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CURED BUT NOT WELL: LONG TERM SURVIVORSHIP AFTER ALLOGENEIC HAEMATOPOIETIC STEM CELL TRANSPLANTATION

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Cured but not well: Long term survivorship after allogeneic haematopoietic stem cell transplantation
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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”I think that after discharge I found getting back to normal the most burdensome. […] that period literally brought me to my knees, because it’s only then that you realize what you’ve been through.”

Survivor after allogeneic haematopoietic stem cell transplantation

To the patients

POPULAR SCIENCE SUMMARY OF THE THESIS

The first step towards recovering from a bone marrow transplantation is realizing that your life will never be the same again[1]. As physicians we can provide the patient a detailed and accurate list of side effects, the risk of dying and the estimated benefits of the treatment, but life-disrupting event that he or she is about to encounter can hardly be described. Instead, most cancer patients simply trusts us healthcare workers with their lives and undergoes the transplant unaware of the life-altering long-term effects that it will likely have[2].

After the battle for life and death is over, the cured patients must find a way to resume living, despite any long-term consequences they might experience. By hindering social interactions and ability to work, fatigue and cognitive impairments often stand in the way and are described by many as the most debilitating consequences of cancer treatment. Imagine an overwhelming tiredness that leaves you exhausted on your bed for an hour after taking a shower. Since sleep does not make it go away, it is there from the moment you wake up until you go to sleep, every day for several years. At the same time, you may experience that your thoughts are slow, and your memory is blurred. You are unable to plan the simplest tasks, such as cooking a meal or brushing your teeth in the morning. While previously able to sustain an academic full-time work, you now find that halfway into your workday a wall of tiredness prevents any further concentration.

The new immune system that has been donated can often largely adapt to the recipient body, allowing transplantation doctors to reduce drugs that suppress inflammation. This contrasts with an organ transplantation, where the patient must take immunosuppressive drugs for the rest of his or her life. Sometimes, however, the adaptation fails, leading to damaging inflammation because the transplanted immune system cannot accept the new body and reacts against it, believing it is a foreign threat that should be eliminated. This is called graft-versus host disease and can affect most organs, resulting in hardened sclerotic skin, dry inflamed eyes, pain from the mouth and difficulties in breathing, among other symptoms. We believe that this process can also affect the brain, damaging the communication between nerve cells, causing fatigue and cognitive problems.

We have studied a new type of treatment for graft-versus host disease, called mesenchymal stromal cells. These cells are normally distributed in all body organs and are thought to help control the immune system, shutting off inflammation when it is no longer needed, to prevent organ damage. More than half of the patients that received this treatment improved in their symptoms, despite previous treatments being ineffective.

The rest of the thesis was dedicated to understanding if graft versus host disease affects the brain. We measured immune system activity in the brains of bone marrow transplanted patients with fatigue and found increased activation in a type of cells called T-cells, that can give rise to graft versus host disease. We confirmed that patients with fatigue also had troubles with memory and planning abilities and showed that activity was abnormally low in a part of their brains called the prefrontal cortex, considered important for intelligent

thinking. Importantly, all of this was associated with a poor quality of life and inability to resume full-time employment. As fatigue cannot yet be demonstrated on an x-ray or quantified in a blood sample, it is often forgotten among the multitude of parameters that requires attention at every visit to the transplantation clinic. In addition to advancing the understanding of how the brain reacts to a bone marrow transplantation, we therefore hope to directly help the suffering patients, by shedding light on their condition and providing instruments to measure these symptoms.

Despite these findings, many questions remain. We do not know to what extent our findings determine if the patients become fatigued or not. Many other factors may also be important, such as the psychological reaction to the life-threatening disease. Since we have only tested the patients once, we cannot be sure if the altered immune system activity we have detected cause fatigue or vice versa, the so-called chicken-and-egg problem. The treatment with mesenchymal stromal cells needs to be repeated in a larger study that also includes patients treated with placebo or “sham treatment”.

The goal of future studies must be to develop ways to prevent or treat fatigue and chronic graft versus host disease, so that transplanted patients can leave the long and winding hospital corridors and venture into the open landscape that is the rest of their lives.

ABSTRACT

Improved donor matching and supportive care have reduced short term complications after allogeneic haematopoietic stem cell transplantation (HSCT), leading to a growing number of survivors. However, despite being cured of leukemia many patients struggle to return to a normal life due to persistent chronic graft versus host disease (cGvHD), fatigue and cognitive dysfunction. Failure to develop tolerance to the grafted immune system underlies cGvHD and we thus hypothesized that MSC treatment may be effective when the first lines of treatment has failed. Further, a similar mechanism in the brain may disrupt neural communication and underlie persistent fatigue and cognitive symptoms. Consequently, this thesis aimed to study MSC treatment of cGvHD, as well as central nervous system (CNS) function, neurobiology, and immunology in patients with fatigue after HSCT.

In **study I**, 11 patients that were refractory to or did not tolerate first-line treatment for cGvHD were administered repeated infusions of allogeneic MSC. Responses were seen in six patients according to the National Institutes of Health scale. Responding patients had a pre-treatment immune phenotype with increased naïve lymphocytes and infusion triggered short-term increases in naïve T-cells and regulatory T-cells. Further, CXC-motif Chemokine Ligand 9 and 10 are potential biomarkers of response, as they decreased in responders and increased in non-responders during the study.

Study II, III and IV recruited 27 patients in haematological remission with (n=14) or without (n=13) self-reported fatigue confirmed with validated questionnaires, 1-5 years after HSCT. Metabolic, neurological, and psychiatric diseases were excluded. Fatigue associated with worse quality of life and reduced employment. Further, computerized testing focusing on memory and executive function demonstrated cognitive impairments. Subsequent functional near infra-red spectroscopy showed reduced prefrontal cortex activity during cognitive challenges, and impaired responses to a single dose of methylphenidate, compared to the non-fatigued patients. Lumbar punctures were performed and immune activity in the cerebrospinal fluid was assessed by proteomic analyses, mRNA sequencing and flow cytometry. Cognitive dysfunction was associated with reduced factors involved in immune regulation, neurogenesis, and synapse function, supported by mRNA-expression suggestive of reduced cell-cell adhesion and noradrenergic neuron differentiation. Flow cytometry demonstrated increased activated T-cells in patients with fatigue and cognitive dysfunction.

In summary, cGvHD is the manifestation of an imbalance between inflammatory and regulatory factors after HSCT, where the stroma plays a major role. MSC treatment can restore this balance by inducing regulatory phenotypes in innate and adaptive immune cells, as demonstrated by the clinical responses seen in study I. We show that dysregulated immune activation after HSCT also occur in the CNS. Deficits in stromal-derived, reparative, and trophic factors in liquor, characterized patients with fatigue and cognitive dysfunction, perhaps by impairing cortical activity. This thesis emphasizes the need to understand the stroma-immune crosstalk, to design targeted therapies for debilitating, currently untreatable inflammatory conditions.

LIST OF SCIENTIFIC PAPERS

I. Treatment of Chronic GvHD with Mesenchymal Stromal Cells Induces Durable Responses; a Phase II Study. **Boberg E***, von Bahr L*, Afram G, Lindström C, Ljungman P, Heldring N, Petzelbauer P, Garming Legert K, Kadri N and Le Blanc K. Stem Cells Transl Med, 2020 Oct.
*Equal contribution

II. Mental fatigue after allogeneic hematopoietic stem cell transplantation is associated with cognitive dysfunction, but not central nervous system inflammation. **Boberg E**, Kadri N, Winterling J, Davies LC, Björklund A, Msghina M, Iacobaeus E, Le Blanc K. Haematologica, 2020 Jun.

III. Reduced prefrontal cortex and sympathetic nervous system activity correlate with fatigue after HSCT. **Boberg E**, Iacobaeus E, Sklivanioti Greenfield M, Wang Y, Msghina M, Le Blanc K. Bone Marrow Transplantation, 2022 Mar.

IV. Cognitive dysfunction after HSCT associate with reduced regulatory activity of adult neurogenesis and immune system activity. **Boberg E**, Kadri N, Hagey D, El Andaloussi S, Iacobaeus E, Le Blanc K. *Manuscript*

OTHER RELEVANT PUBLICATIONS

Short and Long Term Clinical and Immunologic Follow up after Bone Marrow Mesenchymal Stromal Cell Therapy in Progressive Multiple Sclerosis-A Phase I Study. Iacobaeus E, Kadri N, Lefsihane K, **Boberg E**, Gavin C, Törnqvist Andrén A, Lilja A, Brundin L, Le Blanc K. J Clin Med. 2019 Dec.

Tissue immune profiles supporting response to mesenchymal stromal cell therapy in acute graft-versus-host disease-a gut feeling. Gavin C, **Boberg E**, von Bahr L, Bottai M, Törnqvist Andrén A, Wernerson A, Davies LC, Sugars RV, Le Blanc K. Stem Cell Res Ther. 2019 Nov.

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CONTENTS

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ATP	Adenoside Tri-Phosphate
aGvHD	Acute GvHD
cGvHD	Chronic GvHD
BAFF	B-cell Activating Factor
BBB	Blood Brain Barrier
BH	Benjamini Hochberg
BMI	Body Mass Index
CANTAB	Cambridge Neuropsychological Test Automated Battery
CCL	Chemokine C-C motif Ligand
CD	Cluster of Differentiation
CFU	Colony Forming Unit
CNS	Central Nervous System
CRP	C-reactive Protein
CSF	Cerebrospinal Fluid
CXCL	CXC-motif Chemokine Ligand
DAMP	Damage Associated Molecular Pattern
DC	Dendritic Cell
DLI	Donor Lymphocyte Infusion
ECF	Extracellular Fluid
ECP	Extracorporeal Photopheresis
EDA	Electrodermal Activity
EDL	Electrodermal Level
EDR	Electrodermal Responses
ELISA	Enzyme-linked immunosorbent assay
EV	Extracellular Vesicles
FACT-BMT	Functional Assessment of Cancer Therapy – Bone Marrow Transplant
fMRI	Functional MRI
FNIRS	Functional Near Infra-Red Spectroscopy

GDS	Global Deficit Score
GR	Glucocorticoid Receptor
GvHD	Graft Versus Host Disease
GvT	Graft versus Tumour
HAD	Hospital Anxiety and Depression scale
HCQ	Hydroxychloroquine
HLA	Human Leucocyte Antigen
HMGB1	High mobility group box 1
HSC	Haematopoietic Stem Cell
HSCT	Allogeneic Haematopoietic Stem Cell Transplantation
IBMIR	Instant Blood Mediated Inflammatory Reaction
IDO	Indoleamine Dioxygenase
IL	Interleukin
IL-1RA	IL-1 receptor antagonist
IQ	Intelligence Quotient
mHA	Minor Histocompatibility Antigen
miRNA	Micro RNA
MAC	Membrane Attack Complex
MHC	Major Histocompatibility Complex
mPFC	Medial Prefrontal Cortex
MFS	Mental Fatigue Scale
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
MSC	Mesenchymal Stromal Cell
NK-cell	Natural Killer cell
P1, P12	Passage 1, Passage 12
PAMP	Pathogen Associated Molecular Pattern
PDGF	Platelet Derived Growth Factor
PET	Positron Emission Tomography
PMN	Polymorphonuclear leucocyte
SAS	Subarachnoid Space

TF	Tissue Factor
Tfh-cell	Follicular Helper T-cell
TGF	Transforming Growth Factor
Th	T-helper cell
TNF	Tumour Necrosis Factor
Treg	Regulatory T-cell
TSPO	Translocator Protein
VCAM-1	Vascular Cell Adhesion Molecule 1
VRS	Virchow-Robin Space

1 LITERATURE REVIEW

1.1 THE FIRST IMMUNE THERAPY

Allogeneic Haematopoietic Stem Cell Transplantation (henceforth abbreviated as HSCT, since autologous transplantation is not discussed in this thesis) is the first developed and still by far the most frequently used immunotherapy for malignant diseases. It attempts to overcome one of the emerging hallmarks of cancer: the ability to evade immune destruction[3].

Since its first formulation by Paul Ehrlich in 1909, the theory that the immune system not only constantly surveys the body for external pathogens, but also for internal alterations in cellular homeostasis has prevailed and been extensively studied[4]. Such an "immune surveillance" is postulated to eliminate most cells that undergo malignant transformation and play an important role in protecting us from cancer[4]. A malignancy develops through the acquisition of mutations that provide its cells with a survival advantage and suppresses normal growth limiting mechanisms[3]. In this process, neo-antigens are formed, that consist of mutated versions of normally occurring proteins. Furthermore, by growing uncontrollably, a malignant clone gives rise to surrounding tissue damage, releasing damage associated molecular pattern (DAMP) molecules to the environment, and activating surrounding macrophages and dendritic cells (DCs)[3]. These innate antigen presenting cells can potentially activate T-cells of the adaptive immune system that recognize the neo-antigens, thus eliciting an immune reaction[5]. Cytotoxic T-cells and natural killer (NK-) cells appear to be especially detrimental to tumour growth, and their activation can result in rapid tumour cell destruction, a mechanism currently utilised in the development of novel cancer therapies, such as check point inhibitors and chimeric antigen receptor T-cells[3,6].

A successful tumour thus needs to overcome immunological barriers, and it is demonstrated that tumours can even turn the adverse immune reactions to their advantage[3]. Inflammation results in angiogenesis, production of growth factors and degradation of the extracellular matrix, thus increasing available nutrients, providing space, and helping migration of metastatic cancer cells. Emerging tumours can evolve to reap these benefits, while protecting themselves from immune destruction through decreased surface antigen expression as well as recruitment and promotion of regulatory T-cells (Tregs), tumour associated macrophages and myeloid derived suppressor cells[3].

The theory of immune evasiveness as a hallmark of cancer development states that in a patient with clinically evident cancer, a selection process has already taken place, where tumour cells with the ability to evade that person's immune defence have emerged[3,5]. By simultaneously eradicating most of the remains of the tumour and replacing the patient's immune system with one from a healthy donor, the HSCT procedure attempts to re-establish immune surveillance, leading to long term survival[7].

1.2 ALLOGENEIC HAEMATOPOIETIC STEM CELL TRANSPLANTATION

Originally attempted in the 1950:s as a means to save victims exposed to toxic levels of radiation or chemotherapy, the bone marrow transplantation procedure was not successful until the Major Histocompatibility Complex (MHC) was discovered in the 1970:s[8,9]. Before that, the inability to select immunologically matched donors led to detrimental rejections and immunological reactions, killing most of the patients exposed to the treatment[10].

An HSCT procedure consists of several steps. First a conditioning regimen, consisting of chemotherapy and/or radiation, is administered. The purpose of the conditioning is to create physical space for the graft in the recipient bone marrow stroma, to suppress the donor immune system in order to prevent rejection, and to eradicate as many as possible of the remaining tumour cells[11]. After the conditioning, the graft is administered as an intravenous infusion of Cluster of Differentiation (CD)34+ haematopoietic stem cells (HSCs) mixed with naïve and memory lymphocytes and other immune cells[12]. Concomitantly, immunosuppressive treatments are administered to dampen the expected immunological reactions, thus preventing detrimental rejection and acute graft versus host disease (GvHD)[13]. The HSCs will home to the bone marrow niche where they engraft and re-establish haematopoiesis in the recipient. Full haematopoietic reconstitution takes at least one year, with the adaptive immune system, consisting of the B-cell and T-cell compartments, being the slowest[14,15]. Since donor T-cells play an important role in the defence against infections and in supporting the growing HSCs, recipients of T-cell depleted grafts have an increased risk of fatal infections and graft failure[16,17].

