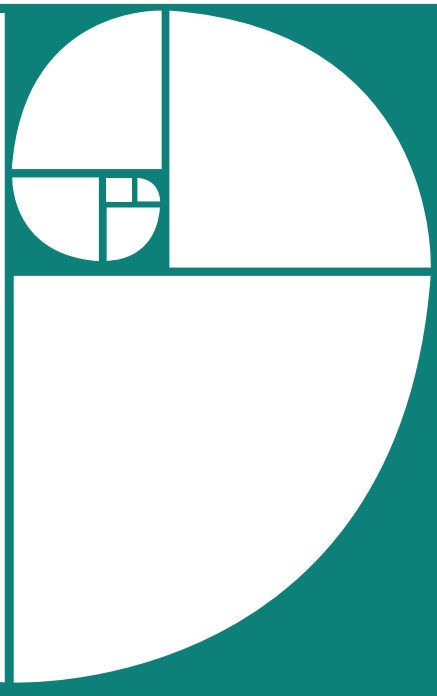
Improving and extending treatment modalities in Graft versus Host Disease

Lotte van der Wagen



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Improving and extending treatment modalities in Graft versus Host Disease

Verbeteren en uitbreiden van behandelmogelijkheden voor Graft versus Host Ziekte
(met een samenvatting in het Nederlands)

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door

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geboren op 19 januari 1983
te Ede

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General introduction

Lotte van der Wagen

INTRODUCTION TO THIS THESIS

The only curative treatment option for many hematological malignancies still remains allogeneic stem cell transplantation (SCT). In recent years we have seen an enormous increase in the use of immune therapy for treating both hematological and solid cancers. As checkpoint inhibition and CAR T-cells have assumed enormous proportions, allogeneic stem cell transplantation has been for decades the only cellular immune therapy available. Since its first use in 1957 (1) vast progress has been made in the SCT field. From being highly experimental since its first use, allogeneic stem cell transplantation is now the standard of care for many hematological malignancies as well as non-malignant diseases such as aplastic anemia and severe immunodeficiencies (2, 3). However despite all accomplishments, today we still face largely the same problems as in the early years of allogeneic SCT, that is Graft versus Host Disease (GVHD). The first description of GVHD, then named 'Runt disease' was made by Billingham and colleagues who described a syndrome after bone marrow transplantation characterized by rash and diarrhea (4). Later on, the discovery of Human Leukocyte Antigens (HLA) and its role in immunological reactions proved to be vital to perform human stem cell transplantations and showed to be of major importance for preventing GVHD (5, 6).

The early goals of SCT mainly comprised of recovery of hematopoiesis in the bone marrow after intensive chemotherapy and/or irradiation. However later the Graft versus Leukemia (GvL) effect became apparent and renewed interest in the field (7).

Despite all improvements in knowledge, the complexity of the human immune system and all its components has not yet solved the fundamental challenge in 2019 of retaining the GvL effect whilst abolishing GVHD.

Acute GVHD

Acute GVHD is a complication of allogeneic SCT that occurs when donor immune cells, mostly T-cells, recognize the transplant recipient as non-self, thereby initiating an immune response. Clinical symptoms of acute GVHD comprise of a maculopapular rash, nausea, diarrhea and liver enzyme increase. Incidence of acute GVHD ranges widely from 9-70% dependent on donor characteristics, HLA matching, gender disparity, hematopoietic graft source, prophylaxis, conditioning regimen etc. As the incidence varies, so does severity of acute GVHD. Data from the Center for International Blood and Marrow Transplant Research (CIBMTR) showed in 2010 that GVHD is fatal to approximately 15% of allogeneic SCT recipients (8). First line treatment of acute GVHD relies heavily on corticosteroids, however steroid refractory (SR) acute GVHD patients have a somber prognosis

with a mortality rate of more than 90% (9). Grading severity of acute GVHD is performed by means of the 'Glucksberg criteria' as described in Table 1 and 2 (10).

Stage	Skin	Liver	Gut
0	No rash	Bilirubin <33 µmol/L	<500 ml diarrhea/day or nausea
1	Maculopapular rash involving <25% of total skin surface	Bilirubin 33-50 µmol/L	500-999 ml diarrhea/day
2	Maculopapular rash involving 25-50% of total skin surface	Bilirubin 51-102 µmol/L	1000-1499 ml diarrhea/day
3	Maculopapular rash involving >50% of total skin surface	Bilirubin 103-254 µmol/L	1500-1999 ml diarrhea/day
4	Generalized erythroderma with bullous formation (possible desquamation)	Bilirubin ≥255 µmol/L	≥2000 ml diarrhea/day or severe abdominal cramping with or without ileus

TABLE 1. Staging of acute GVHD per organ system

Overall grade	Skin	Liver	Gut
I	Stage 1-2	Stage 0	Stage 0
II	Stage 3 OR	Stage 1 OR	Stage 1
III	Stage 0-3 AND	Stage 2-3 OR	Stage 2-4
IV	Stage 4 OR	Stage 4	-

TABLE 2. Grading of acute GVHD based on organ stage

Pathophysiology of acute GVHD

Pathophysiology of acute GVHD is complicated and still not fully understood. Generally accepted is the underlying concept that there are different phases in GVHD. Initially there is a 'damage' phase, where tissues are inflamed, e.g. mucosal barrier loss in the gastro-intestinal tract, due to chemotherapy or radiotherapy. This damage leads to a release of inflammatory cytokines such as Tumor Necrosis Factor (TNF), interleukin-1 (IL-1) and IL-6 (11-14). Also the gut microbiome is an important player in the pathogenesis of acute GVHD. Use of antibiotics leads to loss of beneficial microbiome and less diversity. Microbiota-derived pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) can further initiate inflammatory signals (15-18).

The next phase in the development of acute GVHD comprises of cytokine driven cellular differentiation. Both inflammatory cytokines and PAMPs, e.g. lipopolysaccharides (LPS), can activate antigen-presenting cells (APCs) of the host, which eventually leads to activation of T-cells (13). However, also other cell subsets such as innate lymphoid cells (ILC) are involved in this process (19). Donor T-cells can be activated by host APCs through direct antigen presentation or by donor APCs through alloantigen recognition (20, 21). These T-cells can proliferate and differentiate towards different T-helper subsets, mainly Th1, Th2 and Th17 that can mediate organ specific GVHD (22).

In the final phase, T-cells and inflammatory cytokines induce damage to the epithelial cells of mainly the skin, liver, gastro-intestinal tract and lungs. The damaged mucosal cells can then leak LPS that can amplify the destruction of the epithelium. Furthermore LPS can recruit APCs (monocytes and macrophages) that will produce new pro-inflammatory cytokines thereby contributing to a negative cycle and finally resulting in apoptosis of cells (11, 23).

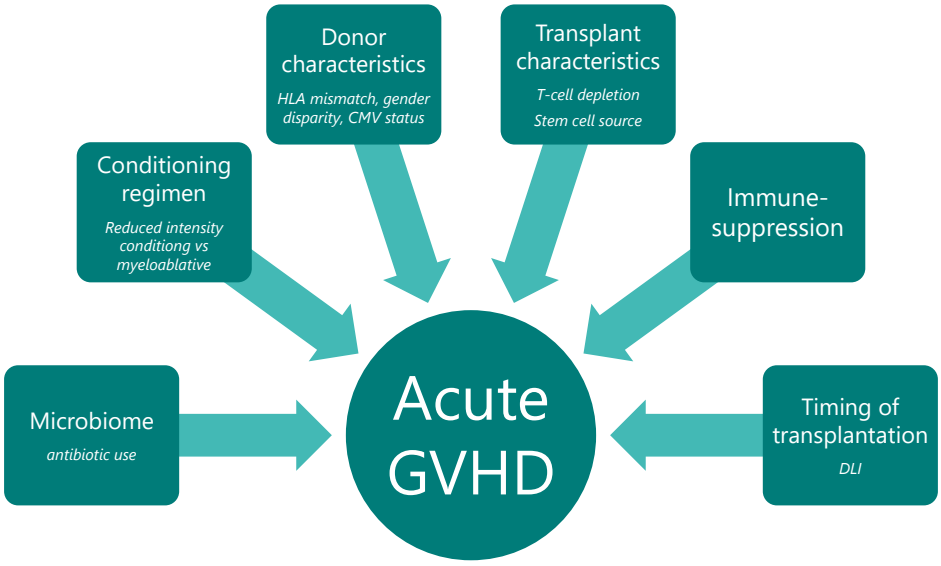


FIGURE 1.
Different factors that influence the development of acute GVHD.
CMV: cytomegalovirus; DLI: Donor Lymphocyte Infusion; HLA: Human Leukocyte Antigen;

Understanding the different phases and mechanisms of acute GVHD pathophysiology will allow us to influence certain factors (Figure 1). By altering the initiation 'damage' phase and manipulating the cellular differentiation that is central in the second phase, the risk of developing GVHD could be decreased.

Current treatment options for acute GVHD

Choice of treatment is dependent on disease severity. However from grade II GVHD onwards the mainstay of treatment still relies heavily on corticosteroids that induce a general immune suppression with a response rate of approximately 33-51% (24-26). Nationally and internationally there was no consensus on second line treatment of steroid refractory acute GVHD patients. Therefore the Dutch SCT community created a consensus guideline for the treatment of acute and chronic GVHD (27). An overview of possible treatment options for acute GVHD is given in Table 3, however choice of agent is dependent on availability, experience and patient characteristics.

