Patient Advice and Liaison Service (PALS) offers confidential advice, support and information on health-related matters. They also provide information about the NHS complaint procedure. You can ask for details of your nearest PALS at your GP surgery, hospital or even phone NHS 111. Please ask your research doctor if you would like more information on this.

In the unlikely event that you are harmed by taking part in this study, compensation may be available.

If you suspect that the harm is the result of the Sponsor's (University College London) or the hospital's negligence, then you may be able to claim compensation. After discussing with your research doctor, please make the claim in writing to Professor Alejandro Madrigal, the Chief Investigator for the research, at the Anthony Nolan Research Institute, Royal Free Hospital, Pond Street, NW3 2QG. The Chief Investigator will then pass the claim to the Sponsor's Insurers, via the Sponsor's office. You may have to bear the costs of the legal action initially, and you should consult a lawyer about it.

Thank you for taking the time to read this patient information sheet.

Centre Number:

Study Number:

Patient Identification Number for this study:

CONSENT FORM

Title:
Vitamin D and immune responses in haematopoietic stem cell transplantation

Chief Investigator: **Professor Alejandro Madrigal**

Please initial all the boxes

1. I confirm that I have read and understand the information sheet dated 8 September 2017 version 1.3 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from Anthony Nolan, from regulatory authorities and or from the NHS Trust, where it is relevant to my taking part in this research. I give permission to these individuals to have access to my records. ☐

4. I agree to my GP being informed of my participation in the study. ☐

5. I confirm that blood samples and data provided may be used for continuing studies in the future to pursue a better understanding of this disease and its treatment (Please let us know if you want the samples disposed of after the current programme). **Yes** ☐ **No** ☐

6. I agree to take part in the above study. ☐

_____ _____ _____

Name of Participant Signature — Date

_____ _____ _____

Name of Person taking consent — Signature — Date

Appendix 3 – Rights and Permissions

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Date: Wednesday, 19 February 2020 14:25
To: Jose Ros Soto <JoseRos.Soto@anthonynolan.org>
Subject: Re: Permission for using image

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From: HotSpot Energy <jwilliams@hotspotenergy.com>
Organization: HotSpot Energy
Date: Tuesday, 3 March 2020 22:04
To: Jose Ros Soto <JoseRos.Soto@anthonynolan.org>
Subject: RE: Permission for thesis

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From: Marketing Help <marketinghelp@bio-techne.com>
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From: "Anderson-Cable, Lindsey" <lindsey.anderson-cable@siemens-healthineers.com>
Date: Friday, 6 March 2020 17:08
To: Jose Ros Soto <JoseRos.Soto@anthonynolan.org>
Cc: "Griffith, Rhys" <rhys.griffith@siemens-healthineers.com>
Subject: RE: ELISA

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That sounds very interesting!

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Thank you, and good luck with your thesis.

With best regards,
Lindsey Anderson-Cable

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From: Elizabeth Sandler <esandler@aaas.org>
Date: Thursday, 20 February 2020 17:13
To: Jose Ros Soto <JoseRos.Soto@anthonynolan.org>
Subject: TERMS OF USE - SCI TRANSL MED - THESIS USE

Re: Figure 2 from Paczesny et al.,
Science Translational Medicine 06 Jan 2010:
Vol. 2, Issue 13, pp. 13ra2
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Supporting data

	1st sample	2nd samples	3rd samples	4th sample
Acute GvHD *(N)*	16	12	8	6
SR chronic GvHD *(N)*	5	5	5	4
SR Acute GvHD *(N)*	8	5	2	1

Table 5-1: Number of patients with the correspondent sample taken in each cohort

De novo acute GvHD

Vitamin D and GvHD biomarkers in aGvHD compared to controls

	Day 0 ***(N=16)***	**AN controls** ***(N=28)***	**Healthy HSCT controls** ***(N=8)***
25(OH)D^3 (nmol/L)*	38.9 (26.3 - 75.9)	50 (20.4 – 79.2) *p* =0.21	39.9 (22.8 -155.3) *p* =0.95
Elafin (ng/ml)*	25 (7.9 – 503.4)	17.3 (6.6 - 2442) *p* =0.15	15.7 (7.9 -32.2) *p* =0.05
ST2 (ng/ml)*	71.1 (14.6 - 366)	15 (3.3 -24.3) *p* <0.001	54.7 (18.1 -190.6) *p* =0.85
REG3α (ng/ml)*	55.3 (4.1 – 907.8)	10.2 (3.5 – 23.3) *p* <0.001	51.3 (13.4 – 81.1) *p* =0.63

**median (range); p values after comparing the aGvHD populations with each control cohort (Mann-Whitney test)*

Table 5-2: Comparison of 25(OH)D^3 and biomarkers between aGvHD patients and each study control group