The potency of the HSCT as an immunological treatment is evident from the acute and chronic GvHD that remain important causes of mortality and morbidity after the procedure[18]. A retrospective study of over 2000 HSCT recipients in 1990 clearly demonstrated that grafts from identical twins (syngeneic transplantation), while having no human leucocyte antigen (HLA) disparity and thus no GvHD, had the highest incidence of relapse, equal to that resulting from modifying the graft by depleting T-cells[19]. In addition, in HSCT, presence of GvHD was demonstrated to result in a significantly reduced relapse rate[19]. These findings confirmed the existence of a clinically relevant T-cell mediated graft versus tumour (GvT) reaction that reduce the risk of relapse beyond what can be achieved with chemo- and radiotherapy alone. The GvT reaction is thus the theoretical benefit that the clinician is seeking, when recommending an HSCT over an autologous or syngeneic transplant for certain high risk haematological malignancies. Further support to this concept comes from the success of administering donor lymphocyte infusions (DLI), where additional lymphocytes from the donor are given to the recipient without prior conditioning, in order to suppress an emerging relapse after HSCT[20].

Both allo-reactive naïve and memory T-cells, as well as NK-cells and possibly allo-antibodies produced by B-cells, are able to mediate the GvT reaction[20]. Thymic selection ensures that T-cell receptors (TCR) on naïve T-cells do not have affinity for self-peptides bound to self-MHC. However, they are still potentially reactive against non-self-peptides bound to self-

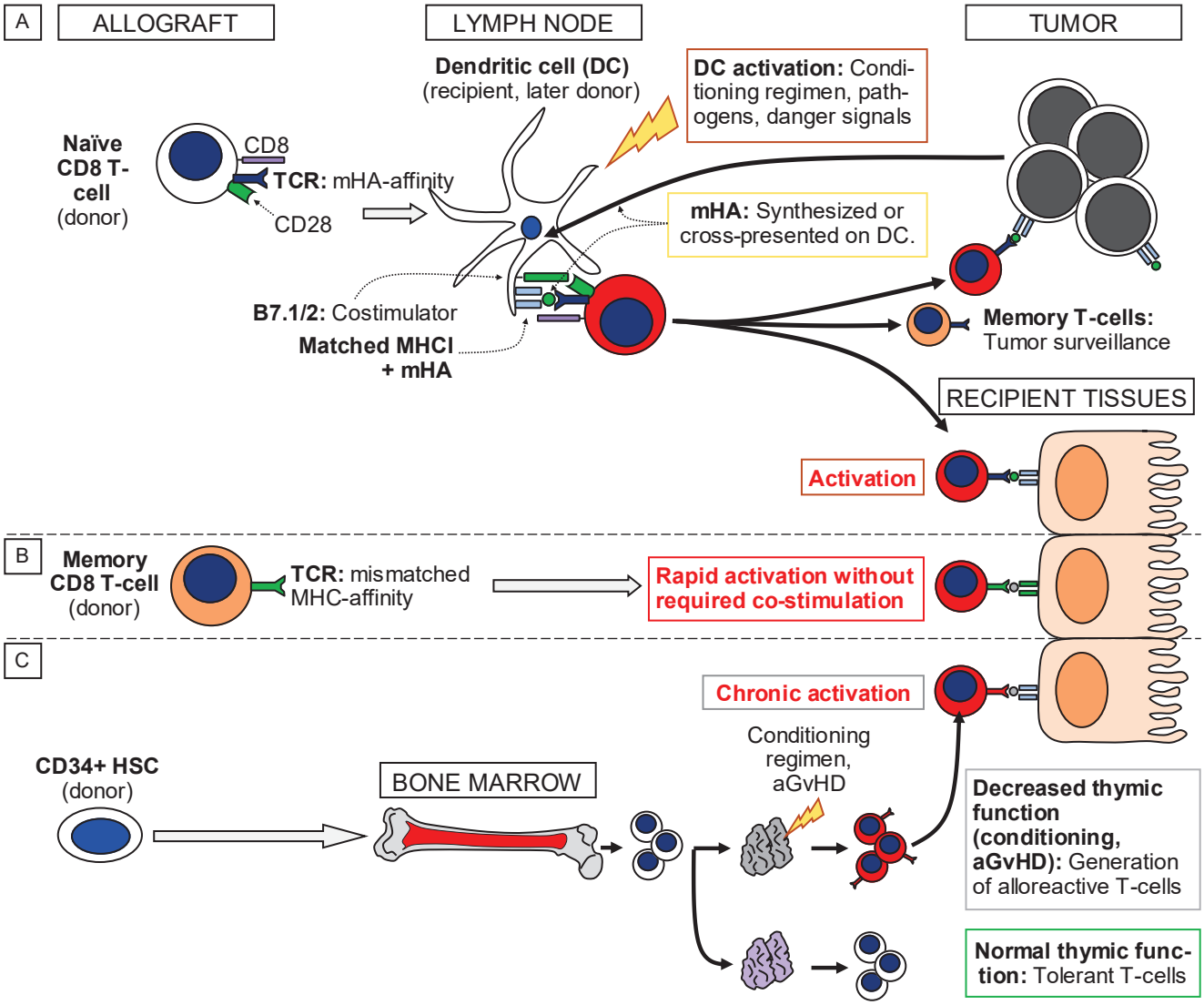


Figure 1: Immunological mechanisms leading to GvT reaction and GvHD: A) The conditioning regimen and pathogens cause tissue damage and inflammation, activating DCs. HLA-matched transplantation: The donor T-cells that have mHA-affinity have normally never been activated (except for H-Y antigens in a female donor) and are therefore naïve. The naïve donor T-cells are activated by DCs that express mHAs on MHC class I. Recipient DCs can synthesize the mHA peptides themselves, while donor-derived DCs cross-present them from tumour or tissue. Activation generates GvHD or GvT reactions. If signs of relapse are detected, DLI can be administered to supply more naïve T-cells and enhance this process. **B)** HLA-mismatched transplantation: Memory T-cells reactive against mismatched MHC will be present in the graft. Since memory T-cells do not require co-stimulation, they will rapidly activate, causing severe, early onset GvHD and GvT reactions. **C)** Immune reconstitution and cGvHD: T-cell progenitors develop from HSCs contained in the graft. They undergo thymic selection and develop tolerance, thus not likely contributing substantially to GvT. Decreased thymic function due to toxicity from the conditioning regimen, aGvHD or older age, impair thymic selection, possibly contributing to cGvHD pathogenesis. In addition, a thymic dysfunction causes limited T-cell repertoire and persistent susceptibility to opportunistic infections.

MHC as well as to non-self-MHC regardless of the bound peptide. Due to the reduced risk of severe GvHD, clinical evidence is in favour of finding a fully MHC-matched donor, preferably a sibling[21]. In MHC matched transplantation, the GvT and GvHD reactions are dependent on minor histocompatibility antigens (mHAs) that stem from genetic disparities between donor and recipient caused by single nucleotide polymorphisms (figure 1A)[20,22]. Since it is unlikely that the donor T-cells have previous experience with these antigens, the mHA-reactive T-cells in the graft are naïve. Naïve T-cells require APCs expressing co-stimulatory molecules in order to become active, resulting in delayed and less potent activation[20]. An exception is when transplanting from a female donor to a male recipient. The Y-chromosome contains male mHAs that the female may have been previously exposed to, resulting in mHA-reactive memory T-cells and more severe GvHD[23,24]. In MHC mismatched transplantation (figure 1B), memory T-cells in the graft that were previously activated by foreign antigens can recognize the non-self MHC present in the recipient. Since memory T-cells can be activated without priming by antigen presenting cells, mismatched transplantations result in strong GvHD and GvT effects[20]. Coord blood transplantation and use of younger donors mitigate this effect to some extent, since they contain a more naïve T-cell compartment[25,26]. Recently, post-transplant administration of T-cell suppressive agents, such as cyclophosphamide, have enabled haplo-identical MHC-mismatched transplantation by eliminating expanding allo-reactive memory T-cell clones shortly after graft infusion[27].

Increased understanding of the potent immunological GvT effect after HSCT has led to the development of reduced intensity conditioning regimens, where lower doses of chemotherapy and radiation are administered, focusing on creating immunological space for the graft rather than eliminating tumour cells[28]. These reduced regimens enable HSCT to be performed in older, more fragile individuals.

1.3 GRAFT-VERSUS-HOST DISEASE

During the follow-up after HSCT, a delicate balance must be maintained, where enough immunosuppression is administered to prevent severe GvHD, without removing the important GvT effect. This is usually achieved through a combination of early single methotrexate doses and continuous calcineurin inhibitor treatment that is gradually tapered during the first 6-12 months after transplant[29]. Meanwhile, a new immune system with donor origin is developed in the recipient. Tissue damage caused by the conditioning regimen and by bacterial infections during the first 3-4 weeks releases pro-inflammatory cytokines, resulting in potent activation of lymphocytes in the graft with TCR affinity for major and/or mHA. If they are not sufficiently suppressed, they will cause acute GvHD (aGvHD), usually manifesting as acute onset of severe diarrhoea, liver necrosis and/or severe skin ulcerations within 3 months after transplant[30].

During the first years after HSCT, central and peripheral tolerance to the recipient tissues develops in the transplanted immune system. If immune tolerance is impaired however, another insidious form of GvHD, termed chronic GvHD (cGvHD), can develop. While the pathogenesis of aGvHD is quite well understood, and highly dependent on the activation of

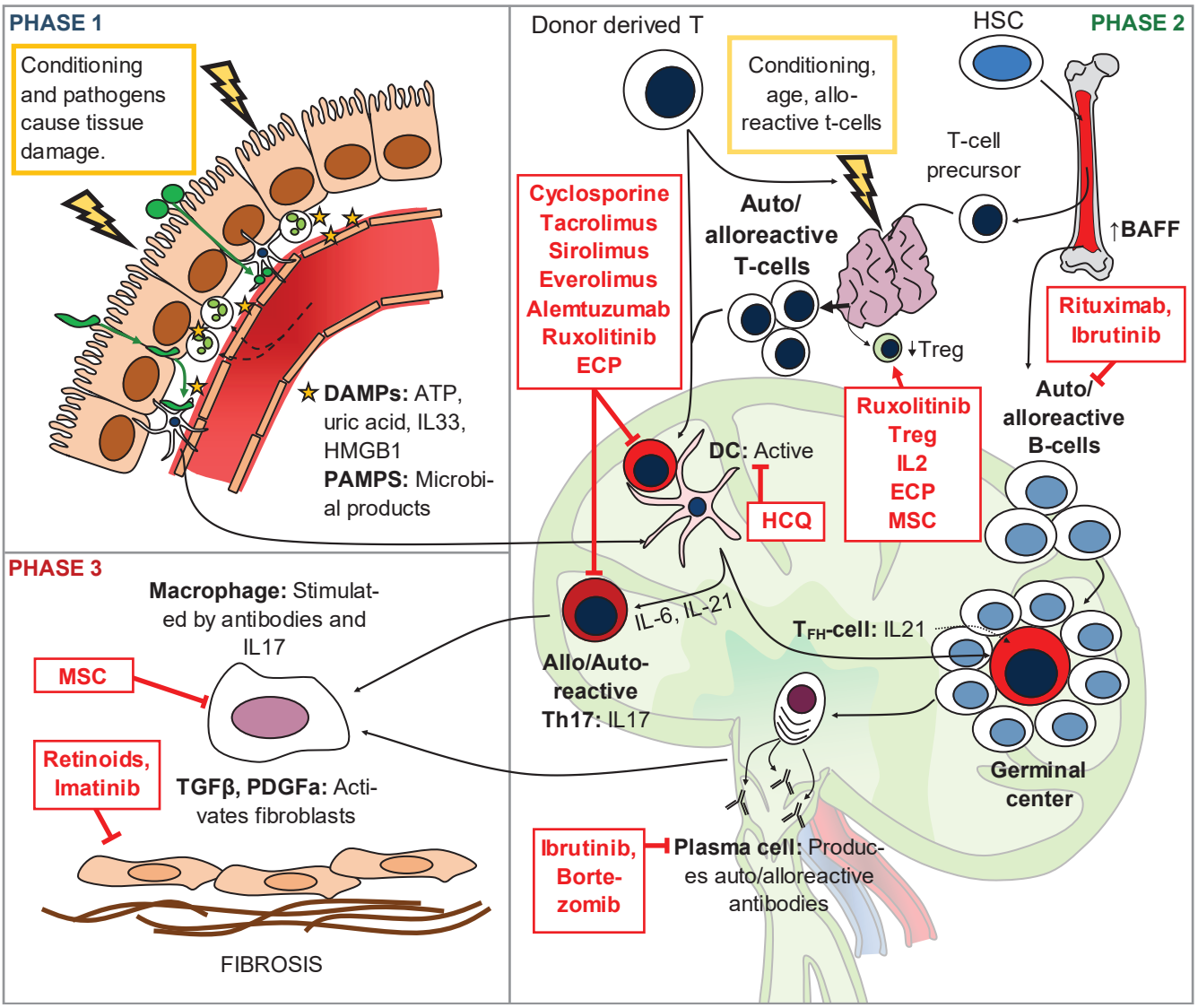


Figure 2: Pathogenesis of cGvHD and treatment targets. cGvHD is thought to develop in in three phases. In phase 1, tissue damage caused by conditioning, infections and aGvHD lead to the release of DAMPs, and allows translocation of bacteria and fungi, carrying PAMPs. PAMPs and DAMPs activate innate immune cells leading to inflammation and more tissue damage. In phase 2, activated DCs stimulate alloreactive T- and B-cells to differentiate into Th1, Th17 and Th2 subtypes. Thymic damage from the conditioning regimen and alloreactive T-cells impair positive and negative selection, leading to autoreactive T-cells and reduced Treg numbers. Tfh-cells produce IL-21 and generate germinal centres resulting in plasma cells producing allo- and auto-antibodies. In phase 3, IL17 and likely allo-antibodies activate macrophages to release PDGFα and TGFβ that stimulate fibroblasts to convert to matrix-producing myofibroblasts, leading to fibrosis and sclerosis.

naïve and memory T-cells caused by systemic inflammation and exposure to allo-antigens, the mechanisms resulting in cGvHD are much less known and likely involves many other components of the immune system[31]. The development begins with the acute inflammation caused by the conditioning regimen, infections and aGvHD (figure 2)[31,32]. This leads to production of DAMPs and pathogen associated molecular patterns (PAMPs) as pathogens cross the disrupted epithelial membranes. The thymus is also damaged in this process, disrupting central immune tolerance by hampering the positive and negative selection of maturing donor T-cells[31,32]. Alloreactive T-cells seem especially detrimental to thymic function, since cGvHD does not manifest after autologous transplantation, where instead thymopoiesis leads to new and diverse T-cell repertoire[33]. Further, the reduced thymic production of Tregs, as well as decreased levels of regulatory B- and NK-cells result in diminished peripheral tolerance due to imbalance between auto- and alloreactive effector cells and their regulatory counterparts[31,32]. The final step in the reaction is the maturation of fibroblasts into matrix producing myofibroblasts, stimulated by platelet derived growth factor (PDGF) α and transforming growth factor (TGF) β, causing fibrosis and sclerosis[31,32]. The resulting disease is characterized by progressive chronic low-grade inflammation and sclerosis, that can affect most organs[34].

1.3.1 Diverse GvHD treatments with limited efficacy

The first line treatment of both acute and cGvHD is high doses of systemic corticosteroids, gradually tapered during weeks to months after treatment initiation[35,36]. The addition of calcineurin inhibitors is frequently made, due to their steroid sparing effects. These regimens are well supported by randomized clinical trials, but frequently fail to achieve remission[37,38]. For steroid-refractory patients, evidence to suggest how to proceed is scarce. A multitude of immunomodulating agents can be attempted for refractory GvHD, targeting T-cells, B-cells, plasma cells, DCs, fibrosis, cytokines as well as agents with multiple targets (Figure 2)[39]. However, few randomized controlled trials exist that demonstrate benefit over treatment with corticosteroids and calcineurin inhibitors. Ruxolitinib was recently demonstrated to induce higher overall response rates and failure-free survival in two phase III randomized open-label trials of patients with steroid refractory aGvHD and cGvHD[40,41]. However, overall survival was not significantly different between the treatment and control groups. Further, mesenchymal stromal cell (MSC) infusions have demonstrated efficacy in inducing remission in a single arm phase III trial in pediatric patients and a randomized phase III trial in adults with steroid refractory aGvHD, but not yet for cGvHD[42,43]. Other treatment recommendations are based on phase II trials and retrospective analyses[39]. Notably, a single-arm trial of ibrutinib for steroid refractory cGvHD demonstrated meaningful responses and led to approval by the Food and Drug Administration in the United States for this indication[44].