Treatment	Dose	% response	Survival	SORT level	Ref.
MSC	1-2 x 10^6 cells/kg on day 1, 8, and 22	72% overall response (39% CR)	50% (6 months)	B	(28)
		50% CR	44% (1 year)	B	(29)
ATG (ATGAM, horse)	6x every other day 30 mg/kg/day *or* 12 days 15 mg/kg/day	57%	45% (6 months)	B	(30)*
ATG (Thymoglobulin, rabbit)	5 consecutive days 3mg/kg/day	56%	55% (6 months)	B	(31)**
Mycophenolate mofetil	2dd 20mg/kg	60% (CR at day 28)	64% (9 months)	B	(32)
		55%	30% (2 years)	C	(33)
Etanercept	2x per week 25 mg sc. during max 8 weeks	46%	Not reported	C	(34, 35)
Ruxolitinib	start 2 dd 5 mg oral, if no severe toxicity increase after 3 days to 2 dd 10 mg	82%	79% (6 months)	C	(36, 37)
PUVA With isolated GVHD of the skin	Through dermatologist	Not reported	Not reported	C	(38)
Infliximab	1x/week 10mg/kg	59%	Not reported	C	(39)

TABLE 3. Treatment options for steroid refractory acute GVHD
Adapted from Table 4 from (27).
ATG: Anti Thymocyte Globulin; CR: Complete Resolution; MSC: Mesenchymal Stromal Cells; PUVA: Psoralene-UV-A-radiation; SORT: Strength of Recommendation Taxonomy (40)
* Disregarded some older retrospective studies with worse response rates and overall survival
** Rabbit-ATG combined with methylprednisolone does not improve outcomes compared to methylprednisolone as single agent

Chronic GVHD

Chronic GVHD can be a devastating complication of allogeneic SCT affecting approximately 30-70% of patients (41). In contrast to acute GVHD it does not only affect the skin, gastro-intestinal tract, liver and lungs, but can affect any organ or tissue. Risk factors for developing chronic GVHD are (42-46):

- Prior acute GVHD
- Stem cell source: Peripheral Blood Stem Cell (PBSC) grafts give a higher risk than bone marrow (BM) or cord blood (CB) grafts
- HLA mismatch
- Sex disparity between donor and recipient, mainly female donors to male recipients
- Older age of both donor and/or recipient
- Viral serostatus (CMV and EBV)

Diagnosis of chronic GVHD can be made according to the standardized NIH criteria. Chronic GVHD is present when there is at least one diagnostic manifestation or at least one distinctive manifestation confirmed by a biopsy or specific test (e.g. pulmonary function test), see Table 4 (47).

Grading of chronic GVHD is notoriously difficult, however can now be performed more uniformly worldwide following the NIH Consensus Development project as depicted in Figure 2 (47). This has shown its use in comparing outcomes of clinical studies.

Depending on the extent and severity of chronic GVHD patients have increased morbidity and mortality as well as an impaired quality of life (48-51). Tragically we still often replace a life-threatening disease as leukemia for another debilitating illness.

Organ or Site	Diagnostic (sufficient to establish the diagnosis of chronic GVHD)	Distinctive[1] (seen in chronic GVHD but insufficient alone to establish a diagnosis)
Skin	Poikiloderma Lichen planus-like features Sclerotic features Morphea-like features	Depigmentation Papulosquamous lesions
Nails		Dystrophy Longitudinal ridging, splitting or brittle features Onycholysis Pterygium unguis Nail loss (usually symmetric, affects most nails)
Scalp and body hair		New onset of scarring or non-scarring scalp alopecia (after recovery from chemoradiotherapy) Loss of body hair
Mouth	Lichen planus-like changes	Xerostomia Mucoceles Mucosal atrophy Ulcers Pseudomembranes
Eyes		New onset dry, gritty, or painful eyes Cicatricial conjunctivitis KCS Confluent areas of punctate keratopathy
Genitalia	Lichen planus like features Lichen sclerosus like features Females: Vaginal scarring or clitoral/labial agglutination Males: Phimosis or urethral/ meatus scarring or stenosis	Ulcers
GI tract	Esophageal web Strictures or stenosis in the upper to mid third of the esophagus	
Liver		
Lung	Bronchiolitis obliterans diagnosed with lung biopsy BOS[3]	Air trapping and bronchiectasis on chest CT

TABLE 4. Signs and symptoms of chronic GVHD (*continued*)

	Other features or unclassified entities[2]	Common (seen with both acute and chronic GVHD)
	Sweat impairment Ichthyosis Keratosis pilaris Hypopigmentation Hyperpigmentation	Erythema Maculopapular rash Pruritus
	Thinning scalp hair, typically patchy, coarse or dull (not explained by endocrine or other causes) Premature gray hair	
		Gingivitis Mucositis Erythema Pain
	Photophobia Periorbital hyperpigmentation Blepharitis (erythema of the eyelids with edema)	
	Exocrine pancreatic insufficiency	Anorexia Nausea Vomiting Diarrhea Weight loss Failure to thrive (infants and children
		Total bilirubin, alkaline phosphatase > 2 ULN, ALT > 2 ULN
	Cryptogenic organizing pneumonia Restrictive lung disease[4]	

		Diagnostic (sufficient to establish the diagnosis of chronic GVHD)	Distinctive[1] (seen in chronic GVHD but insufficient alone to establish a diagnosis)
Other	Muscles, fascia, joints	Fasciitis Joint stiffness or contractures secondary to fasciitis or sclerosis	Myositis or polymyositis[5]
	Hematopoietic and immune		
	Other		

TABLE 4. Signs and symptoms of chronic GVHD (*continued*)

Adapted from Table 1 from Jagasia *et al*, National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease: I. The 2014 Diagnosis and Staging Working Group report. Biol Blood Marrow Transplant. 2015 (47). DOI: (10.1016/j.bbmt.2014.12.001. Creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND)

ALT: alanine aminotransferase; AIHA: autoimmune hemolytic anemia; BOS: Bronchiolitis Obliterans Syndrome; ITP: idiopathic thrombocytopenic purpura; ULN: Upper Limit of Normal.

1: In all cases, infection, drug effect, malignancy, or other causes must be excluded.

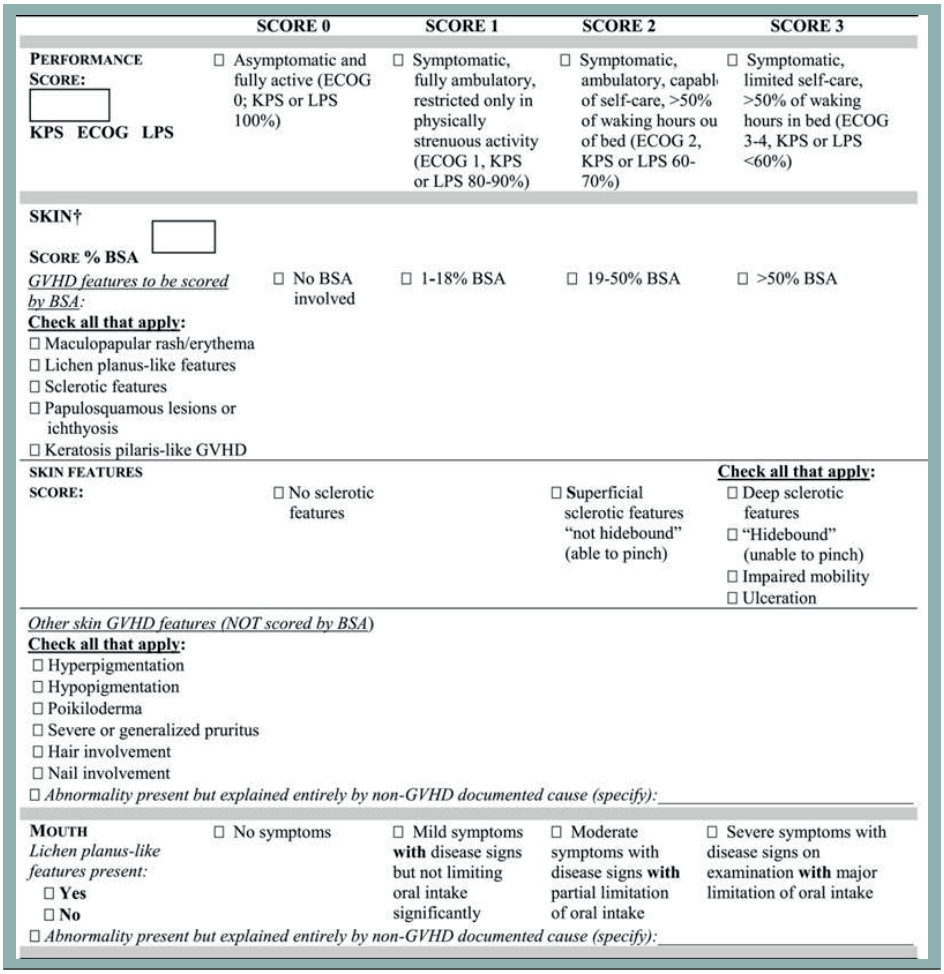
2: Can be acknowledged as part of the chronic GVHD manifestations if diagnosis is confirmed.

3: BOS can be diagnostic for lung chronic GVHD only if distinctive sign or symptom present in another organ

4: Pulmonary entities under investigation or unclassified.

5: Diagnosis of chronic GVHD requires biopsy.

	Other features or unclassified entities[2]	Common (seen with both acute and chronic GVHD)
	Edema Muscle cramps Arthralgia or arthritis	
	Thrombocytopenia Eosinophilia Lymphopenia Hypo- or hyper-gammaglobulinemia Autoantibodies (AIHA, ITP) Raynaud's phenomenon	
	Pericardial or pleural effusions Ascites Peripheral neuropathy Nephrotic syndrome Myasthenia gravis Cardiac conduction abnormality or cardiomyopathy	