Level of 25(OH)D^3 and biomarkers in aGvHD

	Day 0 ***(N=16)***	**1 month** ***(N=12)***	**3 months** ***(N=8)***	**6 months** ***(N=6)***	***p* value****
25(OH)D^3 (nmol/L)*	38.9 (26.3 - 75.9)	40.5 (19 – 76.2)	43.5 (27 – 91.5)	40.6 (24.4 - 79)	*p* = 0.94
Elafin (ng/ml)*	25 (7.9 – 503.4)	23.3 (11.3 – 108.3)	30.8 (15.3 – 2227.5)	20 (7.7 – 847.9)	*p* = 0.27
ST2 (ng/ml)*	71.1 (14.6 - 366)	44.1 (11.1 – 256.1)	25.3 (8 – 172.4)	103.4 (8.1 – 388.6)	*p* = 0.53
REG3α (ng/ml)*	55.3 (4.1 – 907.8)	81.9 (18.6 – 581.1)	75.3 (23.6 – 147.5)	38.6 (24.5 – 106.2)	*p* = 0.99

**median (range); p values derived from Kruskal-Wallis test*

Table 5-3: Level of 25(OH)D^3 and biomarkers in aGvHD at different time points

	Day 0 – 1 month	**Day 0 - 3 months**	**Day 0 - 6 months**
25(OH)D^3	*p*=0.53	*p*=1	*p*=0.75
Elafin	*p*=0.12	*p*=0.16	*p*=0.75
ST2	*p*=0.94	*p*=0.58	*p*=0.92
REG3α	*p*=0.059	*p*=0.50	*p*=0.50

p values derived from Wilcoxon rank sum test

Table 5-4: Comparison of 25(OH)D^3 and biomarkers between baseline and follow-up time points in patients with aGvHD

Relationship between grades of aGvHD and 25(OH)D^3/biomarkers

	Day 0		*1 month*	
	I-II ***(N=10)***	**III-IV** ***(N=6)***	**0-II** ***(N=10)***	**III-IV** ***(N=2)***
25(OH)D^3 (nmol/L)*	38.9 (29.4 – 75.9)	39.8 (24.3 – 66.5)	41.6 (27.8 – 76.2)	23.3 (19 – 27.6)
	p = 0.83		*p* = 0.032	
Elafin (ng/ml)*	28.4 (13.6 - 503.4)	29.4 (7.9 - 31.9)	23.3 (11.5 – 108.3)	20.1 (11.3 – 28.9)
	p = 0.45		*p* = 0.67	
ST2 (ng/ml)*	47.3 (14.6 – 243.6)	180.4 (31.8 - 366)	41.1 (11.1 – 206.4)	136.1 (16 – 256.1)
	p = 0.051		*p* = 0.83	
REG3α (ng/ml)*	44.1 (4.1 - 382)	279.5 (26.5 – 907.8)	81.9 (18.6 – 581.1)	86.8 (79.6 – 93.9)
	p = 0.12		*p* = 0.81	

median (range); p values derived from Mann-Whitney test

Table 5-5: Correlation of vitamin D and biomarkers with aGvHD grade at baseline and 1 month

In addition, when this population was broken down into 3 different categories (no GvHD, mild and severe aGvHD), there was a weak association between 25(OH)D^3 levels and GvHD severity: 25(OH)D^3 was higher in patients who achieved CR (no aGvHD) compared to mild and severe grades (47.6 *vs* 38.3 *vs* 23.3, *p*=0.077). None of the remaining variables investigated showed statistically significant differences.