1.4 MESENCHYMAL STROMAL CELLS

1.4.1 The stroma is not simply a scaffold

Defined as the connective tissue, adipose tissue, lymphatics and blood vessels that surround and support the "functional" parenchyma of internal organs, it is understandable that the stroma could be considered a mere passive scaffold[45]. Indeed, producing extracellular matrix to provide structural support is probably its best characterized function. However, the mesenchymal cells in the stroma, a diverse population of fibroblasts, pericytes and progenitor cells, also play an active role in immune modulation and tissue repair[45]. Consequently, dysregulation of stromal cells can lead to chronic inflammation and fibrosis, suggesting the stroma as a novel therapeutic target in currently incurable diseases such as systemic sclerosis, pulmonary fibrosis, multiple sclerosis (MS) and cGvHD[46].

1.4.2 Stem cells in the stroma?

The term MSC is used to describe a heterogeneous population of cells that fulfil certain criteria after culture on a plastic surface[47]. The *in vivo* characteristics of MSCs remain somewhat elusive, but they are believed to reside in the perivascular space in most organs (figure 3A), and several physiological roles have been proposed. MSCs were initially discovered in 1968 as a small subset of bone marrow cells (1:100 000 – 1:10 000 cells) that could differentiate along osteogenic, chondrogenic and adipogenic lineages *in vitro*, and were consequently named Mesenchymal *Stem* Cells in 1995[48–50]. Their suggested *in vivo* role was to replace tissues of mesenchymal origin (muscle, adipose, tendons, stroma, cartilage and bone), similar to how the HSCs continuously replace blood cells[51]. However, it took about 40 years before it was finally demonstrated that MSC-like cells fulfil true stem cell criteria, i.e., can be serially transplanted *in vivo* while retaining the ability to differentiate to osteoblasts, reticular cells and adipocytes, comprising a complete bone marrow niche able to support haematopoiesis (figure 3A)[52–54]. However, a demonstration that the MSCs found in other organs (such as adipose tissue and placenta) have stem cell characteristics is still lacking. Neither has it been proven that MSCs give rise to other mesenchymal tissues such as skeletal muscle, myocardium, or endothelium *in vivo*. In an effort to align the terminology with the current understanding of the physiological properties of the cells, a change in nomenclature to Mesenchymal *Stromal* Cells was proposed in 2005[55].

1.4.3 Orchestrators of inflammation

Apart from the stem cell properties of a small subset of MSCs in a polyclonal cell culture, a large number of studies have demonstrated that MSCs produce paracrine factors that regulate the activity of both innate and adaptive immune cells *in vitro*[56,57]. Due to their perivascular location, they are well suited to detect surrounding inflammation, and the local cytokine milieu can license MSCs to become either pro- or anti-inflammatory[56]. Pro-inflammatory stimuli, such as tumour necrosis factor (TNF) α and interferon-γ stimulate MSCs to produce indoleamine deoxygenase, prostaglandin E2, hepatocyte growth factor and TGFβ. These

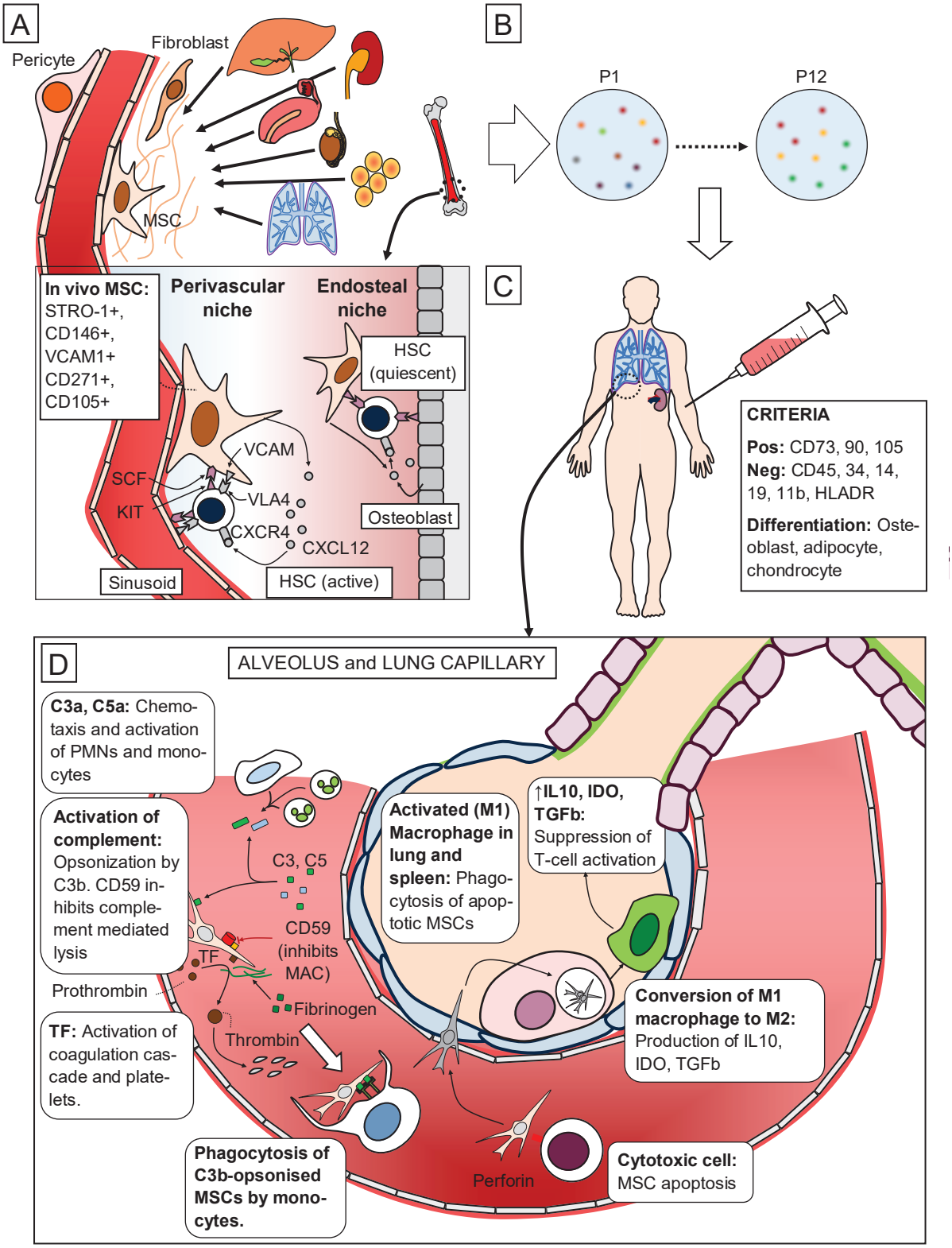


Figure 3: MSC in vivo and therapeutic mechanisms. A) MSCs are thought to reside in the vascular walls of blood vessels in many organs, with a similar phenotype as pericytes. In the bone marrow, MSCs support HSCs through cytokine secretion and cell-cell contact. **B)** MSCs can be harvested from several organs including bone marrow, adipose tissue, and placenta. No single specific antigen exists to isolate MSCs. Instead, harvested mononuclear cells are cultured on a hard, plastic surfaces, resulting in formation of CFUs of cells that fulfil predefined criteria by the International Society for Cell and Gene Therapy. These cells are expanded in several passages to yield a clinical product. With prolonged expansion, a few of the initial clones will eventually dominate the expanded product. **C)** After infusion the MSCs are opsonized by the complement system, leading to phagocytosis. In addition, MSCs express TF that activates the coagulation system and coat the MSCs in fibrin. This reaction, termed IBMIR, recruit neutrophils and monocytes. The MSCs are protected from MAC-mediated cell lysis by CD59 but activated cytotoxic cells may kill the MSCs using perforin. Apoptotic MSCs are then phagocytosed by monocytes that become anti-inflammatory.

factors in turn are known to suppress T-cell activation, promote Treg conversion, and to polarize macrophages towards an anti-inflammatory M2 phenotype. On the other hand, low levels of TNFα and interferon-γ, as well as activation of toll like receptor 4 on the MSC surface by the bacterial cell wall component lipopolysaccharide results in a pro-inflammatory phenotype with increased production of chemokines such as CXC-motif Chemokine Ligand (CXCL) 9, 10, and chemokine C-C motif ligand (CCL) 3, 4 and 5. These chemokines attract and activate lymphocytes, granulocytes, and monocytes. Additionally, several other mechanisms of immune regulation by MSCs have been discovered, such as release of extracellular vesicles (EVs), micro RNA (miRNA), and direct cell-cell contact mediated by expression of cellular adhesion molecules, galectin-1, and programmed death ligands 1 and 2[58–62].

Inflammation serves to preserve tissue homeostasis in the face of external threats and internal tissue damage. Therefore, an active, coordinated program is initiated early after an inflammatory response begins, and functions to eventually limit the extent of immune activation by inhibiting recruitment and promoting apoptosis of granulocytes, and by inducing macrophage-mediated efferocytosis with subsequent release of reparative cytokines such as TGFβ[63–65]. After resolution of inflammation an expansion of regulatory and memory lymphocytes takes place in the so called "post-resolution phase"[64]. It is speculated that MSCs participate in the orchestration of immune activity, both by enhancing early immune responses to pathogens and by later suppressing excessive inflammation to promote resolution.

1.4.4 Medicinal cells

Due to their low expression of MHC class I and II and their ability to suppress T-cell activation, MSCs can be effectively administered in both autologous and allogeneic settings[66]. Further, their self-renewal capacity allows them to be readily expanded ex vivo, to obtain adequate amounts for therapeutic efficacy (figure 3B-C)[67]. Preclinical studies of local injection and systemic infusion of culture-expanded MSCs have been very promising: MSCs were demonstrated to promote both tissue regeneration (in traumatic brain injury, myocardial infarction, osteoarthritis and wound healing) as well as immune regulation in acute (sepsis, acute respiratory distress syndrome, aGvHD) and chronic (rheumatoid arthritis, systemic lupus erythematosus, MS, diabetes mellitus) inflammatory disorders[68–78].

Unfortunately, it has been challenging to translate these findings to the clinic. Among the first trials to demonstrate the feasibility of MSC treatment for inflammatory conditions in humans were a case report of a 9-year-old boy with steroid refractory grade IV aGvHD and a subsequent multi-centre phase II trial where 39 out of 55 patients responded[79,80]. Since then, a large number of phase I/II clinical trials have been conducted, where MSC treatment have been applied to various inflammatory conditions[81]. However, despite promising results, only a handful of prospective, randomized phase III trials have been conducted, demonstrating efficacy for aGvHD, diabetic ulcers and chronic fistulas in Crohn's disease (table 1). Possible explanations for the discrepancy between preclinical and clinical trial outcomes include[82]; 1)

differences in tissue compatibility (often allogeneic MSCs in clinical trials, mostly syngeneic MSCs in preclinical studies), 2) predominantly thawed MSCs in clinical trials and fresh MSCs in preclinical studies, and 3) lower cell dose in clinical trials (typically 1-2 million cells/kg vs 50 million cells/kg iv in preclinical studies).

Table 1: Prospective randomized phase III trials of MSC treatment including a control arm

Indication	Primary endpoint	Primary end point met?	Other results	Reference
Systemic treatment				
Steroid refractory aGvHD	Complete remission for 28 consecutive days	No	Improvement in liver GvHD, high grade GvHD and in children.	Kebriaei et al[83]
Steroid refractory aGvHD (+basiliximab)	Overall response at day 28	Yes	Longer failure free survival. Lower risk of cGvHD.	Zhao K et al[84]
Local injections				
Cartilage defects in knee. Local injection of MSC+hyaluronate compared to micro-fracturing.	Improvement according to arthroscopic evaluation after 48 weeks.	Yes	Less pain after 3-5 years.	Lim H-C et al[85]
Complex perianal fistula in Crohns disease	Remissin at week 24	Yes		Panés J et al[86]
Complex perianal fistulas, no IBD	Fistula healing at week 24-26	No		Herreros M D et al[87]
ALS. Intrathecal injection.	Slower disease progression at week 28	No	Improvement in CSF biomarkers.	Cudkowicz M et al[88]
Heart failure, endomyocardial injection.	Feasibility/safety at 2-year follow-up	Yes	Improved LVEF, 6 min walk test and clinical symptom score.	Bartunek J et al[89]
Heart failure, endomyocardial injection or sham procedure.	Composite endpoint.	No		Bartunek J et al[90]
Foot ulcer and critical limb ischemia.	Not stated	Better wound healing and painless walking.	No differences in pain relief and amputation.	Debin L et al[91]

IBD: Inflammatory Bowel Disease, LVEF: Left Ventricular Ejection Fraction

As a medicinal product, MSCs need an assay to determine their potency. Unlike traditional pharmaceutical compounds, that consist of well-defined molecules with known pharmacodynamic and pharmacokinetic properties, MSCs are living cells whose metabolism and structure vary over time. In addition, MSCs from different sources vary in cytokine expression, procoagulant activity and differentiation potential[92–94]. Due to their adaptive nature, the efficacy and in vivo therapeutic mechanisms of MSC treatment are likely dependent on both donor and recipient properties and may vary depending on underlying

disease and individual patient characteristics[95]. MSC-secreted factors and co-culture experiments to determine in vitro T-cell suppression do not correlate with clinical efficacy[96,97]. A functional potency assay would guarantee that despite the inherent heterogeneity, all patients treated with any MSC therapy receive an equally potent drug. Another method of decreasing heterogeneity is to limit the number of MSC donors. However, a retrospective study suggested that MSCs of lower passage seem to yield better clinical outcomes[98]. In addition, Remestemcel-L, a commercial MSC product created from extensive culturing of cells from a single donor, yielding upwards of 10 000 doses, did not meet its primary endpoint of treating steroid refractory aGvHD, while a later academic trial of MSCs harvested in passage 4-5 for the same indication did (table 1)[43,83].

1.4.5 MSC fate after infusion - Curing by being eaten?

MSCs, being tissue resident stromal cells, are not normally in contact with blood[99]. Since their only natural exposure to blood occurs at sites of injury, such contact is programmed to elicit the coagulation cascade and an inflammatory process, ultimately resulting in wound healing. Unlike endothelial cells, that are equipped with powerful anticoagulant properties, MSCs will therefore cause activation of the coagulation cascade after intravenous infusion[99]. This reaction, which was first observed in trials of pancreatic islet and hepatocyte transplantations through the portal vein, is termed the instant blood mediated inflammatory reaction (IBMIR, figure 3D)[100]. This cascade is initiated by binding of factor VII to tissue factor (TF) expressed on the stromal cell surface, resulting in activation of the coagulation cascade[101]. In addition, complement activation causes membrane attack complex (MAC) formation, as well as chemotaxis for granulocytes and monocytes through the cleavage products C3a and C5a, ultimately resulting in cell death and thrombosis[102]. MSCs express low levels of TF on their surface and have been shown to initiate IBMIR[99,103]. However, bone marrow MSCs also express complement inhibitors CD46, CD55, CD59, and haemostatic regulators tissue factor pathway inhibitor, tissue plasminogen activator and prostacyclin synthase, effectively limiting MAC formation and thrombosis[57]. These properties might explain the lack of clinically relevant thrombotic events seen after MSC infusions[104].