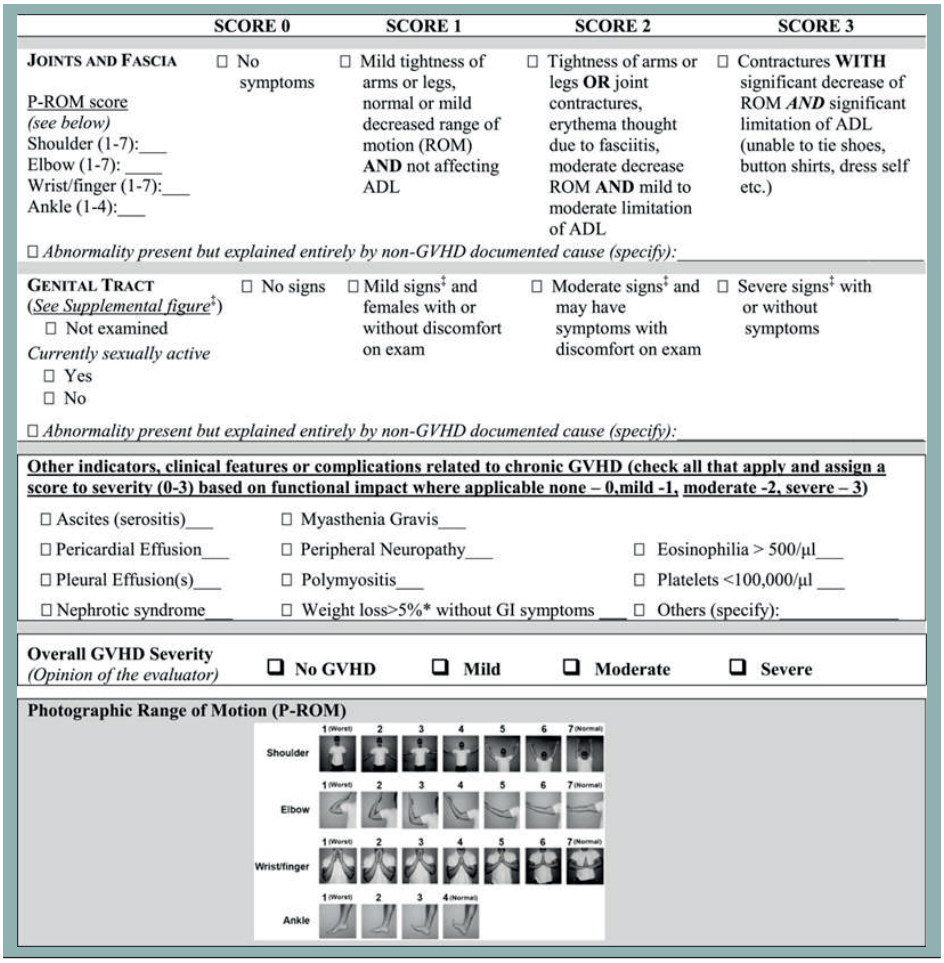


	SCORE 0	SCORE 1	SCORE 2	SCORE 3
PERFORMANCE SCORE: ☐ **KPS ECOG LPS**	☐ Asymptomatic and fully active (ECOG 0; KPS or LPS 100%)	☐ Symptomatic, fully ambulatory, restricted only in physically strenuous activity (ECOG 1, KPS or LPS 80-90%)	☐ Symptomatic, ambulatory, capabl of self-care, >50% of waking hours ou of bed (ECOG 2, KPS or LPS 60-70%)	☐ Symptomatic, limited self-care, >50% of waking hours in bed (ECOG 3-4, KPS or LPS <60%)
SKIN† ☐ **SCORE % BSA**				
GVHD features to be scored by BSA: **Check all that apply:** ☐ Maculopapular rash/erythema ☐ Lichen planus-like features ☐ Sclerotic features ☐ Papulosquamous lesions or ichthyosis ☐ Keratosis pilaris-like GVHD	☐ No BSA involved	☐ 1-18% BSA	☐ 19-50% BSA	☐ >50% BSA
SKIN FEATURES SCORE:	☐ No sclerotic features		☐ Superficial sclerotic features "not hidebound" (able to pinch)	**Check all that apply:** ☐ Deep sclerotic features ☐ "Hidebound" (unable to pinch) ☐ Impaired mobility ☐ Ulceration
Other skin GVHD features (NOT scored by BSA) **Check all that apply:** ☐ Hyperpigmentation ☐ Hypopigmentation ☐ Poikiloderma ☐ Severe or generalized pruritus ☐ Hair involvement ☐ Nail involvement ☐ *Abnormality present but explained entirely by non-GVHD documented cause (specify):* _____				
MOUTH *Lichen planus-like features present:* ☐ **Yes** ☐ **No**	☐ No symptoms	☐ Mild symptoms **with** disease signs but not limiting oral intake significantly	☐ Moderate symptoms with disease signs **with** partial limitation of oral intake	☐ Severe symptoms with disease signs on examination **with** major limitation of oral intake
☐ *Abnormality present but explained entirely by non-GVHD documented cause (specify):* _____				

FIGURE 2. (*continued***)**

	SCORE 0	SCORE 1	SCORE 2	SCORE 3
EYES *Keratoconjunctivitis sicca (KCS) confirmed by ophthalmologist:* ☐ **Yes** ☐ **No** ☐ **Not examined**	☐ No symptoms	☐ Mild dry eye symptoms not affecting ADL (requirement of lubricant eye drops ≤ 3 x per day)	☐ Moderate dry eye symptoms partially affecting ADL (requiring lubricant eye drops > 3 x per day or punctal plugs), **WITHOUT** new vision impairment due to KCS	☐ Severe dry eye symptoms significantly affecting ADL (special eyeware to relieve pain) **OR** unable to work because of ocular symptoms **OR** loss of vision due to KCS
☐ *Abnormality present but explained entirely by non-GVHD documented cause (specify):*				
GI Tract ***Check all that apply:*** ☐ Esophageal web/ proximal stricture or ring ☐ Dysphagia ☐ Anorexia ☐ Nausea ☐ Vomiting ☐ Diarrhea ☐ Weight loss ≥5%* ☐ Failure to thrive	☐ No symptoms	☐ Symptoms without significant weight loss* (<5%)	☐ Symptoms associated with mild to moderate weight loss* (5-15%) **OR** moderate diarrhea without significant interference with daily living	☐ Symptoms associated with significant weight loss* >15%, requires nutritional supplement for most calorie needs **OR** esophageal dilation **OR** severe diarrhea with significant interference with daily living
☐ *Abnormality present but explained entirely by non-GVHD documented cause (specify):*				
LIVER	☐ Normal total bilirubin and ALT or AP < 3 x ULN	☐ Normal total bilirubin with ALT ≥3 to 5 x ULN or AP ≥ 3 x ULN	☐ Elevated total bilirubin but ≤3 mg/dL or ALT > 5 ULN	☐ Elevated total bilirubin > 3 mg/dL
☐ *Abnormality present but explained entirely by non-GVHD documented cause (specify):*				
LUNGS** **Symptom score**:	☐ No symptoms	☐ Mild symptoms (shortness of breath after climbing one flight of steps)	☐ Moderate symptoms (shortness of breath after walking on flat ground)	☐ Severe symptoms (shortness of breath at rest; requiring 0_2)
Lung score: **%** FEV1 ☐	☐ FEV1≥80%	☐ FEV1 60-79%	☐ FEV1 40-59%	☐ FEV1 ≤39%
Pulmonary function tests ☐ Not performed				
☐ *Abnormality present but explained entirely by non-GVHD documented cause (specify):*				

FIGURE 2. (*continued***)**



	SCORE 0	SCORE 1	SCORE 2	SCORE 3
JOINTS AND FASCIA P-ROM score *(see below)* Shoulder (1-7):___ Elbow (1-7): ____ Wrist/finger (1-7):___ Ankle (1-4):___	☐ No symptoms	☐ Mild tightness of arms or legs, normal or mild decreased range of motion (ROM) **AND** not affecting ADL	☐ Tightness of arms or legs **OR** joint contractures, erythema thought due to fasciitis, moderate decrease ROM **AND** mild to moderate limitation of ADL	☐ Contractures **WITH** significant decrease of ROM ***AND*** significant limitation of ADL (unable to tie shoes, button shirts, dress self etc.)
☐ *Abnormality present but explained entirely by non-GVHD documented cause (specify):*___				
GENITAL TRACT (*See Supplemental figure*‡) ☐ Not examined *Currently sexually active* ☐ Yes ☐ No	☐ No signs	☐ Mild signs‡ and females with or without discomfort on exam	☐ Moderate signs‡ and may have symptoms with discomfort on exam	☐ Severe signs‡ with or without symptoms
☐ *Abnormality present but explained entirely by non-GVHD documented cause (specify):*___				

Other indicators, clinical features or complications related to chronic GVHD (check all that apply and assign a score to severity (0-3) based on functional impact where applicable none – 0,mild -1, moderate -2, severe – 3)

☐ Ascites (serositis)___	☐ Myasthenia Gravis___	
☐ Pericardial Effusion___	☐ Peripheral Neuropathy___	☐ Eosinophilia > 500/µl___
☐ Pleural Effusion(s)___	☐ Polymyositis___	☐ Platelets <100,000/µl ___
☐ Nephrotic syndrome___	☐ Weight loss>5%* without GI symptoms ___	☐ Others (specify):_____

Overall GVHD Severity *(Opinion of the evaluator)* ❑ **No GVHD** ❑ **Mild** ❑ **Moderate** ❑ **Severe**

Photographic Range of Motion (P-ROM)

FIGURE 2. Adapted from Figure 1 from Jagasia et al, National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease: I. The 2014 Diagnosis and Staging Working Group report. Biol Blood Marrow Transplant. 2015 (47). DOI: (10.1016/j.bbmt.2014.12.001. Creative Commons Attribution-Non Commercial-No Derivatives License (CC BY NC ND)

Pathophysiology of chronic GVHD

Chronic GVHD seems to be an even more complex disease then acute GVHD as it affects almost all organs and tissues in humans. Mouse models have shown to often be insufficient to mimic the entire spectrum of disease. However, general consensus in the pathophysiology is to divide the development into three phases (41, 52).

The first phase is characterized by early inflammation caused by tissue injury. Conditioning regimens, infections and acute GVHD can all trigger DAMPs (e.g. RNA, DNA, chromosomal HMGB1, extracellular matrix materials, ATP, and uric acid) and PAMPs (e.g. LPS) to be released (52, 53). This leads to increased antigen presentation by APCs (monocytes, macrophages and dendritic cells) and B-cells. This leads to activation of T-cells and neutrophils, contributing to endothelial injury and platelet activation (Figure 3).

The second phase is characterized by chronic inflammation, thymic injury and dysregulation of both B- and T-cells. The alloreactive B- and T-cells are primed by the APCs and then expand and skew into Th1, Th2 and Th17 phenotypes. Inflammation is maintained by production of auto- and alloreactive T-cells that have escaped thymic selection (54). These Th17 cells seem especially important as suppression of Interleukin 17 producing cells has been shown to reduce skin, liver and salivary gland chronic GVHD (55). The process is worsened by thymic injury both by the conditioning regimen and alloreactive T-cells (56). Hereby there are less regulatory T-cells formed and less autoreactive T-cells removed, leading to a loss of regulatory cell populations that contributes to chronic GVHD (57).

Finally the third phase is characterized by fibrosis leading to the clinical phenotype with sclerosis. Activated macrophages produce platelet-derived growth factor α (PDGF-α) and transforming growth factor β (TGF-β) that activate fibroblasts (58). In turn the activated fibroblasts produce extracellular matrix collagen and biglycan that cross links collagen leading to fibrosis and sclerosis in the patient. Plasma cells, activated by B-cell activating factor (BAFF) contribute to this fibrosis by producing immunoglobulins that cause deposits in tissues leading to more damage and fibrosis (59).

This current model of the pathophysiology of chronic GVHD is summarized in figure 3, reproduced from (41). These insights also offer new ways to intervene in this complex iatrogenic disease.