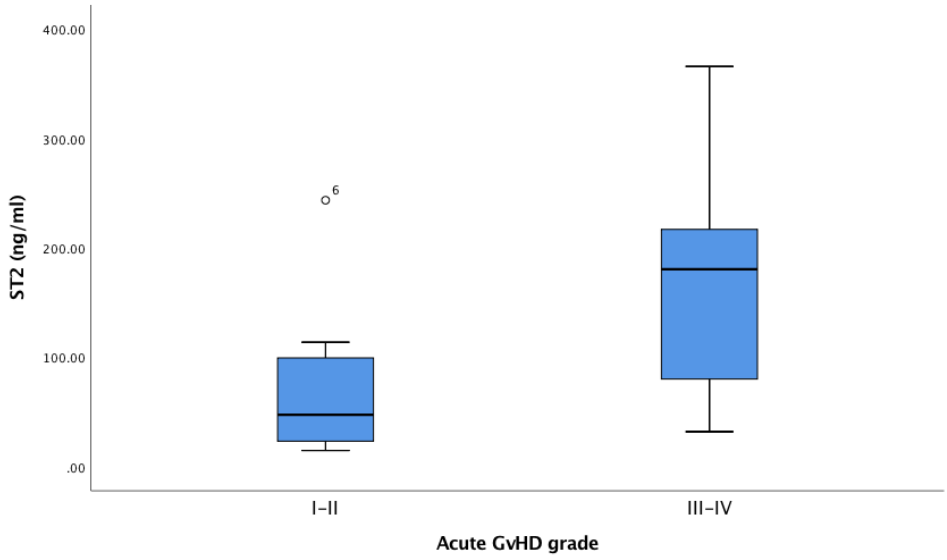

Figure 5-1: Levels of ST2 per aGvHD grade category at diagnosis

Note that patient *number 6* in Figure 5-1 had very high levels of ST2 at baseline. He had stage 3 skin GvHD (borderline grade II-III) that could potentially explain this finding.

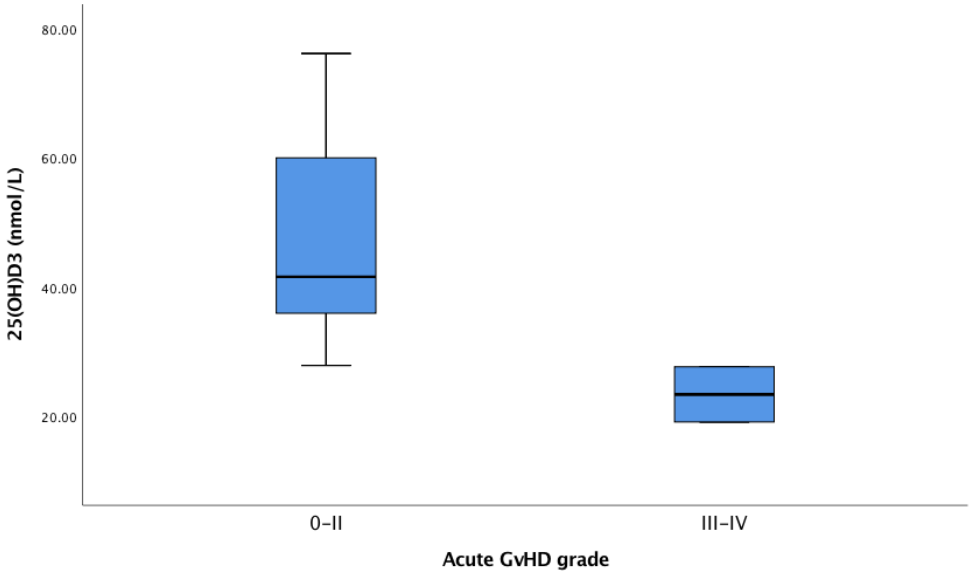

Figure 5-2: Levels of 25(OH)D[3] per aGvHD grade category at 1 moth post-treatment

Correlations

Correlation tests were run in order to explore associations between variables including 25(OH)D^3, GvHD biomarkers, platelet count and baseline ECOG: There was a strong positive correlation between elafin and REG3α at baseline (ρ=0.65, *p*=0.012), but this could not be reproduced at later time points (See Figure 5-3).