Despite protection from complement mediated lysis, MSCs are short lived in the circulation, and can no longer be detected 6 hours after intravenous infusion in humans[105]. Studies in mice suggest that most of the infused MSCs are trapped in the lung, through either an active or a passive process[106,107]. This puts MSCs in close contact with alveolar macrophages, which can engulf them in a process termed efferocytosis. Opsonisation by complement as part of the IBMIR help mediate this process[108].

Based on animal models, one proposed mode of action of MSC-mediated immunosuppression has been that they home to sites of inflammation and release paracrine factors that support immune regulation and tissue repair[95]. However, the pulmonary entrapment and poor long-term engraftment of MSCs contradict this theory in humans[109]. Instead, recent evidence suggest that phagocytosis or efferocytosis by macrophages are important mediators of MSC immunosuppressive action (figure 3D)[65]. In septic mice and

aGvHD patients, monocytes and macrophages engulf MSCs and adopt an anti-inflammatory phenotype, releasing immunosuppressive mediators such as TGFβ, indoleamine deoxygenase and interleukin (IL)-10[110,111]. This novel mode of action may be similar to how the foetus is immunologically accepted during pregnancy. It has been demonstrated that small pieces of the foetal syncytiotrophoblast are continuously shed from the placenta to the peripheral blood and trapped in the maternal lung during pregnancy[112]. These pieces, termed "syncytial knots", are engulfed by lung macrophages through efferocytosis, promoting a similar anti-inflammatory phenotype as described after MSC treatment[113]. Therefore, the effect of systemic MSC infusion may be to strengthen physiological inhibition of alloreactivity.

Importantly, the ability of recipient derived cytotoxic cells to induce apoptosis in MSCs prior to phagocytosis was strongly correlated to clinical outcome after aGvHD treatment, suggesting a novel potency assay for MSC treatment[110]. A reasonable hypothesis would be that inducing apoptosis in MSCs before administering them could bypass this requirement and increase their potency. However, recent preclinical data suggest that viability of administered MSCs is important for their efficacy to treat experimentally induced colitis[114]. Another important question is whether the anatomical location of MSC efferocytosis plays a role. Does entrapment in the lung vasculature inhibit immune regulation in target organs, or do secreted cytokines by affected pulmonary macrophages exert systemic effects? Injecting syngeneic MSCs intraperitoneally or subcutaneously was demonstrated to be superior to iv delivery in resolving experimentally induced colitis[114]. However, promotion of an anti-inflammatory phenotype in pulmonary macrophages is potentially sufficient to prevent foetal rejection and improve sepsis outcome after MSC delivery[111,113].

1.5 THE CENTRAL NERVOUS SYSTEM AFTER HSCT

Even though the immune reconstitution in peripheral blood after HSCT is well characterized, not as much is known about the degree and kinetics of tissue-resident immune cell replacement[115]. Peripheral blood and bone marrow chimerism are poor surrogate markers, as demonstrated by a recent study in which patients with 100% donor chimerism at these sites had remaining recipient-derived tissue resident T-cells, up to 1000 days after HSCT[116].

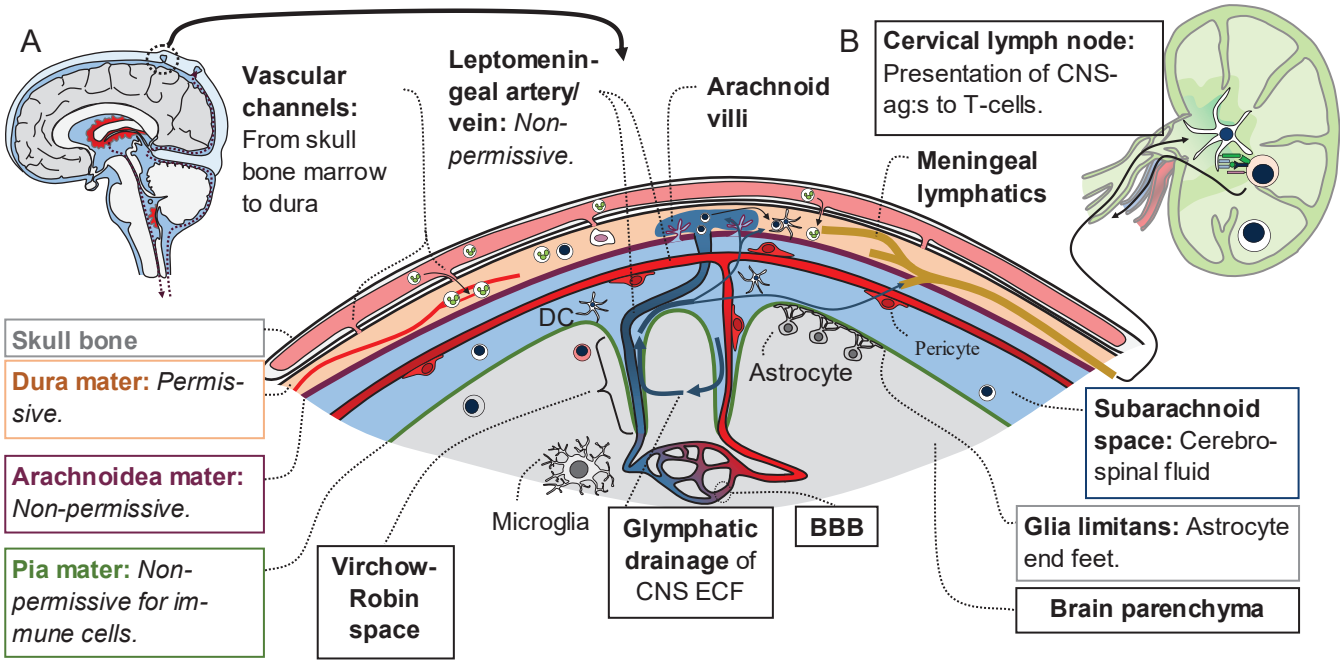
1.5.1 Replaced microglia?

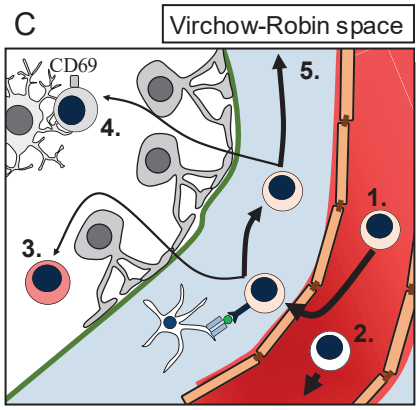
Microglia are the primary resident immune cells of the brain parenchyma. They produce cytokines that regulate inflammation and neuronal survival, and act as phagocytes to prune synapses and clear debris[117,118]. They are also important contributors to central nervous system (CNS) disease pathology, as overactivation cause production of neurotoxic reactive oxygen species and inflammatory cytokines[118]. Microglia populate the brain from the yolk sac during foetal development and normally slowly self-renew during adult life, independent of bone-marrow derived monocytes[117]. However, after HSCT, depleted recipient microglia are partially replaced by donor-derived macrophages, with a higher degree of replacement occurring after myeloablative conditioning[119,120]. The bone-marrow derived macrophages adapt a microglia-like phenotype but retain a transcription signature that is distinct from the yolk-sac derived counterparts[121].

1.5.2 Immune surveillance of the CNS

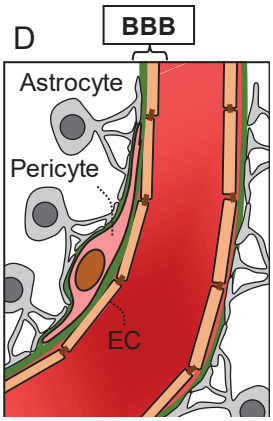
The brain parenchyma is devoid of lymphatic vessels and presentation of CNS antigens to the adaptive immune system consequently require alternative routes. It has recently become evident that the circulation of cerebrospinal fluid (CSF) plays a central role. CSF enters the brain parenchyma from the perivascular space alongside arterioles that penetrate from the subarachnoid space (SAS)[122]. Subsequently, it is mixed with brain extracellular fluid (ECF) containing antigens and drained back into the SAS along exiting venules (figure 4A). Finally, the brain-derived antigens are drained by meningeal lymphatic vessels terminating in cervical lymph nodes, and by the arachnoid villi to the systemic circulation (figure 4B). In addition, specialised hubs have recently been discovered in the vicinity of the venous sinuses in the dura, containing antigen-presenting cells[123]. Through an unknown mechanism, antigens cross the arachnoidea mater close to these hubs and are presented to T-cells arriving through the venous endothelium guided by chemokines such as CXCL12 and CCL19 (figure 4A)[123]. In addition to antigen presentation, these hubs recruit neutrophils, monocytes and B-cells directly from the bone marrow in the overlying skull bone through specialised channels that penetrate down to the dura[124,125]. The immune cells recruited along this route are more immunoregulatory compared to the inflammatory counterparts recruited from the peripheral blood[124].

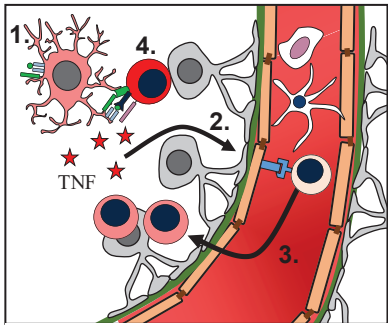
Naïve T-cells are largely not permitted to cross the blood brain barrier (BBB). Instead, the CNS is continuously surveyed by effector and memory T-cells that migrate through the endothelium into the brain parenchyma. In the Virchow-Robin space (VRS), a continuation





1. CM/EM T-cell
2. Naïve T-cell
3. CM/EM T-cell reactive to CNS ag
4. Resident T-cell
5. CM/EM T-cell without affinity to CNS ag





1. Activated microglia produce TNF
2. The TNF activates BBB endothelium (VCAM1)
3. T-cells, DCs, monocytes enter through BBB
4. The activated microglia activate entering T-cells (CD80+MHCII)

Figure 4: Immune surveillance of the CNS. The CNS is considered immune privileged. In contrast to other organs, the capillaries of the brain parenchyma are lined with endothelial cells connected with tight junctions. This non-permissive endothelium makes up the BBB together with the Glia limitans (basement membrane and astrocyte end-feet). The arachnoid mater consists of a layer of cells separating the dura from the SAS containing CSF. During CNS inflammation, this layer can become more permissive and allow entry of innate immune cells. Vascular channels from the bone marrow in the skull provide a direct route for migration of innate immune cells to the meninges. **A)** CNS-derived antigens are transported to the cervical lymph nodes through meningeal lymphatic vessels that drain the CSF, which in turn receives them from the brain parenchyma along the paravenous glymphatic route. **B)** In the lymph nodes, DCs present brain-derived antigens to naïve T-cells. **C)** In contrast to naïve T-cells, activated T-cells (effector or central memory) can migrate across the endothelium of vessels in the VRS. Once the T-cells enter the CSF in the VRS they interact with DCs. T-cells specific for brain-derived antigens presented on DCs in the VRS cross the glia limitans and cause inflammation in the brain parenchyma. In addition, a small subset of activated T-cells infiltrates the parenchyma and become resident T-cells. One function of these T-cells is to interact with microglia and support their maturation. **D)** The BBB surrounds brain capillaries and consist of specialised endothelial cells and the glia limitans. Pericytes maintain BBB integrity and inhibit influx of T-cells and macrophages. E) During acute GvHD, microglia are activated and release TNF that upregulate VCAM1 on the BBB endothelium, allowing entry of T-cells, monocytes and DCs to the brain parenchyma. The activated microglia activate invading T-cells using upregulated MHCII and co-stimulatory CD80.

of the SAS located in between vessels and the glia limitans (figure 4A), the T-cells interact with DCs that present brain-derived antigens[126]. Only those T-cells with TCRs that have high affinity for brain-derived antigens are allowed to enter the brain parenchyma, the rest follow the flow of CSF back into the blood stream (figure 4C)[126]. A small subset of T-cells have recently been demonstrated to become reprogrammed in situ into brain-resident T-cells, and play a key role in microglia maturation and subsequent pruning of synapses[127]. The pericytes aligning the vascular walls are important for maintaining BBB integrity and for inhibiting influx of both T-cells and macrophages during CNS inflammation[128,129]. Pericyte function, in turn, is controlled by cytokines such as TGFβ[130].

These recent discoveries challenge the prevailing notion of the CNS as "immune-privileged" and suggest that activation of alloreactive T-cells against CNS-derived mHAs may take place in either cervical lymph nodes or the above described "dural hubs", subsequently leading to CNS inflammation. However, while not entirely immune-privileged, the delicate neural communication in the brain still appear to be protected by a regulating cross-talk between the meningeal stroma and the bone marrow in the overlying skull bone[131].

1.5.3 GvHD in the CNS?

CNS involvement in both aGvHD and cGvHD have been suggested, although they are considered rare entities[132–134]. Reported symptoms can be severe, and include headaches, altered personality, cognitive dysfunction, focal neurological deficits and seizures, often associated with tapering of immunosuppression[133,135]. Mechanistically, studies in rodents and primates have demonstrated activation of microglia and donor CD8+ T-cell infiltration during aGvHD[136–138]. The activated microglia release TNFα that in turn activates endothelial cells in the BBB to recruit immune cells to the brain parenchyma. The initiating mechanism for microglia activation was not elucidated in these studies but inflammatory cytokines are known to trigger widespread microglia activity in rodents and humans[139,140]. Importantly, these mechanistic studies were performed using MHC-mismatched transplantation and the CNS infiltrates were dominated by memory T-cells[137]. Because of the MHC mismatch, the infiltrating memory T-cells were likely reactive against non-self MHC, rather than against brain derived mHA. This contrasts with the clinical situation, where the majority of HSCT donors are MHC-matched, and presentation of brain-derived mHA should therefore be required, likely through one of the pathways outlined above. A damaged BBB caused by the conditioning regimen did not explain the T-cell influx, since no T-cell infiltration was detected after syngeneic transplantation[137]. Recently, lower doses of alloreactive T-cells were transplanted to mice, to study cGvHD in the CNS[141]. Late infiltration of donor bone marrow derived macrophages and replacement of CD8+ T-cells with low levels of CD4+ T-cells, as well as high levels of interferon-γ were distinguishing features of CNS cGvHD, paralleled by cognitive dysfunction[141]. In addition, transplanting with MHC-II deficient grafts eliminated the symptoms, suggesting a role of T-cell interaction with the infiltrating donor macrophages[141]. The study suggest that low grade T-cell activation in the brain and disturbed microglia function can contribute to cognitive impairment after HSCT that contrast to the

dramatic symptoms associated with aGvHD in the CNS[142,143]. While GvHD outside the CNS has been associated with cognitive symptoms after HSCT, the association is not exclusive, suggesting that the brain may be selectively targeted by the disease process[144]. Therefore, it is also important to consider CNS-specific immune regulation at the BBB, as well as the newly discovered sites of antigen presentation at cervical lymph nodes and the dural hubs[122,129,131].

1.6 PSYCHIATRIC IMMUNOLOGY – ARE FATIGUE AND COGNITIVE DYSFUNCTION AFTER HSCT SYMPTOMS OF DYSREGULATED IMMUNITY?

Since the 1970s the number of annual HSCTs performed worldwide has increased rapidly and in 2012, over 30 000 such transplants were performed[145]. At the same time, improved treatment and prophylaxis of GvHD and infections, as well as less toxic conditioning regimens and more accurate donor to recipient matching has improved survival dramatically[12,146]. Currently, more than 80% of those that survive 2 years after HSCT become long term survivors[147,148]. Taken together, these developments have led to a rapidly growing number of survivors after haematological cancer and HSCT worldwide, and increased interest in the long term morbidities affecting quality of life of these individuals[149].