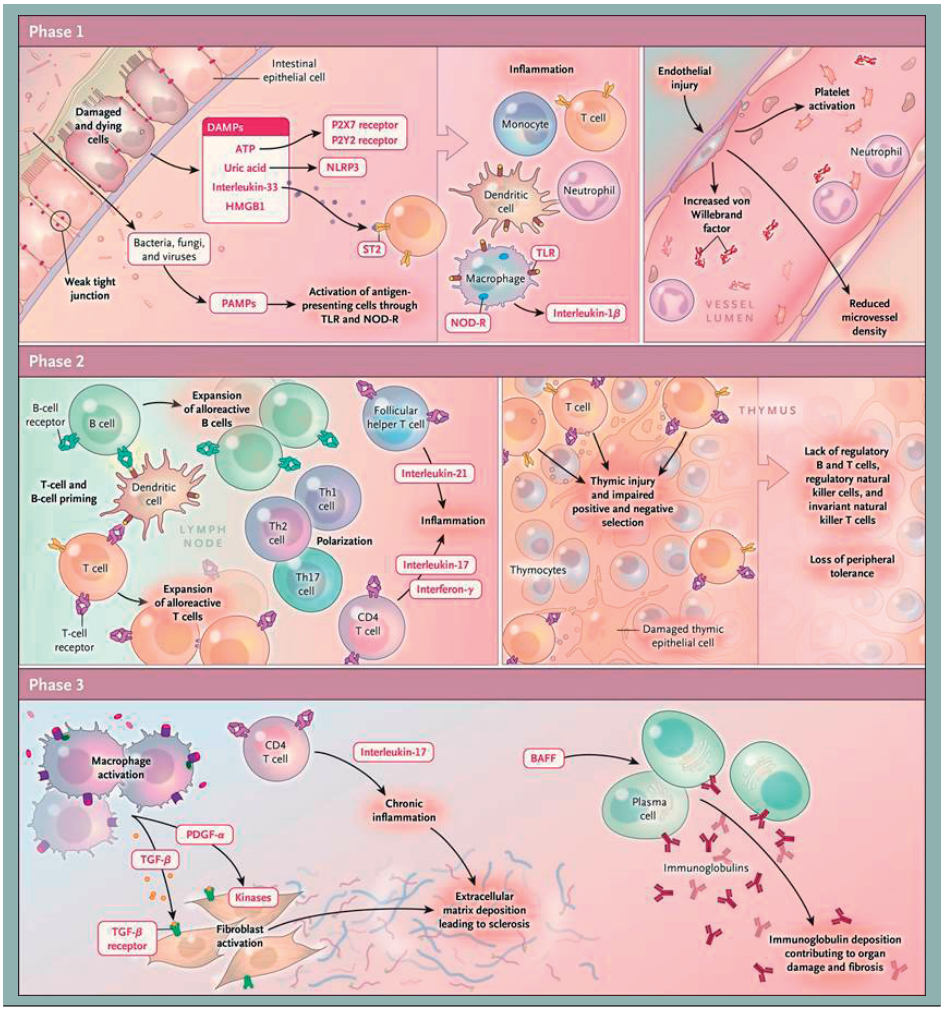


FIGURE 3. Reproduced with permission from Zeiser R, Blazar BR. Pathophysiology of Chronic Graft-versus-Host Disease and Therapeutic Targets. N Engl J Med. 2017;377(26):2565-79. (41), Copyright Massachusetts Medical Society.

Current treatment options for chronic GVHD

As the pathophysiology is being unraveled, new compounds are available for the treatment of chronic GVHD. However, first line treatment consists mostly of (a combination) of corticosteroids, calcineurin or mammalian Target Of Rapamycin (mTOR) inhibitors. Affected patients often require long term use of these immunosuppressive drugs which is associated with the development of side effects and hampered quality of life. When first line therapy fails, there are several second line options however, as is the case with acute GVHD, no international consensus exists on the preferred order. Also as patients with severe sclerotic GVHD rep-

resent a relatively small subgroup, many available compounds were originally developed for other diseases and only later discovered to be of interest for these patients. An overview of treatment options is provided in table 5 based on the Dutch consensus guideline (27).

Treatment	Dose	% response	Survival	SORT level	Ref.
Extracorporal Photopheresis (ECP)*:	12-24 weeks	64-70% (CR+PR)	70-78% (1 year)	A	(60, 61)
Rituximab	375mg/m^2 iv 1x/week during 4 weeks	27-66% (CR+PR)	84% (1 year)	B	(62-64)
Imatinib	100-200mg/day	30-79% (CR+PR)	84% (1,5 years)	B	(64-67)
Rituximab + nilotinib	RTX 375mg/m^2 iv 1x/week during 4 weeks, followed by nilotinib 2dd200mg during 6 months	71% (8% CR, 63% PR)	96,5% (1 year)	B	(68)
MSC	1-4 infusions of 0.6 x10^6 cells/kg bodyweight	74% (21%CR, 53%PR)	78% (2 years)	B	(69)
Mycophenolate mofetil	2x/day 1000mg (median dose Onishi *et al.* 1500mg/day)	7/11** en 6/23 (CR+PR)	Not reported	B	(70, 71)
Ruxolitinib	2x/day 5-10mg	85% (7%CR, 78%PR)	97.4% (6 months)	B	(37)
Ibrutinib	1x/day 420mg	67% (21% CR, 45% PR)	Not reported	B	(72)
Methotrexate	7,5mg/m^2 /week	78% (50%CR, 28%PR)	92% (1 year)	B	(73)
Bortezomib	Dose escalation schedule	5/10 patients PR	Not reported	C	(74)

TABLE 5. Treatment options for refractory chronic GVHD
Adapted from Table 6 from (27).
CR: Complete Resolution; MSC: Mesenchymal Stromal Cells; PR: Partial Resolution; RTX: Rituximab; SORT: Strength of Recommendation Taxonomy (40)

Aims and outline of this thesis

In this thesis we conducted studies with the aim to improve current treatment strategies for patient with acute and chronic GVHD and to provide insight into the complex biology of this iatrogenic disease.

We performed:

- A phase I/II prospective **clinical trial** to determine the efficacy and tolerability of MSC in steroid-refractory acute GVHD patients.
- A phase II prospective **clinical trial** to determine the efficacy and tolerability of the combination of rituximab and nilotinib in steroid-refractory sclerotic chronic GVHD patients.
- **Translational research:**
  - o To identify novel cellular and soluble biomarkers to establish predictive factors for response and overall survival.
  - o To gain insight into MSC properties that influence clinical outcome of steroid-refractory acute GVHD patients.
  - o To apply therapeutic drug monitoring to improve success rates in sclerotic chronic GVHD patients treated with tyrosine kinase inhibitors.

Chapter 1 is a general introduction into allogeneic SCT and its most important complication GVHD.

Chapter 2.1 describes the results of a clinical trial in which steroid refractory acute GVHD patients were treated with MSC. Also it describes the extensive search for plasma and cellular biomarkers to provide a better understanding in the pathophysiology of this disease as well as to provide a more personalized treatment approach.

Published in Leukemia 2015 Sep;29(9):1839-46

In ***chapter 2.2*** we propose the use of non-subjective automated analysis algorithms to analyze flow cytometry data of small cellular subsets in MSC treated acute GVHD patients.

Manuscript in preparation

Chapter 3 focuses on the influence of MSC donor properties on the outcome of steroid refractory acute GVHD patients. These data reflect the importance of markers of immunosuppressive potency of cellular therapies.

Manuscript in preparation

Chapter 4.1 shows the results of a clinical trial performed in patients with sclerotic chronic GVHD that were treated with rituximab followed by nilotinib. The effectiveness of this new combination was tested.

Published in Bone Marrow Transplantation 2018; Oct;53(10):1255-1262

In ***chapter 4.2*** we focus on the importance of therapeutic drug monitoring when using tyrosine kinase inhibitors in patients with sclerotic chronic GVHD.

Published in Bone Marrow Transplantation 2019 Jan 16 (Epub ahead of print)

Chapter 5 reports on the Dutch consensus guideline where also data from this thesis have contributed to certain treatment options.

Published in Nederlands Tijdschrift voor Hematologie 2018;15:54-69)

Chapter 6 reports on the potential beneficial effects of cytomegalovirus infection after (stem cell) transplantation by means of a review of the literature.

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Chapter 7 provides a general discussion that addresses our main findings. Suggestions for future research and the manner in which these should be set up are given followed by a Dutch summary of the outcomes of the studies in this thesis.

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2.1

Biomarker profiling of steroid resistant acute GVHD in patients after infusion of mesenchymal stromal cells

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ABSTRACT

We performed a prospective phase II study to study clinical safety and outcome in 48 patients with steroid-refractory grade II-IV acute Graft-Versus-Host-Disease (aGVHD) treated with Mesenchymal Stromal Cells (MSC). Clinical outcomes were correlated to comprehensive analyses of soluble and cellular biomarkers. Complete resolution (CR) of aGVHD at day 28 (CR-28) occurred in 12 (25%) patients, CR lasting >1 month (CR-B) occurred in 24 (50%) patients. 1-year OS was significantly improved in CR-28 (75% vs. 33% p=0.020) and CR-B (79% vs. 8% p<0.001) versus NonCR patients.

A six soluble biomarker-panel was predictive for mortality (HR 2.924; CI 1.485-5.758) when measured before MSC-administration. ST2 was only predictive for mortality 2 weeks after but not before MSC-administration (HR 2.389; CI 1.144-4.989). In addition, an increase in immature myeloid dendritic cells (mDCs) associated with decreased mortality (HR 0.554; CI 0.389-0.790). Patients had persisting T-cell responses against defined virus- and leukemia-associated antigens.

In conclusion, our data emphasize the need to carefully assess biomarkers in homogenous treatment cohorts. Biomarkers might become an additional valuable component of composite endpoints for the rapid and efficient testing of novel compounds in order to decrease life cycle of clinical testing and improve the success rate of phase II/III trials.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is until today the best curative treatment option for most patients suffering from high risk hematological malignancies as well as from many genetic disorders. Graft Versus Host Disease (GVHD) remains the major complication after allo-HSCT limiting the indications for a wide spread use of allo-HSCT substantially, as GVHD associates with a substantial mortality and morbidity. Many therapies have been tested in steroid-refractory acute GVHD with unfortunately limited success so far (1-4). The recent failure of a large randomized phase III clinical trial testing mycophenolate even in first line in combination with steroids for the resolution of GVHD emphasizes the need for novel drugs for the treatment of GVHD as well as a better selection of patients included into clinical trials (5).