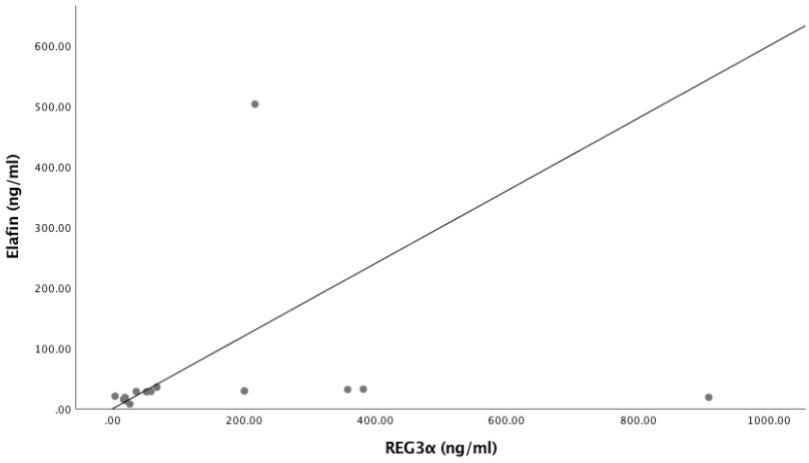

Figure 5-3: Correlation between elafin and REG3α at baseline

Survival

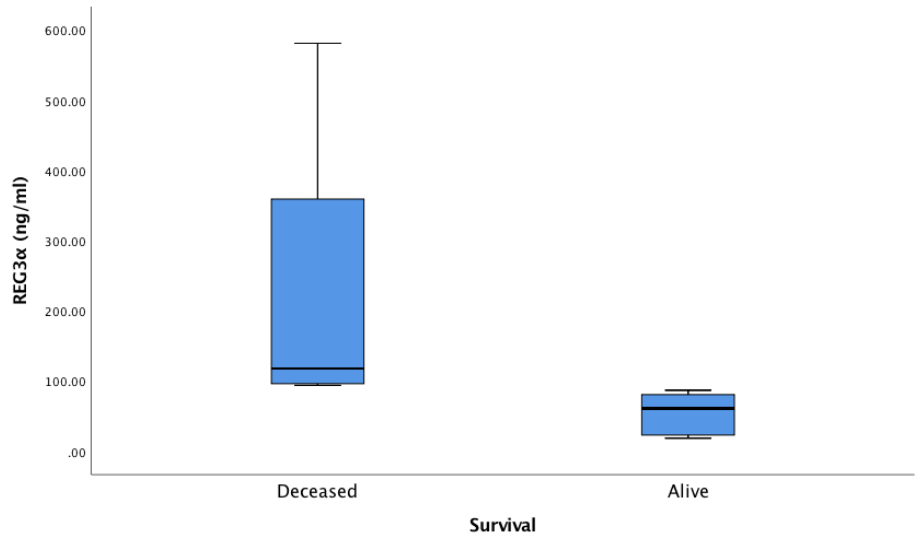

Figure 5-4: Relationship between levels of REG3α at 1 month and survival in aGvHD

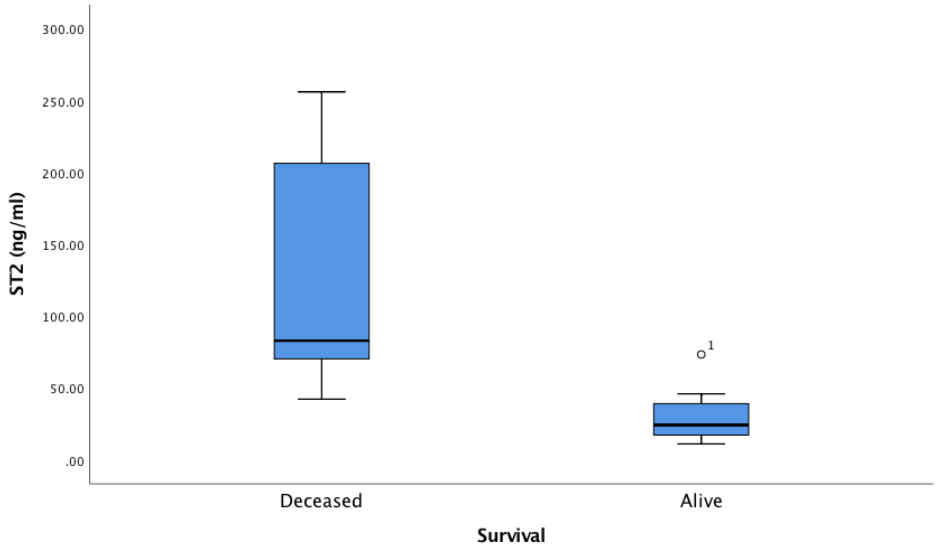

Figure 5-5: Relationship between levels of ST2 at 1 month and survival in aGvHD

Steroid-refractory chronic GvHD

Level of 25(OH)D^3 and biomarkers in SR cGvHD

	Day 0 *(N=5)*	1 month *(N=5)*	3 months *(N=5)*	6 months *(N=4)*	*P value*
25(OH)D^3 (nmol/L)*	46.8 (34.4 – 91.2)	57.6 (27.7 – 78.6)	76.4 (30.7 – 79.7)	48.2 (25.3 – 72.4)	*0.82*
Elafin (ng/ml)*	32.2 (7.5 – 311.2)	117.3 (3.3 – 362.8)	18.2 (5.4 – 367.7)	23.2 (6 – 105.2)	*0.81*
ST2 (ng/ml)*	18.7 (9.1 – 285.7)	35 (19.6 - 1009)	37 (8.2 – 213.8)	48 (26.5 - 111)	*0.58*
REG3α (ng/ml)*	33 (13 – 71.8)	43 (16.5 – 76.2)	30.1 (7 - 184)	38.2 (16.8 – 66.3)	*0.99*

**median (range); p values derived from Kruskal-Wallis test*

Table 5-6: Levels of 25(OH)D^3 and GvHD biomarkers at different time points in patients with SR cGvHD

	Day 0 – 1 month	Day 0 - 3 months	Day 0 - 6 months
25(OH)D^3	*p*=0.50	*p*=0.35	*p*=0.72
Elafin	*p*=0.89	*p*=0.50	*p*=0.27
ST2	*p*=0.043	*p*=0.69	*p*=0.72
REG3α	*p*=0.89	*p*=0.69	*p*=1

p values derived from Wilcoxon rank sum test

Table 5-7: Comparison of 25(OH)D3 and biomarkers between baseline and follow-up time points in patients with SR cGvHD

Correlations

There was a strong positive association between 25(OH)D[3] and elafin at baseline (ρ=0.90, *p*=0.037), also confirmed at 1 month (ρ=0.90, *p*=0.037). See Figure 5-6 and Figure 5-7.