Fatigue and cognitive dysfunction are arguably among the most important of these morbidities[150,151]. Long term follow-up reveals that five years after transplant the symptoms are still as severe as before the treatment, and more pronounced than in healthy controls[150,151]. It is important to remember that the pre-transplant levels of fatigue and cognitive dysfunction are well above pre-morbid levels, due to the intensive treatments for the underlying haematological malignancies that the patients have already undergone. With regards to risk factors, GvHD and depression symptom scores have been associated with fatigue, but the degree of myelotoxicity has not[150]. Similarly, few if any transplant related variables are consistently associated with persistent cognitive decline after HSCT, suggesting that the symptoms are not due to a specific toxicity or side effect of the treatment. However, older patients and patients with lower premorbid intelligence levels are at greater risk suggesting a role of underlying neurocognitive susceptibility[151,152].

1.6.1 What is fatigue?

Being tired is experienced by everyone. It is a predictable consequence of reduced mental and/or physical energy caused by exertion or lack of sleep, relieved by rest. Fatigue on the other hand is a distinct core symptom of overwhelming sense of tiredness and lack of energy, that interfere with daily activities, not relieved after resting - taking a shower can feel like running a marathon[1,155]. It is therefore understandable that fatigue negatively impacts on social, emotional, occupational and financial functioning, ultimately hampering quality of life[156]. Fatigue is prevalent in the general population, affecting upwards of 45% of people, and up to 11% for more than 6 months[157]. It is especially prevalent in patients with chronic diseases, being reported by up to 60% of diabetes patients, 83% of MS patients, 100% of patients with advanced cancer and 30% of cancer survivors[155]. The cause of fatigue associated with chronic disease can be multifactorial and either directly related to the

underlying disease process (for example due to CNS damage in MS or decreased neuromuscular transmission in myasthenia gravis), caused by consequences of the disease (such as anemia or hypercalcemia in malignancy), a side effect of disease treatment (anti-hypertensives, lipid-lowering agents, anxiolytics), or co-existing with the disease without an obvious mechanistic connection[158,159]. In chronic fatigue syndrome the fatigue is a feature of the disease itself.

The neurological mechanisms causing a person with chronic disease to experience fatigue are not known. However, one theory is that fatigue stems from a metacognitive realisation that the brain is failing to exert control over the body's state (i.e., defective allostatic control, figure 5)[153,160,161].

The allostatic/interoceptive system is a closed-loop network in the CNS that attempts to predict demands on internal organs and metabolism and adjusts the set points for homeostatic reflexes according to those predictions (figure 5)[162]. The insular cortex continuously receives information (transmitted by spinal cord visceral afferents, by the vagus nerve, through circumventricular organs, or directly across the BBB) about the current internal state of the body's organs, including blood oxygenation and carbon dioxide concentration, acidity, blood pressure, heart rate, glucose and cytokine concentrations etc. (i.e., interoception) as well as sensory information about the external world[163,164]. The anterior insula processes this information and uses it to predict future body needs[165]. It sends these allostatic predictions to the brain stem and hypothalamus to adjust the set points of the homeostatic reflexes. For example, stress causes heart rate and blood pressure to rise, in anticipation of the need for increased muscle blood flow during fight or flight[166]. Simultaneously, copies of these allostatic predictions are sent to the posterior and mid insula (corollary discharge). These regions will eventually receive the interosensory input that resulted from the modifications and compute a prediction error that is sent back to the anterior insula, which will update its allostatic predictions based on the error[162]. The closed loop is completed when these updated predictions are sent to the brain stem and hypothalamus. The theory postulates that metacognitive areas, probably located in the medial prefrontal cortex (mPFC), monitor the level of prediction error at the anterior insula and uses it to compute interoceptive surprise[153]. For example, after prolonged physical activity, the resulting increased lactic acid concentration cannot be fully restored by adjusting homeostatic reflexes. This failure generates interoceptive surprise that results in tiredness, and the response is rest. Importantly, in this scenario, resting restores muscle function and removes the interoceptive surprise. Fatigue on the other hand, is thought to arise when the surprise is not mitigated by rest and the metacognitive areas detect a chronic lack of mastery over bodily states[153]. This causes a decreased sense of self-efficacy (expectation of personal mastery and control) resulting in negative emotions[167]. An example of functional fatigue is the "sickness syndrome" (social withdrawal, reduced food and water intake, anhedonia and altered cognition) caused by an infection[168]: Systemic inflammatory cytokines affect interoception and organ function, again causing a prediction error that cannot be compensated. In this situation resting will not resolve the problem but can be evolutionary advantageous by limiting contamination and

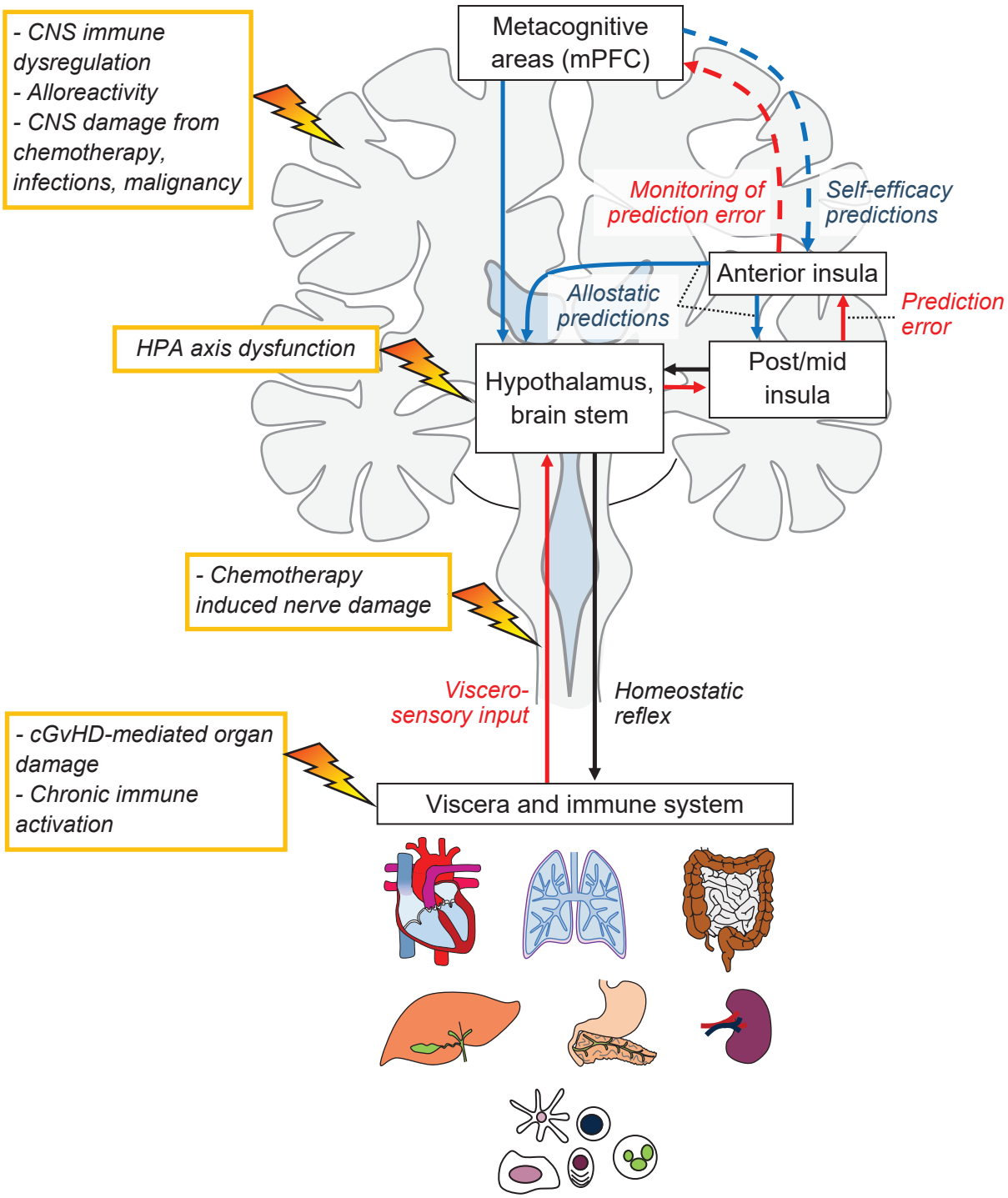


Figure 5: Interoceptive surprise as a neurobiological basis for fatigue in chronic disease. Based on Stephan et al, 2016[153]. The anterior insula continuously receives visceral information about the internal state. This information is integrated with sensory inputs from the external world to generate allostatic predictions that modulate the set points of homeostatic reflexes. The difference between the predicted outcome and the actual resulting alterations in internal state, the prediction error, is communicated back to the insula and is used to adjust the allostatic predictions. The prediction error is continuously monitored by metacognitive brain areas, that generate interoceptive surprise based on failure of allostatic predictions to generate the desired alterations in body state. mPFC also directly influences the hypothalamus and brain stem, modulating HPA axis and cardiovascular responses to psychological stress[154]. Prolonged interoceptive surprise result in fatigue and negative emotions. Failure to generate the desired body state can be caused by either organ damage, immune system dysfunction or neural damage to any part of the circuit. The orange boxes list some plausible mechanisms of such dysfunction after HSCT.

facilitating elimination of the infection by conserving energy to be utilised by the immune system, eventually restoring allostatic control[168]. However, in chronic diseases such as heart failure, MS, or cancer, the interoceptive surprise persist, resulting in chronic fatigue.

Importantly, interoceptive surprise can result from damage or dysfunction of any component in the above-described neural circuit, for example, internal organs, the visceral afferent nerves of the autonomic nervous system or involved CNS regions such as the hypothalamus and cerebral cortex (figure 5)[153,160,161]. Therefore, this theory unifies the multitude of mechanisms suggested to cause fatigue after HSCT as well as in various chronic diseases.

1.6.2 Fatigue and hypothalamic pituitary adrenal axis dysfunction

The hypothalamic pituitary adrenal (HPA) axis regulates the production of cortisol, adrenaline, and norepinephrine, affecting both immune function and behaviour[169,170]. HPA axis dysfunction is implicated in a number of psychiatric diseases[169,170]. Long term breast and ovarian cancer survivors have elevated evening cortisol levels, blunted cortisol responses to psychological stress and downregulated glucocorticoid receptor (GR) responsive genes, suggesting functional GR resistance[171–174].

1.6.3 Fatigue and inflammation

Studies of both rodents and humans has demonstrated that the sickness syndrome is elicited by increases in pro-inflammatory cytokines in the peripheral blood, such as TNFα and IL-6[168,175]. Mechanistic studies have shown that such signals from the periphery reach the CNS through several routes, including direct activation of the afferent vagus nerve, transport across the BBB via carrier molecules, activation of endothelial cells in the BBB and subsequent production of cytokines in the brain as well as cytokine stimulation of brain areas that lack a functional BBB[169]. These mechanisms have led to the hypothesis that after cancer treatment, fatigue is caused by persistent blood inflammation, and cytokine levels have been assessed in patients with leukemia, breast, ovarian, testicular and colon cancer[176]. Before, during and shortly after treatment, IL-6, IL-1 receptor antagonist (IL-1RA), TNFα and C-reactive protein (CRP) have been associated to fatigue, and prospective studies have shown decreases of cytokine levels over time correlating with reductions in fatigue symptoms[177–182]. In long term survivors, mainly elevated levels of IL-1RA and CRP persist and correlate to fatigue[183–189]. A few studies have shown similar results for HSCT recipients[181,190]. The observed correlations between fatigue and peripheral blood cytokine levels are summarized in table 2. Increased leucocyte sensitivity to proinflammatory stimuli is suggested as an underlying mechanism, as lipopolysaccharide stimulation of leucocytes from breast cancer survivors with persistent fatigue causes elevated expression of Nuclear Factor κB and genes encoding IL-6 and IL-1B, as well as increased production of IL-6 and TNFα[171,184].

Table 2: Association between elevations in various peripheral blood cytokines and presence of fatigue before, during and after cancer treatment

Cytokine	Before treatment	During and short term after treatment	Long term after treatment
IL-6	Newly diagnosed AML or MDS[177] Ovarian cancer patients before surgery[178,179]	- Breast cancer patients during chemotherapy[180] - Reduced IL-6 after treatment for ovarian cancer correlates to reduced fatigue, depression and disability[183] - AML and MDS patients undergoing HSCT[181]	- Survivors, mean 63 months after HSCT[190]
sIL-6R			- Increased in survivors ≥2 years after breast cancer diagnosis[184]
IL-1RA	Newly diagnosed AML or MDS[177]	- Early stage breast- or prostate cancer[182]	- Increased in survivors on average 5 years after breast cancer diagnosis[185] - Increased in survivors ≥2 years after breast cancer diagnosis[184] - Testicular cancer survivors 5-20 years post treatment[186]
TNFα	Newly diagnosed AML or MDS[177]		- Survivors, mean 63 months after HSCT[190]
sTNF-RII			- Increased in survivors on average 5 years after breast cancer diagnosis[185]
CRP		- Early stage breast- or prostate cancer[182]	- Breast cancer survivors 3-48 months after treatment[187] - Breast cancer survivors 4 years after diagnosis[188] - Breast cancer survivors 30-39 months after diagnosis[189] - Testicular cancer survivors 5-20 years post treatment[186]

sIL-6R: Soluble IL-6 receptor, sTNF-RII: Soluble tumour necrosis factor receptor II

As the afferent vagus nerve is activated by peripheral inflammatory cytokines it activates its own efferent branch. This reflex activation culminates in the spleen, where specialized T-cells containing choline acetyltransferase reside[191]. These T-cells are activated by norepinephrine from the splenic nerve, and release acetylcholine, which binds to receptors on splenic macrophages, resulting in dampening of their production of inflammatory cytokines[191]. Dysfunction in this "inflammatory reflex" could thus lead to diminished dampening of inflammatory responses with resulting increased peripheral inflammation. Further, the inflammatory signals relayed through the afferent vagus nerve are forwarded to the insula, affecting interoception and causing the behavioural changes of the sickness syndrome, possibly by interrupting the allostatic/interoceptive system[153,192]. Parasympathetic activity can be measured using heart rate variability and studies on healthy individuals have utilized this measurement to demonstrate associations between increased parasympathetic activity and reduced levels of CRP and IL-6, as well as reduced cognitive function in the elderly[193–195]. Chemotherapy is known to cause nerve damage and autonomic nervous system

dysfunction in cancer patients[196]. In breast cancer survivors, decreased heart rate variability correlate to fatigue and peripheral blood IL-6 and CRP levels[197,198].

1.6.4 Fatigue and cognitive dysfunction

In addition to fatigue, cognitive dysfunction is common in cancer patients, limiting work capacity and quality of life[151,199,200]. After HSCT, the trajectories of cognitive decline and fatigue are similar, and the symptoms are correlated[150,151,201]. Metacognitive expectations are held about all cognitive domains[202]. Therefore, cognitive dysfunction may cause fatigue as a result of metacognitive detection of poor CNS network function[160]. The patients commonly experience perturbed executive function, important in novel situations where one cannot rely on instinct or automatic behaviour[144,151,200,203,204]. Instead, these situations are solved through planning of goal-directed behaviour and by disregarding inappropriate stimuli, emotions, or memories, to focus thought. The prefrontal cortex performs such functions and holds an internal construct of reality, encoding and retrieving relevant information from the working memory[205]. Further, it is highly interconnected with other brain areas, to provide top-down regulation of emotions, thought and behaviour[205,206]. Prefrontal dysfunction can lead to irritability, impaired decision-making and lack of insight, seen in psychiatric patients with schizophrenia, bipolar disorder and ADHD[207–210]. Post-mortem and animal studies suggest that the loss of brain volume in the prefrontal cortex of schizophrenia patients is due to loss of synaptic connections rather than loss of neurons, leading to ineffective cognitive processing[207,208]. Breast cancer treatment and cancer-related fatigue has been associated with compensatory hyperactivation of several cortical regions during cognitive challenges, including the prefrontal cortex[211]. Such hyperactivation may represent an attempt of fatigued breast cancer survivors to maintain cognitive function despite CNS dysfunction, at the expense of increased energy expenditure[212]. Cortical and subcortical connectivity changes are also described, and changes in connectivity between insula and dorsolateral prefrontal cortex (possibly involved in top-down regulation of fatigue) associated with reduced fatigue after acupuncture treatment of breast cancer survivors[213].