Mesenchymal Stromal Cells (MSC) have been suggested as an interesting candidate for exploration in patients suffering from acute GVHD refractory to steroids (6-8). MSC are non-hematopoietic cells that reside in the bone marrow and possess multilineage potential (9-11). In 2008, Le Blanc *et al.* were the first to suggest the effectiveness of MSC as a second line therapy in steroid-refractory patients (8) after which several groups have repeated this setup (12-23). Consequently, a phase III trial has been performed testing MSC as compared to placebo. Although the full report is not yet available, preliminary reports suggest that MSC are only active in a subset of patients. While this allowed approval of MSC as second line therapy for children in Canada (24), the widespread use is regarded with caution. It is not well understood which patient categories might be preferentially susceptible to MSC therapy. This dilemma creates the need for a better clinical selection of patients who enter into clinical trials as well as a close biological monitoring linked to clinical trials in order to identify predictors of response in general and to defined therapies.

To date, biomarkers predicting occurrence, resolution or survival in the context of GVHD come from the analyses of very heterogeneous patient groups receiving multiple treatments. A combination of multiple biomarkers such as TNFR1, Reg3α, HGF, Elafin and IL2Rα have been found to predict day 28 posttherapy nonresponse and mortality at 6 months (25). ST2 was found to be predictive for therapy-resistant GVHD not only before initiation of therapy once GVHD occurs (26), but also already at day 28 after umbilical cord blood SCT (27). The latter data suggest that with currently available therapies the fate and therefore the survival chances of a patient are predetermined already very early after allo-HSCT. However, the indicated biomarkers have neither been tested specifically in the context of MSC therapy nor in a prospective cohort. Therefore we performed a phase I/II

trial that aimed to not only assess clinical efficacy of MSC but also allow a comprehensive analysis of known and novel soluble and cellular potential biomarkers in order to predict either resolution of GVHD or survival. In addition, we asked whether the rather general immune suppression by MSC possibly associates with a reduced immune response against defined viral and tumor-associated antigens.

METHODS

Study design

We conducted an open label non-randomized phase II study between January 2009 and July 2012. This clinical trial was registered at www.clinicaltrials.gov (#NCT00827398). Patients were eligible for participation when newly diagnosed with GVHD matching acute grade II-IV and showed progression after 3 days, or no improvement after at least 7 days of treatment with 2mg/kg/day prednisolone. All patients or their legal guardians provided written informed consent. Patients received MSC infusions on day 0 and 8 after enrollment. A third infusion was provided at day 22 when the patient achieved at least a partial response (PR). A fourth dose was infused if complete remission (CR) was not reached after 8 weeks. MSC were dosed to 1-2 x 10^6 cells/kg bodyweight. Previously defined stopping criteria included: progression of aGVHD after MSC infusion in >50% of patients, treatment related mortality within 24 hours of MSC infusion of more than 2 patients in the first 10 patients and occurrence of secondary malignancies.

During MSC therapy all patients received cyclosporine and 2mg/kg prednisolone. When a PR of acute GVHD was achieved, prednisolone was tapered by 15% of the total dose twice weekly.

MSC production

Production of MSC by the GMP-licensed Cell Therapy Facility of the UMC Utrecht is described in the Supplementary Methods.

Assessments

Comparing study outcomes between different studies is complicated due to the use of different outcome definitions. We therefore chose to report our clinical outcomes in two widely used definitions. We reported both complete resolution of GVHD symptoms at day 28 (CR-28) as well as best response (CR-B), complete resolution of all GVHD symptoms for at least 1 consecutive month (Table 1).

Blood samples were drawn at 0, 2, 4, 8 and 16 weeks. Absolute lymphocyte numbers were measured by TruCount (manufacturer's protocol, BD Biosciences).

PBMCs were isolated and cryopreserved until further analysis. Plasma was stored at -80°C. Plasma samples were analyzed for: interleukin (IL)-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17, IL-21, IL-22, IL-23, interferon-gamma (IFNγ), tumor necrosis factor-alpha (TNFα) and hepatocyte growth factor (HGF) using multiplex immunoassays (28). Elafin, interleukin 2 Receptor α (IL-2Rα), Suppression of Tumorigenicity 2 (ST2), TNF receptor I (TNFR1) and Regenerating Islet Derived Protein 3α (REG3α) were measured by ELISA (all from R&D except REG3α from USCN). Antibodies used for FACS analyses are reported in the Supplementary Methods.

To functionally analyze different T-cell subfractions, CD4+ and CD8+ T-cells were stimulated at different time points (0, 4 and 8 weeks after enrollment) with LPS (1μg/ml) and intracellular cytokine secretion was measured (IFNγ, IL-4, IL-10, IL-17) after 8 hours.

Ability to secrete IL-10 and TNFα by dendritic cells (DC) and monocytes was measured by using frozen PBMCs from 2 CR-B patients. Samples were obtained 8 weeks after infusion of MSC and compared to samples from 2 healthy donors as controls. PBMCs were thawed and enriched for HLA-DR+ cells by magnetic beads, according to the protocol from Miltenyi, followed by FACS sorting and stimulated o/n with LPS (1μg/ml). IL-10 and TNFα were measured using multiplex immunoassays.

Mixed lymphocyte reactions (MLR) were performed 2 months after first MSC infusion as described (28). T-cell responses against diverse antigens were tested by IFNγ ELISpot. PBMCs were cultured in culture medium in the presence of 1uM peptides and 100 u/ml IL-2 at a concentration of $5x10^5$c/ml (29). After 5 days, IFNγ ELISpot was performed. Peptides (Supplementary table 1) were purchased from ProImmune (Oxford, UK). Minor antigen mismatches were confirmed by PCR.

Statistics

Statistical analyses were performed using GraphPad Prism 5 for Windows (GraphPad Software), SPSS (IBM Statistics, version 20) and R (for Windows Ri386 3.1.0). Probability of survival was estimated by means of Kaplan-Meier curves and significance of differences with log-rank tests. Patients were censored at time of death or last follow-up. We used Cox proportional hazards models for evaluating time dependent associations of clinical and biological parameters with both response status and 1-year mortality (Supplementary table 2). Parameters with a probability level <20% were considered for multivariate Cox proportional hazards models. In all other cases a probability level of 5% ($p<0.05$) was found to be significant. Unpaired samples T-tests were used for analyzing cellular responses before and after MSC infusion. Risk of infection between CR-B and NonCR-B patients was analyzed by competing risk analysis with death as a competing event.

RESULTS

Patient characteristics, study design and clinical outcome

Between January 2009 and July 2012, 7 children and 43 adults with steroid-refractory grade II-IV GVHD were included. All patients, except 1 who was treated with etanercept, received MSC as first alternative rescue therapy after failing corticosteroids. Two adults who died due to progression of their underlying malignancy within three weeks after inclusion were excluded from further analysis. The first patient was diagnosed with a relapse of his AML on the same day he received his first MSC infusion. The second patient died due to progressive myeloma lesions in the skull causing cerebral compression whilst being treated with MSC for GVHD that occurred after DLI. We found no reason to suspect a causal relation between these progressive malignancies and MSC treatment.

Baseline characteristics of the remaining 48 patients are shown in Table 2. At inclusion, 25% of patients presented with overall GVHD grade II and 75% with GVHD grade III-IV. Main organs affected were gut (87.5%), skin (52.1%) and liver (35.4%) (Table 2). Median cell dose per infusion was 1.8 x 10^6/kg bodyweight (range 0.9-2.5), median number of infusions was 3 (range 1-4). Patients received on average MSC from 1.5 different donors. Median time from diagnosis of GVHD to study enrollment was 14 days (Table 2). As indicated in the protocol all patients were followed up 365 days, however median follow-up time was 150 days, due to high mortality rates in the first months after treatment in the non-responding patients.

Clinical results

Multiple response criteria have been described in multiple studies such as best response at day 28 or overall best response, indicating that no clear consensus has been reported so far (30, 31). Therefore, patients were classified as complete responders either with complete resolution of GVHD symptoms at day 28 (CR-28) as well as best response (CR-B), complete resolution of all GVHD symptoms for at least 1 consecutive month (Table 1).

149 infusions of MSC were performed. No adverse events occurred within 48 hours after infusion of MSC. During the follow-up period a total of 75 Serious Adverse Events (SAEs) were reported of which 36 (48%) were related to infections (Supplementary table 3). More SAEs as well as more infectious SAEs were reported in patients with both CR-28 as well as CR-B when compared to respectively NonCR-28 and NonCR-B. However patients in both CR-B as well as CR-28 groups had a longer follow-up time. Therefore we performed a competing risk analysis (32) of the risk of infection and death between CR-B and NonCR-B patients in

Term	Abbreviation	Definition
28-day Complete Resolution	CR-28	Resolution of GVHD in all involved organs (overall grade 0) at day 28 after first infusion of MSC
28-day Non Complete Resolution	NonCR-28	No complete resolution of GVHD (overall grade ≠ 0) in all involved organs at day 28 after first infusion of MSC
Best Response Complete Resolution	CR-B	Resolution of GVHD in all involved organs (overall grade 0) for at least one consecutive month
Best Response No Complete Resolution	NonCR-B	No complete resolution of GVHD (overall grade ≠ 0) lasting for at least one consecutive month

TABLE 1. Response definitions

the first 100 days. Risk of infection was not different between groups (p=0.54, Supplementary figure 2) whilst the risk of dying was significantly increased in the NonCR-B group (p=0.002).

At day 28, 25% of patients experienced a complete resolution of GVHD related symptoms (CR-28) whilst 50% of patients reached CR-B (Figure 1). Median time from first infusion of MSC to reach CR-B was 53.5 days (range 3-116). Analysis of

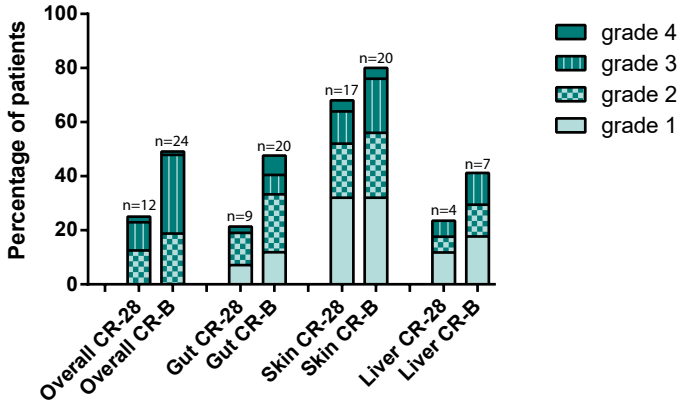

FIGURE 1: Percentage of patients reaching CR-28 or CR-B for overall GVHD and separately per organ system involved: respectively skin GVHD, gut GVHD and liver GVHD.
CR-28, Resolution of GVHD in all involved organs (overall grade 0) at day 28; CR-B, Resolution of GVHD in all involved organs (overall grade 0) for at least one consecutive month; GVHD, Graft versus Host Disease; n, number of patients. Grade represents the overall GVHD grade or grade per organ at enrollment of the study.