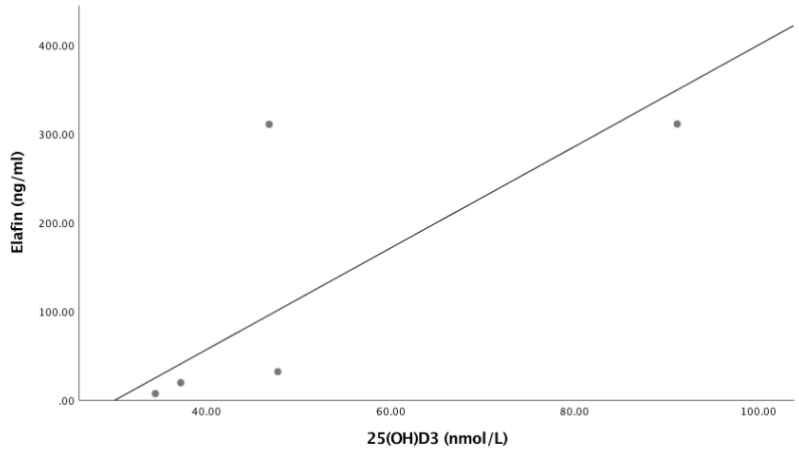

Figure 5-6: Correlation between 25(OH)D[3] and elafin at baseline in SR cGvHD

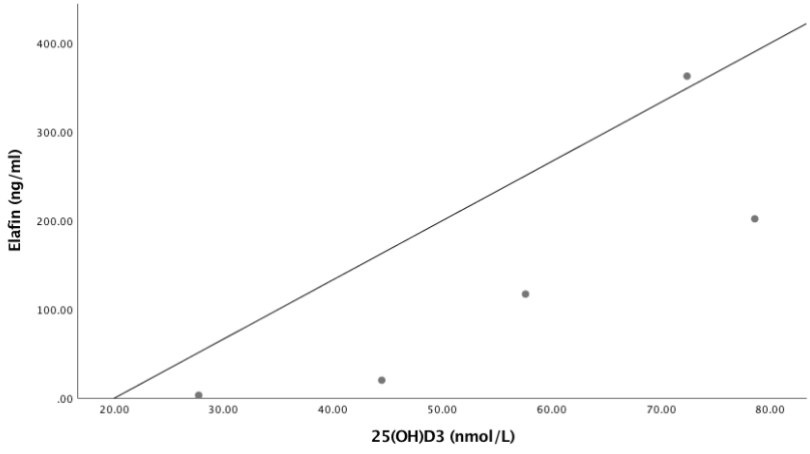

Figure 5-7: Correlation between 25(OH)D[3] and elafin at 1 month in SR cGvHD

Steroid-refractory acute GvHD

Level of 25(OH)D^3 and biomarkers in SR aGvHD

	Day 0 ***(N=8)***	**1 month** ***(N=5)***	**3 months** ***(N=2)***	***P value***
25(OH)D^3 (nmol/L)*	35.6 (23.9 – 62.6)	27.3 (22.2 – 65.2)	42.8 (33 – 52.5)	*0.41*
Elafin (ng/ml)*	114.2 (6.9 – 266.5)	54.6 (10 - 1784)	104.3 (14.2 – 194.5)	*0.67*
ST2 (ng/ml)*	231.7 (93.9 – 483.1)	373.9 (114.5 - 1265)	219.9 (132.1 – 307.7)	*0.66*
REG3α (ng/ml)*	100.4 (57.1 – 304.1)	369.7 (6 – 768.5)	113.8 (49.1 – 178.5)	*0.81*

**median (range); p values derived from Kruskal-Wallis test*

Table 5-8: Levels of 25(OH)D^3 and GvHD biomarkers at different time points in patients with SR aGvHD

	Day 0 – 1 month	**Day 0 - 3 months**	**Day 0 - 6 months***
25(OH)D^3	*p*=0.69	*p*=0.18	-
Elafin	*p*=0.50	*p*=0.66	-
ST2	*p*=0.69	*p*=0.18	-
REG3α	*p*=0.59	*p*=0.66	-

*p values derived from Wilcoxon rank sum test *Not applicable as only 1 patient had the 4th sample drawn*

Table 5-9: Comparison of 25(OH)D^3 and biomarkers between baseline and follow-up time points in patients with SR aGvHD

Correlations

Only a negative correlation was found between the platelet count and ST2 at baseline (r=-0.76, *p*=0.028).