Compared to patients with solid tumours, CNS structure and function in HSCT recipients is less well studied. However, altered white matter integrity in the CNS (possibly compromising connectivity) was detected one year after HSCT and correlated with cognitive performance[214]. In a similar study, regional grey matter reductions were also demonstrated[215]. Further, we recently demonstrated reduced prefrontal cortex activity in response to cognitive challenges in fatigued survivors after HSCT[216].

Long-term follow-up of fatigue symptoms in HSCT recipients demonstrated aGvHD as a risk factor of fatigue at three months post-transplant, and a similar correlation between fatigue and cGvHD one year after treatment[150]. The mechanisms outlined here provide several plausible causes for this epidemiological correlation, all potentially leading to chronic interoceptive surprise and decreased self-efficacy: (1) Peripheral inflammation, caused by GvHD or a disturbed inflammatory reflex. (2) Dysfunctional or activated BBB endothelium resulting in increased influx of immune cells to the CNS[217]. (3) Disrupted organ function or damage to

the peripheral nervous system[132,133]. (4) Damage to the prefrontal cortex or other CNS areas, causing cognitive dysfunction[214–216].

1.6.5 Treatment of cancer-related fatigue

There is no treatment for cancer-related fatigue. A multitude of pharmacologic and non-pharmacologic interventions have been investigated, with varying results[176]. The most promising intervention, where meta-analyses indicate a modest effect, is aerobic exercise, usually accomplished through individualized programmes of gradual increase from modest levels of intensity[218]. Such graded exercise therapy is speculated to restore a sense of mastery over bodily states[153]. The effects of central stimulants modafinil and methylphenidate have varied between studies, and some studies suggest that they are more effective in patients reporting severe fatigue[176]. Selective serotonin reuptake inhibitors do not seem to be effective against fatigue, underlining the distinction to depression. TNFα antagonists etanercept and infliximab have shown positive effects on fatigue in both cancer and psoriasis patients[176].

1.7 CONCLUDING REMARKS – THE MIND AND THE BODY TOGETHER

Being diagnosed with cancer is often a severely distressing event that may cause dissociative psychological reactions and post-traumatic symptoms[219]. In addition, personality traits have been associated with fatigue and quality of life after breast cancer treatments[220,221]. Given these circumstances, one could hypothesize that cancer-fatigue emerges when a person with an underlying vulnerability suffers psychological trauma caused by the disease. In chronic fatigue syndrome, a vigorous debate is ongoing between advocates for a psychological versus a biological explanation of the disorder[222]. The patients, believing that a psychological explanation would render a lot more stigma and likely less resources, strongly support the biological hypothesis. The conflict became very heated in 2011, after a randomized study published in the Lancet demonstrated effects of physical activity and cognitive behavioural therapy, but no effect of the pacing therapy advocated by patients[223]. Despite controversies, researchers should not refrain from incorporating psychological mechanisms into their theoretical framework if that improves understanding of the illness and enable improved therapies. Perhaps a biopsychosocial model taking both the genetic, psychological and social background of the individual, as well as the biological and mental trauma caused by the cancer and its treatments into consideration would solve the unanswered riddle of cancer-fatigue[224]? The hypothesis of defective allostatic control represents such a model, where inability to maintain homeostasis challenges a person's perceived self-efficacy, resulting in tiredness and feelings of hopelessness[225].

2 RESEARCH AIMS

The overall aim of this thesis was to study long-term immunological complications after HSCT. Study I evaluated mechanisms of MSC-treatment of cGvHD. Studies II, III and IV aimed to determine if immune perturbations are associated with fatigue and cognitive dysfunction after HSCT. Study III and IV were designed based on the results of study II.

2.1 SPECIFIC AIMS

To study safety, clinical efficacy, and mechanisms of action of repeated MSC infusions to treat cGvHD, as well as the role of recipient immune status in determining treatment effect (study I).

To assess clinical characteristics, cognitive function, quality of life and employment in patients with fatigue after HSCT (study II).

To measure prefrontal cortex activity during cognitive testing in patients with fatigue after HSCT (study III).

To explore neurobiological disturbances and metabolic derangement in the CNS of patients with fatigue and cognitive dysfunction after HSCT using proteomics and mRNA sequencing of EVs (study IV).

To characterize immune composition and immunological activity in the CNS of patients with fatigue and cognitive dysfunction after HSCT (study II and IV).

3 MATERIALS AND METHODS

3.1 STUDY SUBJECTS (STUDY I-IV)

All studies were conducted in accordance with the Helsinki convention and approved by the regional ethical committee in Stockholm. All subjects provided written, informed consent.

3.1.1 Cohort 1 – refractory cGvHD

In study I, 11 patients with moderate to severe cGvHD, unresponsive to, or not tolerating treatment with steroids and calcineurine inhibitors, were prospectively recruited from the department of Haematology, Karolinska University Hospital. The patients were at least 18 years old and had no evidence of active malignancy.

3.1.2 Cohort 2 – patients with and without fatigue 1-5 years after HSCT

For studies II, III and IV, 27 patients were recruited from the department of Haematology, Karolinska University Hospital (cohort 2). The patients were at least 18 years old and in haematological remission, 1-5 years after HSCT. Exclusion criteria were previous severe psychiatric or neurologic disorder, high doses of sedatives, opioids or neuroleptic drugs, previous total body or CNS irradiation, previous intrathecal chemotherapy, or high doses of corticosteroids. Brain magnetic resonance imaging (MRI) excluded neurologic diseases or significant structural abnormalities. No patients had anaemia, hypercalcemia, or obvious infectious/inflammatory disorders. Subjects were allocated as fatigued (n=14) or non-fatigued (n=13) based on self-reported symptoms and the mental fatigue scale (MFS) score (fatigued: ≥14p, non-fatigued: ≤10p)[226]. A study flow chart is provided in figure 6.

In study III, patients from cohort 2 without contraindications to methylphenidate treatment were included (n=24). Further, data from 27 healthy controls (12 male and 15 female, 22-55 years old), recruited in a previous trial (Sklivanioti et al, manuscript in preparation), was included for comparison.

Study IV included patients from cohort 2 with available CSF samples (n=26).

3.2 STUDY TREATMENTS

3.2.1 Clinical MSC treatment (study I)

Procurement of the clinical MSC product was accredited by the Swedish National Board of Health and Welfare under Swedish law 2008:286 (Cell- och vävnadslagen) (approvals number 952/2009, 6.3.3-8874/2011, 6.1.3-9791/2013 and 6.1.3-16411/2015). Donors provided written, informed consent before the procedure.

The MSC production process for study I was identical to Le Blanc et al 2008[80]. Briefly, bone-marrow mononuclear cells from healthy donors were separated by density gradient. Washed cells were resuspended and plated in culture flasks with 10% foetal calf serum. When the

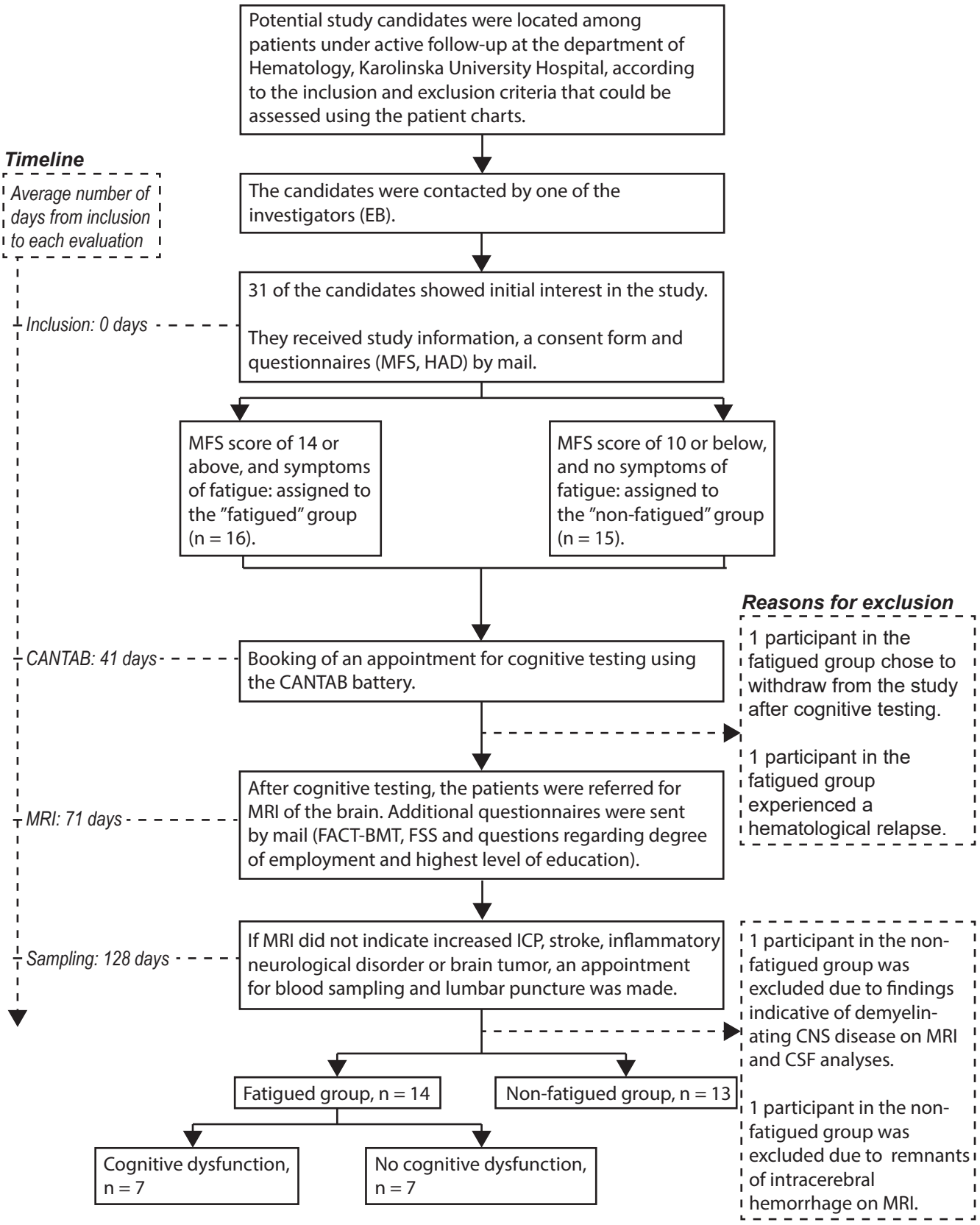


Figure 6: Flow chart for included patients in study II-IV. *Figure from Boberg et al, Haematologica 2020*

cultures were near confluence (>80%), the cells were detached by treatment with trypsin and plated in new flasks for 1-4 passages. The final cell product was cryopreserved.

Release criteria for clinical use included absence of visible clumps, spindle-shape morphology, absence of contamination by pathogens, viability greater than 95%, and immune phenotyping proving expression of CD73, CD90, and CD105 surface molecules (>90%) and absence of CD34, CD45, CD14, and CD35.

After thawing, washing, and counting, $2x10^6$ MSCs per kg was infused every 4-6 weeks, for a minimum of 6 doses. Responding patients were administered an additional 1-3 doses.

3.2.2 Methylphenidate treatment (study III)

To evaluate prefrontal cortex and sympathetic nervous system effects of increased dopamine and noradrenaline concentrations, a single dose of 30mg short-acting methylphenidate was administered to all subjects in study III after the first session of cognitive testing. A 30-minute break without any food or beverage other than water, allowed for medication uptake. The cognitive testing was repeated after the break.

3.3 CGVHD DIAGNOSIS AND GRADING

In study I, cGvHD was evaluated using the 2014 National Institutes of Health criteria[34]. Evaluations were performed every three months during MSC treatment, with a final evaluation 1 year after treatment.

3.4 SELF-ASSESSMENT OF SYMPTOMS (STUDY II)

Questionnaires with validated Swedish translations were selected. Brief instruments were prioritized to avoid respondent fatigue and increase clinical utility[227]. The MFS is a self-reporting questionnaire, that incorporates affective, cognitive and sensory symptoms, duration of sleep and daytime variation[226]. The Fatigue Severity Scale assesses the impact of fatigue on different aspects of functioning[228]. Quality of life was measured using the Functional Assessment of Cancer Therapy – Bone Marrow Transplant (FACT-BMT) scale, and depression and anxiety using the hospital anxiety and depression scale (HAD)[229,230]. The reliability of the selected instruments, as measured by Cronbach's Alpha (a measurement of the correlation between the different items within an instrument assessing the same property) were determined as adequate (0.67 – 0.94)[226,230–233]. Further, all instruments were previously validated against other methods of assessing similar symptoms[226,230–233].

3.5 MEASURING COGNITIVE FUNCTION

Objective testing is regarded as the gold standard for assessing cognitive function in cancer patients, since self-reported cognitive symptoms tend to correlate more strongly to mood and fatigue, than to cognitive test results[234]. Further, to increase clinical utility, we wanted a test that could be administered without formal neuropsychologial training. Finally, we prioritized evaluating cognitive domains known to be dysfunctional in cancer survivors[234]. Thus, five

tests were selected from the computer-based Cambridge Neuropsychological Test Automated Battery (CANTAB), a suite of language-independent and culture-free tests that are applicable to both elderly and young populations[235,236]. The selected tests were sensitive to frontal and temporal lobe dysfunction, evaluating executive function, visual memory and attention/processing speed. Administration was conducted on a computer with a touch screen in a disturbance-free environment. Normative data, matched by age and premorbid intelligence quotient (measured with the Swedish version of the National Adult Reading Test[237]) were used to convert the CANTAB test scores to z-scores. The z-scores were converted to a deficiency score and the deficiency scores were averaged to obtain a global deficit score (GDS)[234]. Cognitive dysfunction was defined as GDS≥0.5.

In study III, we included three additional paradigms, sensitive to prefrontal cortex dysfunction. The Stroop task evaluates cognitive control and resolving cognitive interference[238]. The outcome variables were median reaction time and proportion of correct answers[239]. During the verbal fluency task, subjects were instructed to generate as many words as possible within 20s, beginning with each of five selected vowels. In the emotion regulation task, five images with negative emotional valence were presented and the subjects were instructed to either passively observe or actively regulate the emotion intensity evoked by the images[240]. The relative difference in subjective emotion rating between the induction and regulation tasks were measured. The Stroop and emotion regulation tasks were administered in a block design, with 30s rest between each block. Testing was performed in a dimly lit and sound isolated room.

3.6 ASSESSMENT OF PREFRONTAL CORTEX ACTIVITY: FNIRS

Functional near infra-red spectroscopy (fNIRS) detects regional brain activity by using near infra-red light to measure the increase in oxygenated haemoglobin resulting from local vessel dilation as a response to increased nerve activity (neurovascular coupling)[241]. Compared to functional MRI (fMRI) and positron emission tomography (PET), fNIRS have less spatial resolution, only measure the cerebral cortex due to limited penetration depth and can only be applied to hairless parts of the head (i.e., the forehead). On the other hand, fNIRS has better temporal resolution, allows the subject to move freely, is relatively inexpensive and does not emit any harmful radiation or noise[242]. Both fNIRS and fMRI measure regional blood oxygenation, and their results are usually correlated[243]. In study III, we used a BioPac fNIR model 1100-V3.2A (fNIR Devices LLC, Potomac, MD, USA) with a 16-channel sensor placed on the forehead to measure regional prefrontal cortex activity during the Stroop, verbal fluency and emotion regulation tasks (figure 7A).