Patients - n (%)	48	(100)
Age - years (range)	44,9	(1,3-68,9)
Child - n (%)	7	(14,6)
Male - n (%)	31	(64,6)
Time from allo-HSCT to enrollment - median days (range)	85	(24-436)
Time from diagnosis aGvHD to enrollment - mean days (median)	14.5	(7-183)
Mean cells per infusion - n x 10^6/kg (range)	1,7	(0,9-2,5)
Primary disease - n (%)		
Myeloid neoplasms	27	(56,3)
Lymphoid neoplasms	16	(33,3)
Non malignant disorders	5	(10,4)
Stemcell source - n (%)		
PBSC	37	(77,1)
BM	4	(8,3)
CB	7	(14,6)
Type of donor - n (%)		
Sibling	10	(20,9)
MUD	38	(79,2)
Conditioning regimen - n (%)		
Myeloablative	17	(35,4)
Nonmyeloablative	31	(64,6)
Overall GVHD grade - n (%)		
grade 2	12	(25)
grade 3	33	(68,8)
grade 4	3	(6,3)
Skin GVHD - n (%)	25	(52,1)
Gut GVHD - n (%)	42	(87,5)
Liver GVHD - n (%)	17	(35,4)
Patients pretreated with 2nd line GVHD agents - n (%)	1	(2,1)

TABLE 2. Baseline characteristics of patients
N: number; PBSC: Peripheral Blood Stem Cells; BM: Bone Marrow; CB: Cord Blood; GVHD: Graft versus Host Disease; MUD: Matched Unrelated Donor

complete overall resolution of GVHD demonstrated that 12 patients who reached CR-B had not reached CR-28. This was largely due to slower resolution of gastro-intestinal symptoms when compared to relatively faster resolution of both skin and liver GVHD symptoms (Figure 1). No new immunosuppressive treatments were initiated in any of the participating patients before response evaluation at day 28 or during the 1 year follow-up.

Multiple clinical parameters including clinical grading of aGVHD were analyzed in a univariate Cox proportional hazards model (Supplementary table 2). However in multivariate analysis aGVHD severity at start of MSC infusion was not significantly associated with either CR-28 or CR-B.

1-year OS after MSC infusion was determined using Kaplan Meier curves. 1-year OS for the entire patient cohort was 44%, OS was significantly improved when comparing CR-28 (75%) vs. NonCR-28 (33% p=0.02) and CR-B (79%) vs. NonCR-B (8%, p<0.001) (Figure 2). Neither a defined donor batch of MSC nor mean infused cell dose of MSC was associated in a Cox-regression analysis with CR-28, CR-B, or OS.

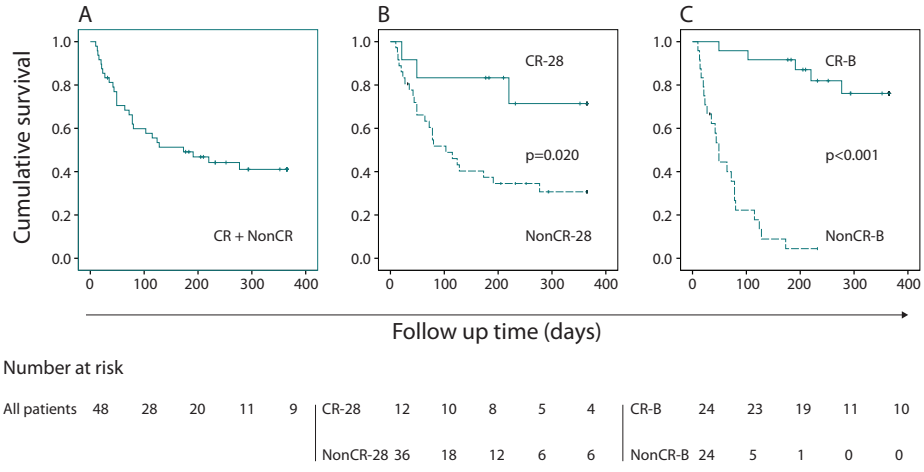

FIGURE 2. A: 1 year Kaplan Meier estimate of overall survival for the entire patient cohort. B: 1 year Kaplan Meier estimate of overall survival according to resolution of GVHD symptoms at day 28 (CR-28) after first MSC infusion.
Survival was superior in the CR-28 patients (75%) compared to NonCR-28 patients (33% , log-rank test p=0.020). C: 1 year Kaplan Meier estimate of overall survival according to best response complete resolution of GVHD symptoms (CR-B) after first MSC infusion. Survival was superior in the CR-B patients (79%) compared to NonCR-B patients (8% , log-rank test p<0.001).

Soluble biomarkers that associate with clinical resolution of GVHD symptoms

Several (combinations of) biomarkers have been associated with higher chances of clinical resolution of GVHD symptoms, resistance to corticosteroids and overall survival (OS) (25, 26, 33) . We therefore tested an extended panel of soluble factors, either previously reported to be involved in GVHD or representative markers of Th1, Th2 or Th17 responses (list of all (bio)markers tested in univariate analysis is available in Supplementary table 2, list of levels of (bio)markers tested available in Supplementary table 4). Although several factors were predictors

in univariate analysis, only the panel of biomarkers proposed by Levine *et al.* in 2012 (IL2Rα, TNFR1, HGF, IL-8, Elafin and Reg3α) (25) was predictive of 1-year OS when tested at time point 0 in both univariate (p=0.005) and multivariate Cox regression analysis (HR 2.924; CI 1.485-5.758) together with age (HR 1.032; CI 1.005-1.059) (Cox regression analysis performed forward stepwise, p=0.05, table 3). Surprisingly, significance was not achieved (either univariate or multivariate) when testing the biomarker panel of Levine for influence on CR-28 or CR-B, whilst the original publication shows strong significance for non-response at day 28 after initiation of GVHD therapy. This could be due to the strong fluctuation in clinical responses seen early after MSC infusion in combination with the relatively low number of only 12 patients, in comparison to the original patient cohorts in which these biomarkers were identified (25, 26, 33-35), reaching CR-28.

ST2 has been reported as strong predictive marker for nonresponse to GVHD therapy at day 28 when measured at initiation and 14 days after initiation of therapy (26). In our cohort, ST2 was not predictive for therapy resistance before infusion of MSC. However, two weeks after the first infusion of MSC, a high amount of soluble factor ST2 correlated with an increased risk of death (HR 2.389; CI 1.144-4.989, table 3), as reported (26), but again no other soluble factors. Significance was in the multivariate analysis not achieved when assessing influence of ST2 on resolution of GVHD (both CR-28 and CR-B), again most likely due to the fluctuating clinical responses in combination with our relatively low number of patients.

	Outcome 1-year mortality			
	Variable	**HR**	**CI**	**p-value**
Day 0	Age	1,032	1.005-1.059	0.02
	Levine biomarker formula	2,924	1.485-5.758	0.002
Day 14	Immature mDC1 * at day 14	0.554	0.389-0.790	0.001
	ST2 * at day 14	2,389	1.144-4.989	0.02

TABLE 3.
Outcome multivariate Cox proportional hazard models on the outcome parameter mortality 1 year after inclusion. All variables that in the univariate analyses (Supplementary Table 2) had a probability level <20% (p-value <0,2) were entered in the multivariate model (method Forward stepwise: PIN 0,05, POUT (0,10). Analyses at day 0 include only univariately tested variables that were known at time point 0. Analyses at day 14 include all univariately tested variables with p-values <0.2. HR: Hazard Ratio; CI: Confidence Interval; mDC1: immature myeloid Dendritic Cells; ST2: Suppression of Tumorigenicity 2

Cellular changes after infusion of MSC

MSC have been suggested to act via regulatory cellular (36-40) subsets. Therefore, a comprehensive panel of cellular subsets as indicated in "methods" was measured before and at predetermined time points after first MSC infusion. The most compelling observation was made for myeloid dendritic cells (mDCs) in the peripheral blood, specifically the immature population. Before MSC treatment, mDCs were decreased as reported (41) when compared to time matched No-GVHD controls (n=5) in both CR-B and NonCR-B patients (Figure 3). After infusion of MSC a swift increase in several DC subpopulations was observed, which was significant already after 2 weeks but also at later time points (Figure 3). We did not find significant differences in Treg numbers either between CR-B and NonCR-B patients or with time matched No-GVHD controls as was reported in a smaller cohort (42). Several groups have related the immature state of DC to the induction of a tolerogenic response of T-cells (43, 44). Therefore, the expression of CD80 and CD86 as indicators of the maturation level of these cells was assessed

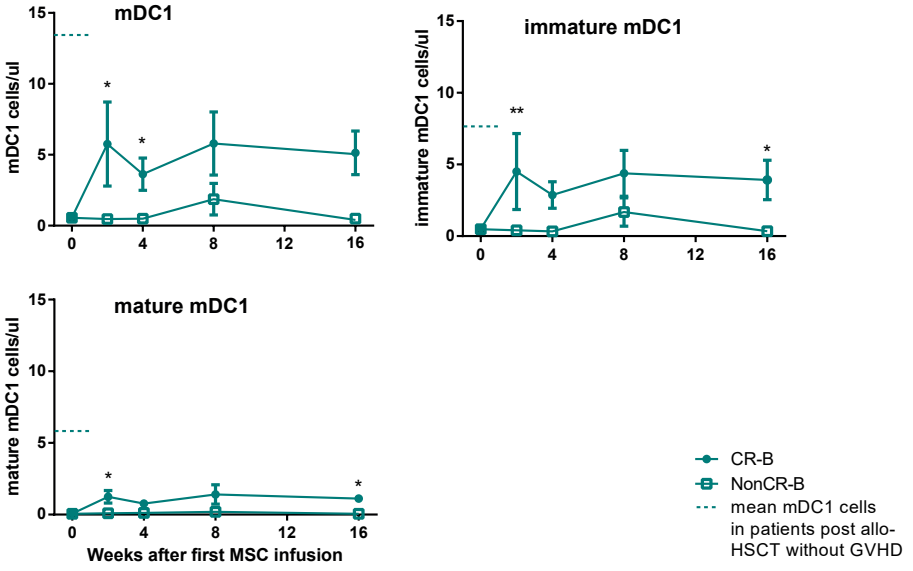

FIGURE 3. Different subsets of myeloid Dendritic Cells measured before first infusion of MSC (time point 0) and at 2, 4, 8 and 16 weeks after first infusion (respectively 44, 44, 36, 32 and 21 samples could be analyzed, the error bars represent the standard error of the mean). Differences between groups were calculated using unpaired samples T-tests. As a comparison we inserted the dotted line that represents the mean of measured samples from patients 6 weeks after allo-HSCT without GVHD as a control group.