De novo vs SR aGvHD

Concentration of 25(OH)D^3 and biomarkers were compared between *de novo* and SR aGvHD (See Table 5-10): even though biomarkers reached higher concentration in SR than *de novo* aGvHD, only the difference in ST2 levels at baseline attained statistical significance (71.1 *vs* 231.7, *p*=0.010) and at 1 month (44.1 *vs* 373.9, *p*=0.006). See Figure 5-8 and Figure 5-9.

	Acute GvHD ***(N=16)***	**SR aGvHD** ***(N=8)***	***p* value****
25(OH)D^3 (nmol/L)*	38.9 (26.3 - 75.9)	35.6 (23.9 – 62.6)	0.2
Elafin (ng/ml)*	25 (7.9 – 503.4)	114.2 (6.9 – 266.5)	0.25
ST2 (ng/ml)*	71.1 (14.6 - 366)	231.7 (93.9 – 483.1)	0.010
REG3α (ng/ml)*	55.3 (4.1 – 907.8)	100.4 (57.1 – 304.1)	0.32

**median (range); **p values derived from Mann-Whitney test*

Table 5-10: Levels of 25(OH)D^3 and GvHD biomarkers in de novo and SR aGvHD at day 0

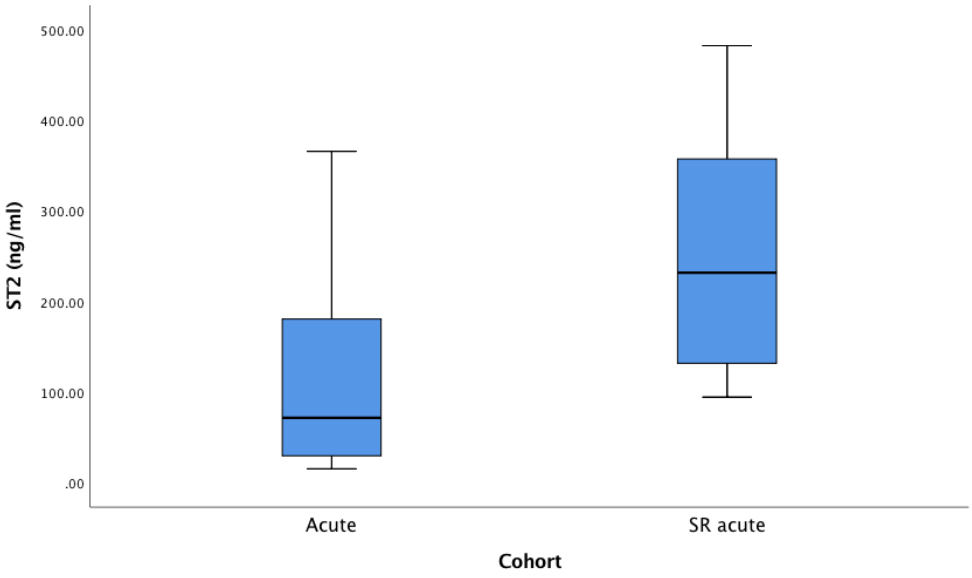

Figure 5-8: Levels of ST2 in de novo and SR aGvHD at day 0

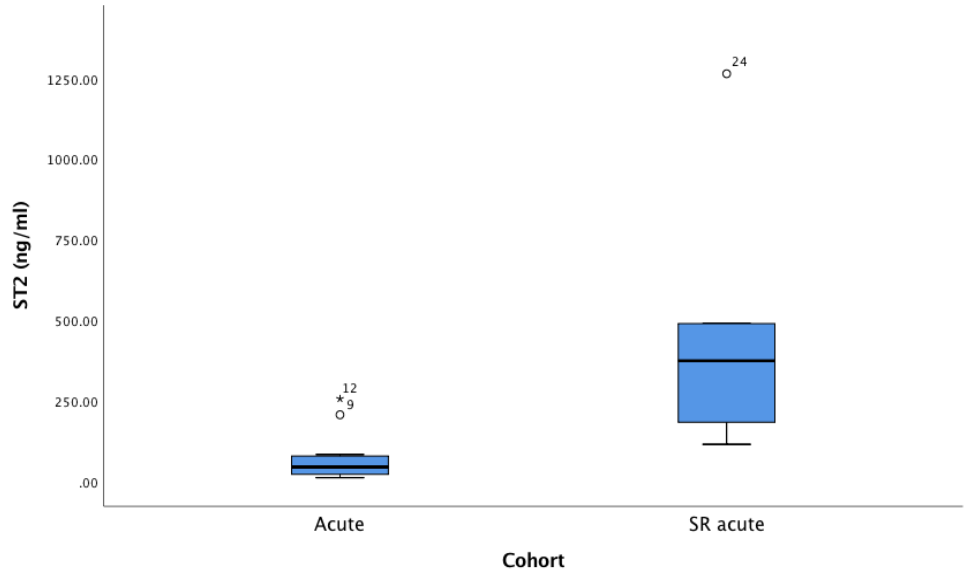

Figure 5-9: Levels of ST2 in de novo and SR aGvHD 1month post-treatment

The three GvHD cohorts

Although the nature of acute and cGvHD differs, comparison between the three study cohorts has been carried out to find out whether there are remarkable differences in the concentration of any study variables that could be linked to the different processes underlying their pathophysiology.

The only remarkable difference was in the levels of ST2 at baseline, higher in the aGvHD setting, mainly in is SR form, than in cGvHD (p=0.012). See Table 5-11 and Figure 5-10.