Because of differences in functional brain activity patterns based on hand preference, all participants were assessed to be right-handed using the Edinburgh Handedness Inventory – short form[244,245]. The raw data was processed using the COBI studio software and Matlab with the package SPM for fNIRS toolbox, to yield beta-coefficients for each channel that allowed for statistical within- and between-group comparisons (figure 7B-D).

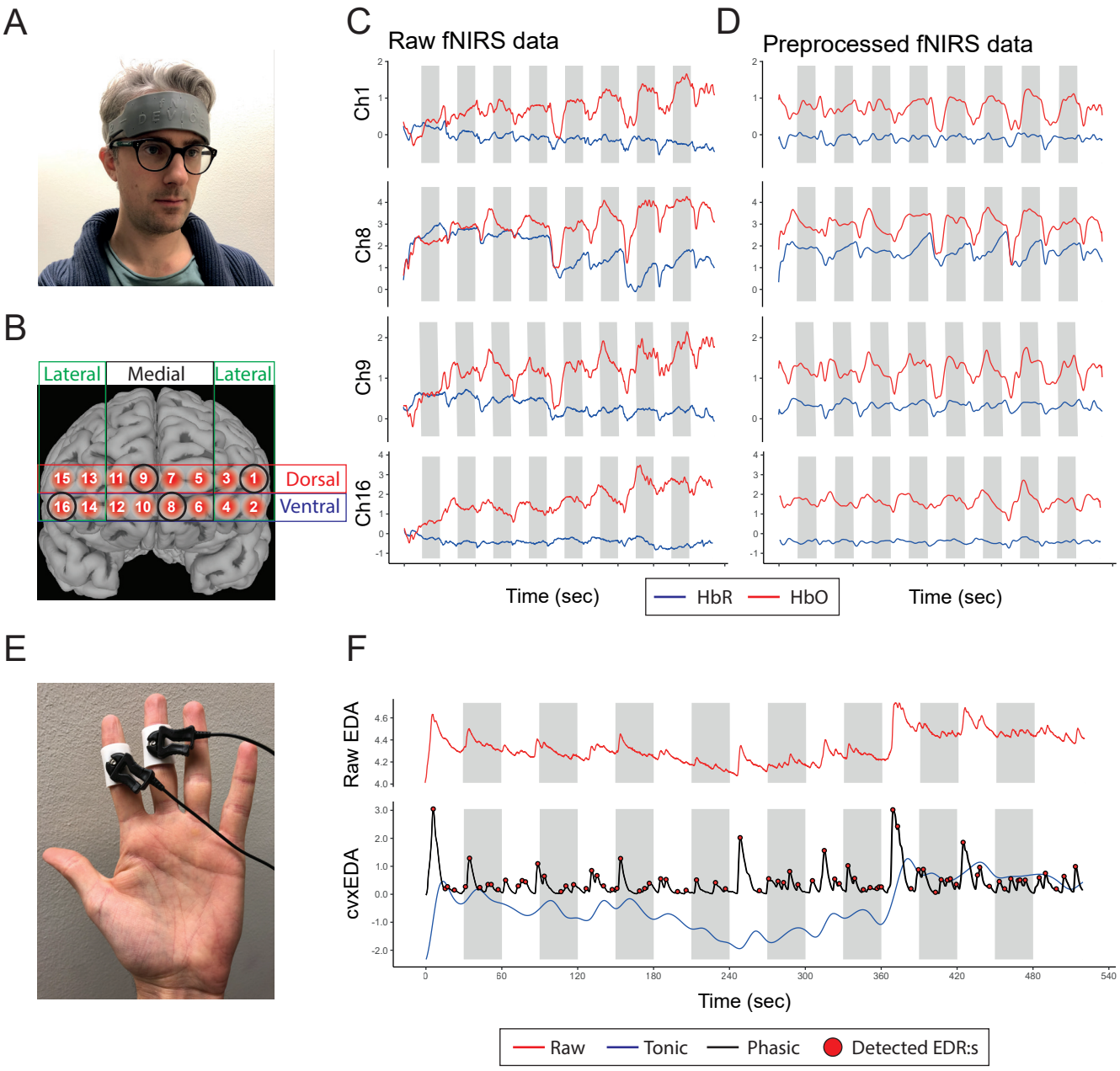


Figure 7: fNIRS and EDA analysis. A) The 16-channel fNIRS headband was centred on each subject's forehead. The person in the picture is the author E.B. **B)** The approximate anatomical location of each of the 16 channels. **C-D)** Examples of fNIRS signals from the Stroop task before and after pre-processing (band-stop filtering and detrending). The grey areas represent each task block and the white areas between represent resting phases. **E)** Placement of Ag-AgCl electrodes for EDA registration on the left middle phalanges of dig 2 and 3. **F)** Example of raw and pre-processed (low-pass filtering, smoothing, and decomposing with cvxEDA) EDA signal from the Stroop task. The red line represents the raw data, the blue line the tonic component and the black line the phasic component. Red dots represent automatically detected EDRs. Ch: Channel, HbR: Deoxygenated Haemoglobin, HbO: Oxygenated Haemoglobin, cvxEDA: convex optimization EDA. *Figure from Boberg et al, Bone Marrow Transplantation 2021*

3.7 ASSESSMENT OF SYMPATHETIC NERVOUS SYSTEM ACTIVITY: EDA

The sympathetic nervous system innervates sweat glands and increased sweat production results in increased conductivity in the skin (i.e., Electrodermal activity, EDA)[246]. In study III, EDA was measured using silver-silver chloride electrodes placed on dig 2 and 3 (figure 7E). Data was recorded using the MP150 data acquisition and analysis system (BioPac). The EDA signal was decomposed into tonic and phasic components (figure 7F). The tonic component (electrodermal level, EDL) reflects overall degree of arousal and the phasic component (electrodermal responses, EDR) either reflects responses to external stimuli (during the tasks) or degree of arousal (during rest). The increase in EDL from rest to task, as well as mean EDR amplitudes at rest (non-specific EDR) and during cognitive tasks were used as outcome variables in study 3.

3.8 BIOLOGICAL SAMPLING AND BIOBANKING

Peripheral blood was separated into plasma and mononuclear cells using centrifugation with Lymphoprep (Alere Technologies AS, Oslo, Norway). CSF was centrifuged to separate cells from supernatant. Plasma and cell-free CSF were stored in -80ºC freezers. Cells from blood and CSF were stored in liquid nitrogen in human AB plasma with 10% dimethyl sulfoxide.

3.8.1 Immune cell composition analysis: Flow cytometry

Flow cytometry allows for measurement of the expression of multiple proteins on each individual cell contained in the analysed sample. Cells are labelled with antibodies specific for the proteins of interest conjugated to fluorochromes. Florescence with a specific wavelength is triggered using lasers and detected. The technique is widely used for classification of cells and detection of biological processes (such as immune cell activation) in clinical diagnostics and research. The number of proteins that can be simultaneously examined is limited by florescent spectrum overlap. Examples of pitfalls in flow cytometry analysis are interrupted flow, poor compensation and presence of doublets (especially in samples with a high cell count and rapid acquisition)[247]. Compensation becomes increasingly difficult as the number of fluorochromes increase. In study IV we employed a panel of 24 different fluorochromes, resulting in a complicated compensation process. Additionally, lack of positive or negative control samples can make it difficult to apply gating correctly for antigens that do not have a clear bimodal distribution (such as CD69)[248]. This became an issue in the flow cytometric analysis of CSF in both study II and IV, as we only had one sample of CSF cells per patient, limiting our ability to construct proper controls. As a mitigation, we used internal biological controls. For example, the threshold for CD3 expression can be set using monocytes in the same sample as they normally do not express that protein.

Flow cytometry analysis of immune cell composition in the peripheral blood (study I and II) and CSF (study II and IV) was conducted. Briefly, cells were thawed and washed with RPMI media supplemented with 10% foetal calf serum and stained for 20 minutes in 4ºC. In study I, the cells were permeabilized after staining to enable intranuclear staining for Ki67 and

FoxP3. Staining for CCR7 in study IV was conducted separately by adding 1ul of antibody to the samples and incubating for 30 minutes in 37°C.

3.8.2 Protein concentration analysis: ELISA and multiplex methods

Enzyme-linked immunosorbent assay (ELISA) is an antibody-based technique to measure the concentration of a single protein with high sensitivity and specificity, considered a gold standard for protein concentration measurement[249]. ELISA was used to quantify B-cell activating factor (BAFF) in study I. To detect a larger number of proteins efficiently and rapidly in a small amount of biologic fluid, multiplex techniques are commonly applied instead[250]. Study I, II and IV utilised different techniques for multiplex protein detection: Bio-plex X-plex from Bio-Rad in study I (using beads with monoclonal antibodies), antibody microarray in study II and Olink proximity extension assay in study III (using oligonucleotide antibody probes and PCR for signal amplification). The Bio-plex X-plex assay generated protein concentrations, while the antibody microarray and proximity extension assays generated LOG2-normalized relative concentrations. Thus, the latter two methods only allowed for between group comparisons (i.e., fatigued vs non-fatigued patients) of each analysed protein but did not allow for between-protein comparisons (i.e., whether the concentration of TNFα was higher than IL-10 in the fatigued patients).

3.8.3 Genetic analyses

miRNA sequencing of plasma was performed using real time PCR in study I and mRNA sequencing of CSF EVs was performed using next generation sequencing in study IV. EVs were separated by centrifugation of CSF at 20 000xg for 30 min[251].

3.9 STATISTICS

3.9.1 Non-repeated within- and between-group comparisons: Study I-IV

Analysed data included clinical variables, cognition, fatigue, cognitive paradigm performance, as well as cellular and protein analyses. Categorical data was compared using Fishers exact test. Continuous variables were first tested for normality using the Shapiro Wilks' test. Normally distributed variables were compared using Student's T-test or one way analysis of variance (ANOVA, post hoc Tukey Honest Significance test with Benjamini Hochberg [BH] correction). Non-normal variables were compared using Wilcoxon rank sum test or Kruskal Wallis test (post hoc Dunn's test with BH correction).

3.9.2 fNIRS channel data: Study III

One sample t-test for activity in each channel. Paired or unpaired t-tests for within- or between-group comparisons.

3.9.3 Multi-omics data analysis: Study I, II and IV

Data from miRNA sequencing and protein multiplex assays were compared using t-tests, and mRNA expression using the Deseq2 package. Gene ontology term analysis was performed

using the statistical overrepresentation test in the Panther classification tool. BH correction for multiple comparisons was applied. A principal component analysis of CSF protein expression was performed in study IV.

3.9.4 Adjustment for confounders: Study II and III

Linear regression was performed to adjust analyses for confounders in study II (exploratory analysis of CSF and blood parameters) and study III (age-adjusted comparisons to healthy controls). The confounders were included as independent variables in the linear models.

3.9.5 Analysis of repeated measures: Study I, III and IV

Linear mixed effects models were applied to repeated measures: Flow cytometry data for each infusion in study I, EDA measurements for each block of cognitive testing in study III (assuming logarithmic habituation over time), and body mass index (BMI) change from transplant to study inclusion in study IV. Cytokine data for each infusion in study I was analysed with repeated measures ANOVA.

3.9.6 Correlations: Study III and IV

Linear regression was used to correlate cytokine concentrations and EDA data to fNIRS channel activity in study III, as well as to correlate BMI to protein concentrations in study IV.

3.9.7 Statistical software

All statistical analyses were performed using R[252].

3.10 ETHICAL CONSIDERATIONS

When study 1 was designed, no established treatment for steroid refractory cGvHD existed, and results from clinical trials of aGvHD suggested efficacy of MSC-treatment, without severe side-effects. Potential downsides to the included patients were limited discomfort during blood sampling and tissue biopsies, repeated time-consuming hospital visits, and to date unknown side effects. These downsides were judged to be outweighed by the possibility of symptom improvement, as well as possible reductions in steroid and calcineurin dose, which would lead to improved quality of life and reduced risk of infections. Being a pilot study, no power calculations were performed.

Studies II, III and IV exposed patients to multiple time-consuming and discomforting investigations. The lumbar punctures were associated with a risk of severe complications including cerebral herniation, infections, and haemorrhage, mitigated by assessing haemostatic parameters and brain anatomy using blood sampling and MRI, prior to the procedure. Additionally, all samplings were performed by experienced physicians and nurses. Further, study III exposed participants to possible side effects of a single dose of methylphenidate. These downsides were judged to be outweighed by the increased understanding of their own symptoms that the participants could receive through participation in the study.

In a broader context, the studies in this thesis could improve understanding of fatigue and cognitive dysfunction and lead to novel targeted treatments for cGvHD, potentially improving the lives of other transplanted patients. Potential mechanisms of fatigue and cognitive dysfunction found here may be explored in patients with other chronic disorders, suffering from similar symptoms.

4 RESULTS AND DISCUSSION

4.1 REPEATED INFUSIONS OF MSCS TO TREAT CGVHD

Among the 11 patients recruited in study I, six responded to MSC treatment according to the national institutes of health classification. Responses were seen mainly in eyes, mouth, and joints, with an increased range of motion in all treated patients except for one. Responding patients were able to reduce immunosuppression, with two patients eventually being entirely free of corticosteroids. No immediate side effects were observed. One patient died of progressive cGVHD after only one infusion, two patients withdrew from the study (after three and seven infusions) due to signs of haematological relapse (increasing CD19 chimerism and increased M-protein levels respectively). Further, there were five cases of grade three infection and two cases of dysplasia (in skin and cervix).

Pre-treatment immune status was predictive of response, with the responding patients having more naïve T-cells (CD4+CD27+CD45RA+ and CD4+CCR7+) and naïve B-cells (CD19+IgD+CD38low) compared to non-responders. The differences remained stable throughout the study and were not affected by MSC treatment. Instead, we observed short term increases in absolute number of naïve T-cells and Tregs after each MSC infusion in the responders, effects that were not present in non-responders. Further, the levels of several cytokines, notably CXCL9 and CXCL10, involved in T-cell homing to inflammatory tissues, decreased in responders and increased in non-responders, throughout the course of treatment[252]. Recipient immune status is suggested as important for MSC efficacy, as induction of apoptosis in MSCs by recipient cytotoxic cells and subsequent macrophage-mediated efferocytosis suppresses inflammation by inducing a regulatory macrophage phenotype[110,111]. CXCL9 and CXCL10 are produced by pro-inflammatory macrophages and contribute to cGvHD pathogenesis by mobilising effector T-cells expressing CXCR3 to the skin[253,254]. The naïve immune status of the responders could potentially be used clinically as a criterion for selecting cGvHD patients suitable for MSC treatment and decreased CXCL9 and CXCL10 levels are potential biomarkers of response.

Important drawbacks of this study are the limited size and the lack of a control group, preventing conclusions about MSC-specific effects.

4.2 FATIGUE AND COGNITIVE DYSFUNCTION AFTER HSCT

4.2.1 Strengths and weaknesses of the chosen study design

Fatigue and cognitive dysfunction are well recognized consequences of many diseases (such as cancer, non-malignant chronic disease, MS, chronic fatigue syndrome and depression), yet the mechanisms causing them are largely unknown. Therefore, to study these symptoms in the context of HSCT it was important to exclude alternative causes, and to find a well-matched reference group.