* $p < 0.05$ ** $p < 0.01$ mDC1: myeloid Dendritic Cells 1; CR-B: Resolution of GVHD in all involved organs (overall grade 0) for at least one consecutive month; GVHD: Graft versus Host Disease; NonCR-B: No resolution of GVHD in all involved organs for at least one consecutive month.

on mDC1 (45). Both mature and immature mDC1 of CR-B patients were increased; however, the majority of the cells were immature (Figure 3) as reported also for mDC1 from healthy donors. Because total mDC1 numbers and measured subsets (mature and immature) are highly correlated, only the immature mDC1 subset was entered in the multivariate Cox regression model. In the multivariate Cox regression analysis an increase in immature mDC1 numbers was the only cellular factor among all others that correlated with a decreased risk of death (HR 0.554, CI 0.389-0.790, table 3).

Although the phenotype of mDC1 from successfully treated patients resembled the phenotype from healthy donors (supplementary figure 3), function might be altered in mDC1 from CR-B patients through direct contact with MSC (46). In order to assess pro-inflammatory or tolerogenic status of mDC1, mDC1 and monocytes, as control for an antigen presenting cell of different origin, from two CR-B patients and two healthy donors were sorted, stimulated overnight with LPS, and TNFα and IL-10 secretion measured by multiplex immunoassays. Selectively TNFα but not IL-10 was detected in mDC1 and monocytes from healthy donors and patients; and the ratio of TNFα secretion was calculated from healthy donors as compared to the patients in each cell fraction. While the capacity to produce TNFα in monocytes was equivalent in CR-B patients when compared to healthy donors as indicated by a ratio close to 1 (1.2-1.6), a marked decrease in the ability to produce TNFα in mDC1 was observed in CR-B patients as reflected by a higher ratio (3.1-115.8) (supplementary figure 4). These data suggest a reduced pro-inflammatory profile of mDC1 but not monocytes in CR-B patients.

MSC treatment does not impair general lymphocyte functionality or responses against viral and tumor-associated antigens

T-cells have been described to be functionally impaired after incubation with MSC (47, 48) and the increase in mDC1 in our cohort suggested that functional T-cells might get additionally tolerized after infusion of MSC via an alternative mechanism. Therefore, the overall proliferation rate of T-cells in patients after allo-HSCT was assessed before and after application of MSC in a Mixed Lymphocyte Reaction (MLR). However, no differences were observed between all groups (CR-B vs. NonCR-B vs. No-GVHD controls, data not shown) suggesting that the general proliferation capacity is not impaired in our cohort.

Next we assessed intracellular cytokines profiles (IL-4, IL-10, IL-17 and IFNγ) in patients with abundant material (n=20) in CD4+ and CD8+ T-cell subsets before and 4 and 8 weeks after infusion of MSC after stimulation with LPS. However, no general skewing in Th1, 2 or Th17 responses was observed in CR-B when compared to NonCR-B patients.

In order to assess whether reactivity of T-cells against individual minor-histocompatibility antigens, viral antigens, or tumor-associated antigens is selectively impaired after infusion of MSC, lymphocytes before and 2 months after MSC infusion, time points at which no significant differences in levels of immune suppression could be observed, were cultured with selected antigens potentially presented within the context of the individual HLA-machinery of each patient. One week later, the production of IFNγ was measured by means of Elispot. Due to HLA and minor histocompatibility antigen restrictions not all patient samples could be tested.

No decrease in the number of reactive cytotoxic T-cells was detected in the CR-B or NonCR-B patients in regard to the reactivity to viral antigens. 7 of 12 patients tested had CMV reactivations at the time of sampling, a significant enhancement of the reactivity to CMV was observed in CR-B patients after MSC treatment reflecting adequate CMV responsiveness despite severe immune suppression (Figure 4). Similarly, activation capacity upon stimulation with different tumor antigens (i.e. hTERT, Wilms Tumor-1, PRAME, proteinase 3 and MUC1) was not impaired in the two groups (Figure 4). No T-cell responses against minor-histocompatibility antigens were detected at any time point in GVHD patients (data not shown, list of all peptides available in Supplementary table 1). In conclusion, our data suggest that achievement of CR-B following MSC administration can improve the response to CMV whilst anti-leukemia reactivity is not impaired over time.

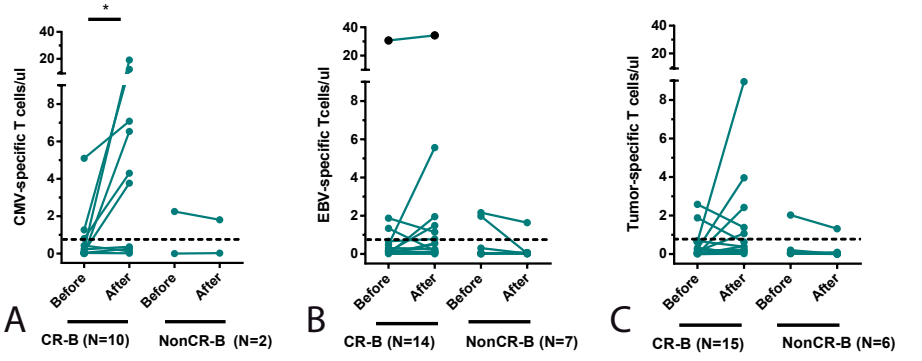

FIGURE 4. T-cell responses against diverse antigens were tested by IFNγ ELISpot. Lymphocytes from before MSC infusion and from time point 2 months after MSC infusion were cultured with selected antigens.

A: A significant increase of CMV reactive T-cells was observed in CR-B patients at time point 2 months after first MSC infusion. B: No differences were detected in the number of EBV reactive T-cells either in CR-B or NonCR-B patients. C: No differences were detected in tumor specific T-cells either in CR-B or NonCR-B patients. Tumor antigens used were hTERT, Wilms Tumor-1, PRAME, proteinase 3 and MUC1 (complete list of all peptides available in Supplementary table 1).

CMV: Cytomegalovirus; EBV: Epstein Barr Virus; CR-B: Resolution of GVHD in all involved organs (overall grade 0) for at least one consecutive month; NonCR-B: No resolution of GVHD in all involved organs for at least one consecutive month; N=number of patient samples.

DISCUSSION

Steroid-refractory aGVHD is still the main contributor to allo-HSCT related mortality and currently there is no standard second line treatment for these patients. We tested the clinical efficacy of platelet lysate cultured MSC and found this treatment to be effective in 50% of patients. Reaching either a complete resolution of GVHD symptoms at day 28 or a complete resolution of symptoms as best response is strongly correlated with an improved 1-year OS compared to NonCR patients. The response rate of 50% is comparable to response rates reached by others with either FCS cultured MSC (8) or platelet lysate cultured MSC (12, 14). Also the 6-month overall survival of the entire patient cohort of 50% is equal to overall survival rates of 50% described in the review of Martin *et al.* in 2012 (31), questioning whether MSC will hold true as promising novel agent for the treatment of GVHD.

A major focus of our comprehensive study was to analyze how to discriminate early between patients benefiting from this costly therapy and patients that might be better off with a different treatment regimen. Multiple biomarkers have been described in clinical cohorts comprised of heterogeneously treated GVHD patients (33, 35, 49). Although a panel of 6 biomarkers as described by Levine *et al.* (25) has been the strongest predictor for OS in our cohort, that is very homogeneous for GVHD treatments prior to enrollment, the Levine-formula surprisingly did not predict resolution of GVHD most likely due to the fluctuating course of the disease. In addition, 2 weeks after initiation of treatment, the Levine-formula was no longer the strongest predictive marker for OS. In line with this alternating success of the Levine-formula, high levels of ST2, a recently described potent marker for therapy-resistant GVHD and death without relapse when measured before and after initiation of treatment (26), was not predictive for OS prior to infusion of MSC. Differences in conditioning regimens (myeloablative vs. nonmyeloablative) between previous validation cohorts for ST2 (26) and our patients might partially have accounted for this observation. However, in our cohort ST2 turned out to be highly predictive 14 days after treatment. Both ST2 and the Levine biomarker panel were not correlated with clinical severity of aGVHD. These data suggest, that described predictive biomarkers of response might need further careful evaluations in rather homogeneous clinical cohorts with defined interventions in prospective studies. Alternatively, in the recent context describing ST2 already at day 28 after umbilical cord blood allo-HSCT as biomarker for the occurrence of aGVHD and TRM (27) it is tempting to speculate that MSC can break the negative predictive value of ST2 and thus rescue more patients when compared to conventional therapies.

A reduction in numbers of DCs has been reported to be associated with GVHD in humans (41). In line with this observation, we report that DCs are not only decreased in numbers during GVHD, but also increase substantially in patients with complete resolution of GVHD. Most strikingly, the increase in immature DC is, in a multivariate Cox regression analysis, an independent predictor of resolution of GVHD after 2 weeks, providing a potential new understanding of MSC immunomodulatory working mechanisms.

Due to limited patient material, functional analyses of mDC1 was restricted to selected patients. However, our data suggest indeed a reduced pro-inflammatory status of mDC1 in line with the observation that MSC can dampen in vitro secretion of pro-inflammatory cytokines like TNFα in antigen presenting cells (46). Strikingly, immature dendritic cells could be observed in gut biopsies of selected patients (J.K. unpublished observation), suggesting that such cells are indeed present in the local microenvironment. Due to the missing control of steroid responding patients as a nature of a phase II trial it is not possible to claim that the observed changes are a consequence of MSC therapy or rather a general phenomenon of resolution of GVHD. However, multiple reports in mice indicate that MSC can push DC directly towards a more tolerogenic state (50, 51). Whether the increase in immature mDC1 is truly a marker that can be used in the clinic needs to be validated in an independent cohort.

A major concern of all therapies for GVHD has been a possible unspecific dampening of the general immune response and therefore inducing a possible risk for relapse of malignancy and/or viral reactivations. As our phase II trial did not have a control group we could not compare the risk of severe infections with or without MSC treatment. We calculated the risk of an infectious SAE per patient per day at risk and this was 0,00009 per patient. Competing risk analysis showed no difference in risk of infection in the CR-B or NonCR-B patients. In a cohort of 11 patients from Lucchini *et al.* (52) MSC treated patients did not have more or more severe viral reactivations upon MSC infusion. We extended these findings by demonstrating in a prospective cohort of 48 patients that MSC treatment maintains tumor reactivity of immune cells and retains adequate CMV responsiveness.