	Acute GvHD ***(N=16)***	**SR cGvHD** ***(N=5)***	**SR aGvHD** ***(N=8)***	***p* value****
25(OH)D^3 (nmol/L)*	38.9 (26.3 - 75.9)	46.8 (34.4 – 91.2)	35.6 (23.9 – 62.6)	0.22
Elafin (ng/ml)*	25 (7.9 – 503.4)	32.2 (7.5 – 311.2)	114.2 (6.9 – 266.5)	0.47
ST2 (ng/ml)*	71.1 (14.6 - 366)	18.7 (9.1 – 285.7)	231.7 (93.9 – 483.1)	0.012
REG3α (ng/ml)*	55.3 (4.1 – 907.8)	33 (13 – 71.8)	100.4 (57.1 – 304.1)	0.19

**median (range); **p values derived from Mann-Whitney test*

Table 5-11: Levels of 25(OH)D^3 and GvHD biomarkers in the three study groups

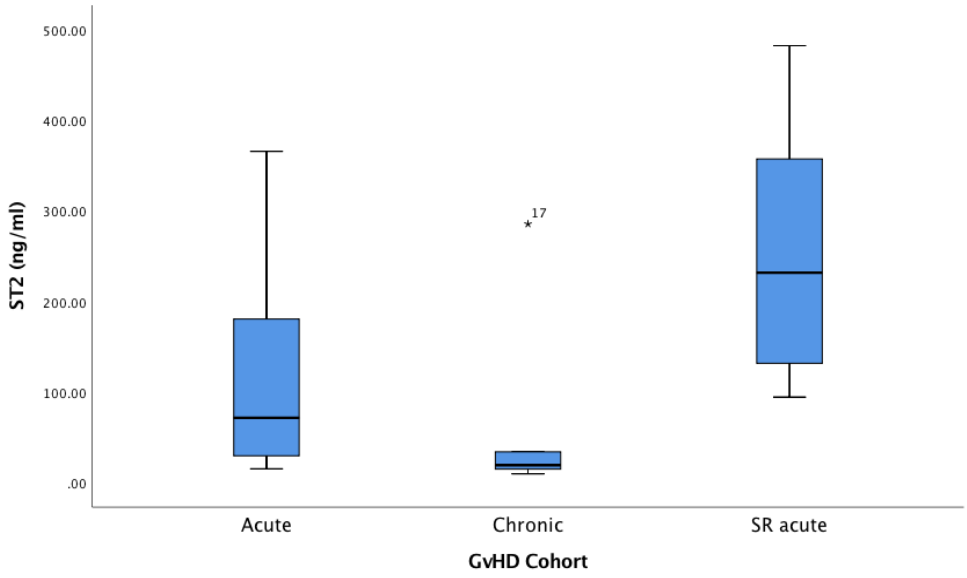

Figure 5-10: Baseline levels of ST2 in the three different study groups

Controls

Patients one month post alloHSCT were recruited as controls groups for this study. As mentioned, half of the them developed GvHD after recruitment and therefore they were removed as control group but analysis was done to explore whether there were any differences in the levels of 25(OH)D^3 and biomarkers that could predict GvHD at this early post-HSCT phase, even before clinical manifestations appear.

Despite the low number of cases in both cohorts, there were significant differences in levels of 25(OH)D^3 and ST2. Vitamin D was higher in healthy HSCT controls compared to those who developed GvHD (39.9 *vs* 22.5, p=0.027). Moreover, ST2 was also higher in healthy HSCT controls than in those with GvHD (54.7 *vs* 24.4, p=0.046). See Table 5-12.