For studies II, III and IV we chose a cross-sectional design, recruiting patients long-term after transplant. This approach has several advantages and disadvantages. The main advantage is practicality and simplicity. We ended up with a group of patients with distinct and meaningful symptoms that persisted even though they were free of haematological disease, and a considerable time had passed since the HSCT. In addition, the recruited control group was well matched in all aspects except for the symptoms of interest. The obvious downside to the cross-sectional approach is that we are unable to determine cause and effect. Specifically, are the perturbations we demonstrated caused by the HSCT procedure, by any therapies administered before transplant or by the haematological disease itself? Additionally, are they the cause of fatigue and cognitive dysfunction, or do they represent a parallel phenomenon caused by a yet unknown disease process? To answer such questions, longitudinal studies are required. However, despite recent improvements many patients still do not become long-term survivors after HSCT[146]. In addition, only about 20-30% of survivors likely end up suffering from long-term fatigue and there are no established risk factors. Consequently, a longitudinal study beginning before transplant would need to include a large number of patients to end up with an adequate group of long-term fatigued survivors, which would make it costly and time consuming. We therefore designed a small exploratory cross-sectional study, aiming to carefully examine relevant patients using multiple resource-intensive analyses to find biomarkers in need of confirmation in such longitudinal studies.

The analyses performed can be roughly grouped into two categories: characterizing disability and searching for pathophysiological mechanisms.

4.2.2 Characterizing disability (study II and III)

Study II demonstrated distinct disabilities in the patients with fatigue: In line with studies on cancer patients, fatigue was associated with a meaningful decrease in quality of life[255–257]. Cognitive function was significantly lower overall ($p=0.0002$) and seven patients in the fatigued group were determined to have cognitive dysfunction ($GDS\geq0.5$) compared to none in the non-fatigued group[233]. The fatigued patients below 65 years of age (the retirement age in Sweden) were unable to sustain full employment (55% vs 93% of full time in the non-fatigued group, $p=0.01$) and the effect was most pronounced in the subgroup with CD, signifying the ability of the objective cognitive assessments to describe relevant disability. Importantly, there were no significant differences between the groups on most individual tests in the cognitive battery. This likely reflects individual differences in cognitive proficiency and necessitates the use of a GDS[233]. To avoid the situation where proficiency in one domain hides disabilities in others due to averaging, a performance that matches or exceeds the reference population ($z\text{-score}\geq0$) on an individual test results in a deficiency score of 0. Thus, the test is only sensitive to disability. In other words, a person with excellent reaction times and planning abilities may still be classified as having a cognitive dysfunction if he or she performs below average on a memory test. This was an important consideration in our study, given the high premorbid intelligence quotient scores in our population (111.1 in fatigued and 115.2 in non-fatigued patients).

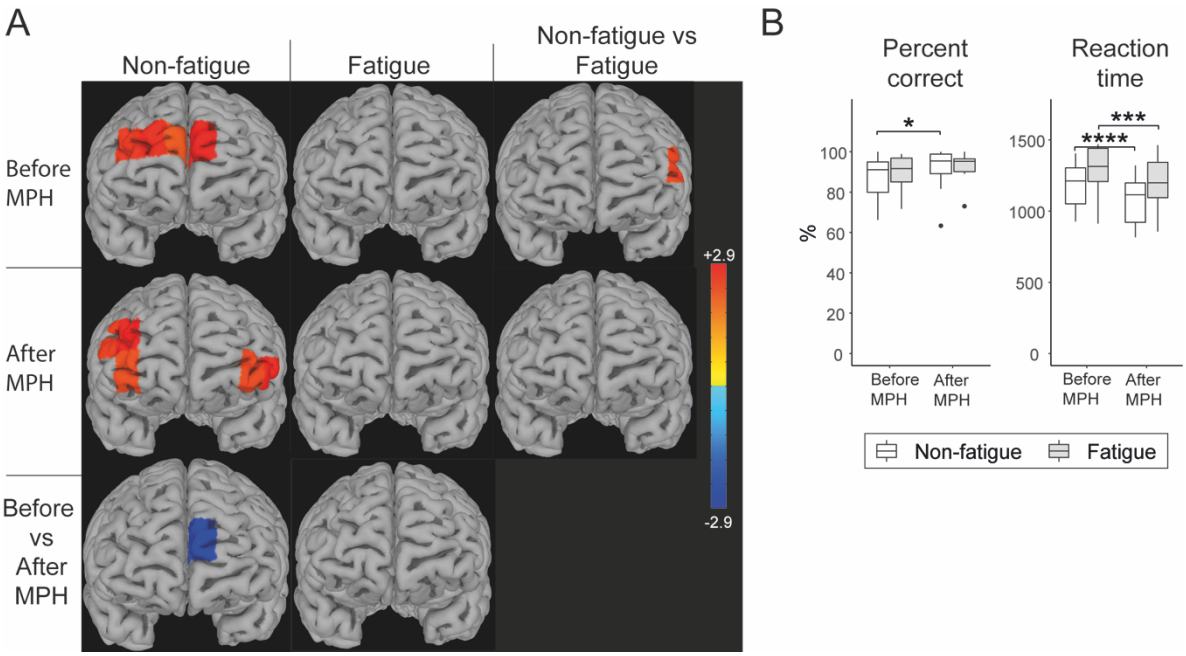


Figure 8: Stroop task results from study III. A) The non-fatigued patients activated dorsomedial channels during the Stroop task, before methylphenidate administration. After methylphenidate, activity shifted to lateral channels. In contrast, fatigued patients did not significantly activate the prefrontal cortex during the Stroop task and methylphenidate administration did affect activity. **B)** Task performance was similar between fatigued and non-fatigued patients before methylphenidate (MPH), and both groups improved reaction time after methylphenidate administration. However, only the non-fatigued patients improved in accuracy after methylphenidate. *Adapted from Boberg et al, Bone Marrow Transplantation 2021*

Given the decreased cognitive abilities in the fatigue group, we designed study III to measure neurologic activity in the prefrontal cortex, important for planning and executive function and often perturbed in cancer survivors[210]. We demonstrated reduced prefrontal cortex activity in the fatigued patients during both the Stroop (figure 8A), verbal fluency and emotion regulation tasks, compared to both non-fatigued patients and healthy controls. These results somewhat contrast previous findings on breast cancer patients where compensatory over-activation in the prefrontal cortex associated with fatigue or chemotherapy, suggesting that HSCT result in a different pattern of CNS injury[210]. As in study I, cognitive performance on each individual test was similar in fatigued and non-fatigued patients (figure 8B), perhaps due to individual differences in proficiency. Preserved cognitive performance at the expense of increased brain activity has been hypothesized as a mechanism leading to fatigue[210]. Such a compensatory overactivation may be located in cortical areas undetectable by fNIRS in HSCT recipients. Future studies should therefore employ fMRI to allow whole brain analysis.

4.2.3 Possible pathophysiological mechanisms (study II and IV)

Based on previous associations of increased peripheral cytokines in fatigued HSCT survivors, graft-versus-host mediated immune activation leading to fatigue or cognitive dysfunction was an important hypothesis when designing our studies[189]. In study II we measured cytokine concentrations and performed a limited flow cytometry analysis of immune cell subsets in the CSF. In addition, we analysed markers of neurodegeneration, BBB damage, intrathecal immunoglobulin production and anaerobic metabolism. None of the studied parameters significantly differed between fatigued and non-fatigued patients, nor between patients with or without cognitive dysfunction. Due to the limited study size, the negative results may have been caused by a lack of statistical power. Another possible explanation is that an acute inflammatory insult after transplant caused persisting neurological damage in the fatigue group, despite the inflammation being resolved. Finally, since the protein microarray used in study II was limited to demonstrating relative between-group differences, it is possible that both patient groups had increased levels of inflammatory cytokines in the CSF, and that perturbed immune regulation caused fatigue and cognitive dysfunction. Such a mechanism can be investigated in future studies by including healthy controls.

In study IV, we expanded the search for a pathophysiological mechanism based on the results of studies II and III. The dysfunctional activation of the prefrontal cortex and previous studies demonstrating loss of white and grey matter in the CNS after HSCT suggested underlying neuronal damage[213,214]. Further, the only clinical parameter that differentiated fatigued from non-fatigued patients in study I was BMI at time of transplant, which was higher in the fatigued group (26.17 vs 23.41, p=0.019). Obesity is associated with inflammation and a known risk factor for decreased cognitive function after cancer treatment[258–260].

Consequently, we performed a multiplex proteomic analysis focusing on neurologic and metabolic markers. We also included immune markers to validate the findings of study I and evaluate immune regulation. The results demonstrated that patients with cognitive dysfunction had reduced levels of several proteins involved in neurogenesis, neuron survival, synapse formation and synaptic plasticity, axon elongation, neural stem cell regulation, CNS trauma and neural damage, and BBB function. Additionally, proteins involved in immune regulation, leucocyte migration and stroma/extracellular matrix such as TGFβ-1, hepatocyte growth factor, colony stimulating factor 1 and CCL19 were also downregulated. BMI remained increased in patients with cognitive dysfunction at time of study inclusion, and no significant change in BMI from transplant to inclusion was evident. Adjusting for BMI did not affect the correlations between cognitive function and CSF protein expression.

The proteomic analysis was complemented by mRNA sequencing of EVs in the CSF. Supporting the proteomic findings, gene ontology term analysis of differentially expressed mRNA between patients with and without cognitive dysfunction suggested downregulated cell-cell adhesion (important for neurogenesis), wound healing and noradrenergic neuron differentiation; and upregulated reactive gliosis and neuroinflammatory response. mRNA and

protein levels were not correlated. However, mRNA content in EVs can differ substantially compared to the cells of origin[261].

Based on the proteomic and mRNA sequencing results, we designed a flow cytometry panel to determine the effects of these differences at the cellular level. The panel included markers of T-cell activation and anergy, monocyte and DC markers, and chemokine receptors. One patient was excluded from the analysis since the CSF erythrocyte count and flow cytometry data indicated peripheral blood cell contamination. The analysis revealed that patients with fatigue and cognitive dysfunction had significantly higher proportion of activated T-cells (about 5% vs 2.5%, p=0.016 for fatigue vs non-fatigue and 0.0087 for cognitive dysfunction vs non-cognitive dysfunction)[262,263]. As the activated T-cell level was inversely correlated to CSF TGF-beta concentration, the intrathecal T-cell activation may result from an inadequate immune regulation in the patients with fatigue and cognitive dysfunction. A pre-clinical study recently suggested that interactions between low levels of alloreactive T-cells and infiltrating bone-marrow derived macrophages can cause cGvHD in the CNS, resulting in cognitive dysfunction[138]. Our data support the existence of such a mechanism in the clinical setting as well.

Microglia mediate continuous synaptic pruning and clearing of CNS debris, important for cognition, and may therefore mediate inflammation-related neurological symptoms[117,264,265]. PET with a radioligand for the mitochondrion-associated translocator protein (TSPO) located in microglia has been developed as a non-invasive technique to measure microglia activity. In addition, synaptic density can be measured with PET using a radioligand for synaptic vesicle glycoprotein 2A. These techniques may be used in future studies in search of a connection between immune dysregulation and cognitive dysfunction in humans.

In addition, the flow cytometry analysis in study IV demonstrated an increased proportion of CD16+ NK-cells, negatively correlated to neurotrophic markers, in patients with cognitive dysfunction. NK-cells have been demonstrated to eliminate neuroblasts in the dentate gyrus of the hippocampus, causing reduced cognitive function in the elderly[266]. Our results therefore suggest that NK-cell mediated cytotoxicity in the CNS contributes to cognitive impairments after aHSCT.

Importantly, study IV demonstrated the feasibility of performing multi-parameter flow cytometry, proteomic analysis and mRNA sequencing to study neuropathology in humans. Flow cytometric cell sorting and subsequent single cell mRNA sequencing are available techniques to scrutinize the function of the dysregulated immune cell subsets in greater detail.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

5.1 THE FUTURE OF MSC TREATMENT FOR CGVHD

A recent trial demonstrated efficacy of ruxolitinib for treating steroid refractory cGvHD, establishing it as an evidence based option in this clinical scenario and raising the question of the proper place for MSCs in the treatment arsenal for cGvHD[41]. Important advantages of MSC treatment are the low incidence of side effects and relative ease of administration (one infusion per month)[104]. Further, it can be speculated that T-cell mediated cytotoxicity, required for MSC efficacy, may be more effective early in the disease course, due to activated T-cells playing a central role in phase 1 and 2 of the disease, compared to in phase 3, which is characterized by extracellular matrix production, macrophage and fibroblast activity[31,32]. Loss of tolerance due to reduced regulatory lymphocyte populations is suggested as important for the cGvHD pathogenesis and MSC treatment may improve tolerance by stimulating Treg expansion[31,32,111]. Taken together, it can be argued that MSCs added to corticosteroids for first-line treatment of moderate-severe cGvHD is appealing from a mechanistic standpoint and that a randomized trial in this scenario has a reasonable chance of demonstrating efficacy, especially in patients with high naïve cell counts.

5.2 THE NEUROBIOLOGY OF FATIGUE AND COGNITIVE DYSFUNCTION AFTER HSCT IN THREE PHASES

In 2016, the Centers for Disease Control and Prevention in the United States, proposed a 3-phase study hypothesising that chronic fatigue syndrome (CFS) is caused by immune-mediated brain dysfunction[267]. Phase 1 was a cross-sectional deep phenotyping of CFS patients to define pathophysiology, phase 2 was a longitudinal validation of the suggested biomarkers to establish end points for intervention, and phase 3 was an early phase intervention study with immunomodulatory agents targeting the validated biomarkers. Our approach to patients with fatigue and cognitive dysfunction after HSCT is very similar. Study II, III and IV can all be said to be part of phase 1. The obvious next step is to recruit patients earlier after HSCT and follow them prospectively, evaluating biomarkers at multiple time points.

Study IV demonstrated impaired immune regulation in the CNS with low levels of stroma-derived cytokines such as TGFβ and VEGF, as well as increased T-cell activation, primarily in patients with cognitive dysfunction. MSCs are known to produce TGFβ and to suppress T-cell activity. Further, systemic delivery of MSCs has shown promise for treating neurologic conditions such as MS and Alzheimer's disease, resulting in increased VEGF, suggestive of restored BBB function[56,268,269]. Given this data, it is reasonable to attempt treating fatigue and cognitive dysfunction after HSCT with MSC infusions. The apparent safety of MSC treatment increases the incentive to use them as treatment for these disabling, but not deadly symptoms[104,268]. The trial of MSC treatment for Alzheimer's disease suggested that repeated low doses of MSCs result in optimal cognitive benefit[268]. To simultaneously increase understanding of neurobiological and -immunological mechanisms, included patients should

be evaluated with PET using ligands for the TSPO (microglia activity) and SV2A (synaptic density) before and after treatment.

5.3 ARE WE SEEING WHAT WE WANT TO SEE?

Personal beliefs, praise from peers, and advancing scientific careers are strong incentives that may have impacted our ability to objectively scrutinize the research findings discussed above. A common theme in all studies in this thesis is that they include a small number of subjects that are measured with a multitude of investigations, resulting in a high likelihood of false positive findings that can be pieced together into seemingly plausible mechanisms. While such exploratory studies have great utility to investigate novel treatments or symptoms, conclusions drawn from their results may be biased by our values. For example, study I did not account for spontaneous improvement in cGvHD, and studies II, III and IV did not consider the alternative explanation of a psychological trauma causing fatigue and cognitive dysfunction. Study design was based on the assumptions that MSCs are effective against cGvHD and that fatigue and cognitive dysfunction are caused by neurobiological defects after HSCT. As human beings, scientists are not entirely neutral, and should not pretend to be[270]. Instead, the scientific community should strive to examine a problem from many angles to reduce such inherent bias, and I endorse other researchers to perform studies that challenges the conclusions drawn herein: The suggested randomized first-line trial of MSC treatment for cGvHD would of course test the assumption of MSC efficacy. Incorporating psychological risk factors such as personality traits or pre-transplant episodes of fatigue in future longitudinal studies of HSCT-related fatigue could demonstrate the relative importance of these mechanisms compared to neuroimmune dysfunction. Finally, it is vital that these studies are adequately powered to be conclusive.

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