	Healthy HSCT controls *(N=8)*	GvHD controls *(N=8)*	*p* value**
25(OH)D^3 (nmol/L)*	39.9 (22.8 – 155.3)	22.5 (12.4 – 42.1)	0.027
Elafin (ng/ml)*	15.7 (7.9 – 32.2)	13.4 (7.7 - 37)	0.83
ST2 (ng/ml)*	54.7 (18.1 – 190.6)	24.4 (11 – 96.5)	0.046 ???
REG3α (ng/ml)*	51.3 (13.4 – 81.1)	43.7 (26.8 – 151.9)	0.83

**median (range); **p values derived from Mann-Whitney test*

Table 5-12: Levels of 25(OH)D3 and GvHD biomarkers in patient 1 month post-HSCT

Patient number	At baseline (day 0)								1 month	3 months	6 months
	Skin	Gut	Liver	aGvHD grade	25(OH)D^3 (nmol/L)	Elafin (ng/ml)	ST2 (ng/ml)	REG3α (ng/ml)			
1	1	0	0	I	34.77	28.16	18.90	51.98	CR	CR	CR
2	1	2	0	III	34.81	29.57	31.83	200.86	CR	CR	Relapse
3	1	0	0	I	63.55	15.75	62.3	16.88	CR	CR	CR
4	2	0	0	I	32.71	35.85	113.73	67.73	No CR	CR	CR
5	2	0	0	I	75.85	28.7	14.63	58.65	CR	CR	CR
6	3	0	0	II	59.58	28.64	243.6	36.28	CR	Relapse	No CR
7	1	0	1	II	46.64	18.81	99.35	19.32	CR	Relapse	-
8	1	0	0	I	29.35	20.94	42.65	4.112	No CR	CR	-
9	1	1	1	II	32.68	32.57	26.35	382.02	No CR	-	-
10	3	0	0	II	31.43	503.43	51.95	217	No CR	-	-
11	1	0	0	I	43.07	13.64	23.14	20.34	No CR	-	-
12	1	4	0	IV	49.65	17.86	164.85	-	No CR	-	-
13	1	1	2	III	44.8	19.02	195.92	907.8	-	-	-
14	0	4	0	IV	66.5	21.87	79.95	-	-	-	-
15	0	3	1	III	29.95	7.97	216.89	26.48	-	-	-
16	1	3	1	III	26.34	31.95	366	358.15	-	-	-

Table 5-13: Baseline characteristics and outcomes of patients with de novo acute GvHD

Patient number	At baseline (day 0)													
	Steroids	Other IS	Skin	Mouth	Eyes	Gut	Liver	Lungs	Joint/Fascia	cGvHD grade	25(OH)D^3 (nmol/L)	Elafin (ng/ml)	ST2 (ng/ml)	REG3α (ng/ml)
1	systemic	CsA	1	2	2	0	0	0	0	Moderate	47.73	32.15	285.68	33.02
2	systemic	CsA	2	0	0	0	2	0	0	Moderate	37.17	19.75	33.46	71.78
3	systemic	CsA	0	2	1	0	1	0	0	Moderate	46.79	310.8	14.37	27.39
4	systemic	CsA	0	2	1	0	0	2	1	Moderate	34.41	7.5	18.7	12.99
5	systemic	CsA + Tac	2	1	0	0	0	0	0	Moderate	91.18	311.2	9.14	71.34

Table 5-14: Characteristics of patients with steroid-refractory chronic GvHD

Patient number	At baseline (day 0)									
	Steroids	Other IS	Skin	Gut	Liver	aGvHD grade	25(OH)D^3 (nmol/L)	Elafin (ng/ml)	ST2 (ng/ml)	REG3α (ng/ml)
1	systemic	CsA	3	2	0	III	62.56	266.5	283.3	88.38
2	systemic	CsA	2	2	0	III	38.95	170.73	483.05	-
3	systemic	CsA	3	3	0	III	38.71	173.42	258.75	304.1
4	systemic	CsA	2	4	0	IV	32.43	101.49	431.95	-
5	systemic	CsA + Etanercept	0	3	0	III	23.9	15	128.96	197.15
6	systemic	none	0	3	0	III	40.9	6.89	93.92	74.1
7	systemic	CsA	2	0	0	III	28.25	126.85	133.85	57.13
8	systemic	CsA	0	4	0	IV	27.53	20.27	204.7	112.41

Abbreviations: IS, immunosuppression, CsA, ciclosporin; Tac, tacrolimus

Table 5-15: Characteristics of patients with steroid-refractory acute GvHD