In conclusion, infusion of MSC associates with a long term OS in 50% of patients suffering from GVHD grade II-IV, a group with otherwise high mortality, and does not impair immune responses to defined viral or tumor-associated antigens. Immature dendritic cells might play a key role in recovery from GVHD by MSC. ST2 is no longer predictive biomarker for a fatal outcome before initiation of therapy and might give rise to the hope, that indeed MSC will hold promise to their potential within the correctly selected patient group. Our data advocate therefore for

a careful re-evaluation of described biomarkers in more homogenous treatment cohorts, which might allow a better selection of compounds for larger clinical trials. Biomarker guided evaluation of defined compounds might therefore be an attractive alternative or addition to composite endpoints in clinical trials evaluating novel drugs for the treatment of GVHD. A prospective clinical trial is currently recruiting (HOVON 112, www.hovon.nl, EudraCTnr 2011-003237-33) in order to assess efficacy of MSC in patients suffering from severe GVHD.

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SUPPLEMENTARY INFORMATION

METHODS

MSC production

Expanded MSC from bone marrow are classified as Advanced Therapy Medicinal Products and manufactured in the GMP-licensed Cell Therapy Facility of the UMC Utrecht. The bone marrow aspirates were obtained from third party non-HLA matched healthy donors as approved by the Dutch Central Committee on Research Involving Human Subjects (CCMO, Biobanking bone marrow for MSC expansion, NL41015.041.12). Either the bone marrow donor or the parent or legal guardian of the donor signed the informed consent approved by the CCMO. Bone marrow was separated using a density gradient centrifugation (Lymphoprep, Axis Shield, Oslo, Norway). MSC are isolated by plastic adherence and expanded using the MC3 systems and α-MEM (Minimal Essential medium) with L-glutamine from Macopharma (Tourcoing, France). In short, 100-300 x 10^6 mononuclear cells in culture medium (α-MEM, 5% platelet lysate and 3.3 IU/ml Heparin) are seeded in a 2-layer CellStack (2-CS) using the seeding set. After 7 days, a medium exchange was performed using the exchange sets, depleting all nonadherent cells. When 80 – 100% confluency was reached (± 10 days), the cells are harvested using trypsin (TrypLE™ Select Enzyme™, Life technologies Corp. Grand Island, NY, USA). The Passage 1 (P1) cells were seeded in CellStacks (2-5 x 10^6MSC/2-CS), medium was exchanged and the cells were passaged after ± 6 days (P2). This procedure is repeated to obtain P3 cells for infusion. The mean number of MSC harvested at P3 was 59 x 10^6 MSC/2-CS. P3 cells were cryopreserved in MACO Biotech freezing EVA bags (Macopharma, Tourcoing,France) in 20 ml 0.9% Sodium Chloride (Fresenius Kabi, Bad Homburg, Germany); 10% CryoSure-DMSO (WAK-Chemie Medical GmbH, Steinbach, Germany); 5% Human Serum Albumin(Cealb, Sanquin, Amsterdam, the Netherlands). P3 cells were cryopreserved using a computer-controlled rate freezer (IceCube 1810, Sy-Lab GmbH, Neupurkersdorf, Austria) in clinical cell dosages ranging from 20 – 200 x10^6 MSC/bag and stored in the vapor phase of liquid nitrogen (< -150 °C)(1). The MSC batches were released if they fulfilled the following release criteria: immunophenotype of the MSC: >70% $CD73^+$ cells , > 70% $CD105^+$ cells, and >70% $CD90^+$ cells, and <10% $CD45^+$ cells and <1% $CD3^+$ cells; sterility tests according to the European Pharmacopeia: negative for aerobic and anaerobe bacteria, fungi and yeast; mycoplasma < 10 CFU/ml and endotoxin < 1 IU/ml (<5 IU/kg/hr). All MSC batches fulfilled the criteria as described by Dominici *et al.* (1) and all MSC were >95 % positive for CD 73/CD 90/CD 105. The MSC bags

were thawed in a water bath at 37 °C, kept on ice while a sample was taken for cell counting and infused intravenously within 1 hour at the clinical wards. The ability of thawed GMP-grade MSC to suppress T-cell proliferation was assessed during the implementation of the MSC production from 5 different donors. Different MSC:Peripheral Blood Mononuclear Cells (PBMC) ratios were assessed as shown in supplementary figure 1. T-cell proliferation was decreased to 38% (mean of all 5 assessed MSC donors when added in ratio MSC: PBMC of 1:1). Cell viability of thawed MSC was assessed for each product throughout the whole study and the median viability was 95% (mean 93,5%, standard deviation 7,6%).

Assessments

FACS stainings were performed with: CD3-PerCP, CD4-PerCP, γδ-TCR-APC, CD80-APC-H7, CD86-PE-Cy7 (BD Pharmingen), CD4-PE, CD8-PerCP, CD19-PerCP, CD14-PerCP, CD141-PE (BDCA3) (BioLegend), CD3-eFluor-450, CD4-PE-Cy7, CD4-Alexa Fluor-780, CD8-APC, CD25-FITC, CD127-PE-Cy7, HLA-DR-FITC, FoxP3-APC, CD20-PacBlue (eBioscience), CD1c-APC (BDCA1), CD303-FITC (Miltenyi). Samples were analyzed with an LSR-II flow cytometer (BD Biosciences) and acquired data were analyzed using FACS Diva software (BD Biosciences).

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	peptide	restriction	sequence
Viral antigens	CMVpp65 (363-373)	A1	YSEHPTFTSQY
	CMVpp65 (495-503)	A2	NLVPMVATV
	CMVpp150 (946-955)	A3	TVYPPSSTAK
	CMVpp65(341-349)	A24	QVDPVAALF
	EBV (259-267)	A2	GLCTLVAML
	EBV BRLF-1 lytic (148-156)	A3	RVRAYTYSK
	EBV EBNA-3A (325-333)	B8	FLRGRAYGL
Tumor antigens	hTERT (540-548)	A2	ILAKFLHWL
	hTERT (973-981)	A3	KLFGVLRLK
	hTERT (461-469)	A24	VYGFVRACL
	WT1 (317-327)	A1	TSEKRPFMCAY
	WT1 (126-134)	A2	RMFPNAPYL
	WT1 (235-244)	A2 & A24	CMTWNQMNL
	PRAME (300-309)	A2 & A24	LYVDSLFFL
	prot3 (169-177)	A2	VLQELNVN
	Muc1 (79-87)	A2	TLAPATEPA
	p53 (264-272)	A2	LLGRNSFEV
Minor antigens	HY	A1	IVDCLTEMY
	HY	A2	FIDSYICQV
	SMCY (950-960) - HY	B7	SPSVDKARAEL
	LRH1	B7	TPNQRQNVC
	UTA2.1	A2	QLLNSVLTL
	SP110	A3	SLPGGTSTPK
	ACC2	B44	KEFEDGIINW
	HA8	A2	PTLDKVLEV
	HA3	A1	VMEPGTAQY
	LBADIR	A2	SVAPALALSPA

SUPPLEMENTARY TABLE 1. List of peptides used for T-cell reactivity analyses. Peptides were purchased from ProImmune (Oxford, UK). Analyses were only performed on samples from patients with appropriate HLA types.

	Outcome CR-28			Outcome CR-B			Outcome 1-year mortality		
	HR	CI	p-value	HR	CI	p-value	HR	CI	p-value
Clinical markers									
Adult vs. children	1.206	0,264-5,507	0.809	0.984	0,364-2,660	0.974	**0.322**	**0,076-1,363**	**0.124**
Age	0.993	0,966-1,021	0.618	1.002	0,982-1,023	0.830	**1.025**	**1,002-1,049**	**0.033**
CMV mismatch	1.205	0,323-4,496	0.781	0.827	0,329-2,078	0.687	0.922	0,417-2,043	0.842
Days from allo-HSCT to enrollment	**1.004**	**1,000-1,009**	**0.078**	1.002	0,998-1,006	0.380	1.000	0,997-1,004	0.851
Days from diagnosis GVHD to enrollment	1.009	0,994-1,024	0.260	**1.011**	**0,998-1,024**	**0.109**	**1.009**	**1,000-1,018**	**0.055**
Donor type (sibling or MUD)	1.571	0,343-7,188	0.560	1.022	0,399-2,619	0.964	1.500	0,567-3,969	0.414
Gut GVHD	0.793	0,174-3,623	0.765	1.184	0,399-3,509	0.761	2.510	0,593-10,625	0.211
Liver GVHD	**0.141**	**0,018-1,095**	**0.061**	**0.430**	**0,160-1,158**	**0.095**	1.496	0,693-3,229	0.305
Mean number of MSC infused	0.776	0,234-2,576	0.678	1.175	0,464-2,972	0.734	**0.470**	**0,211-1,049**	**0.065**
Overall GVHD grade									
grade 2 vs. grade 3	**0.249**	**0,076-0,816**	**0.022**	**0.441**	**0,191-1,022**	**0.056**	1.372	0,550-3,419	0.498
grade 2 vs. grade 4	0.557	0,067-4,636	0.588	0.358	0,045-2,858	0.332	0.758	0,091-6,302	0.797
grade 2 vs. grade 3 and 4	**0.274**	**0,088-0,851**	**0.025**	**0.435**	**0,190-0,996**	**0.049**	1.321	0,533-3,276	0.548
Primary disease									
myeloid neoplasms vs. lymphoid neoplams	0.517	0,107-2,492	0.411	0.573	0,188-1,742	0.326	**3.185**	**1,441-7,042**	**0.004**
myeloid neoplasms vs. non-malignant disorders	**3.330**	**0,853-12,996**	**0.083**	1.657	0,550-4,989	0.369	0.359	0,047-2,768	0.326
Skin GVHD	**0.264**	**0,071-0,976**	**0.046**	0.648	0,287-1,467	0.298	0.950	0,446-2,025	0.894
Stemcell source									
PBSC vs. BM	0.000	0	0.989	0.822	0,187-3,620	0.796	0.708	0,166-3,014	0.640
PBSC vs. CB	1.058	0,232-4,829	0.942	1.557	0,606-3,999	0.357	0.494	0,147-1,654	0.253
Total body irradiation	0.597	0,193-1,853	0.372	0.592	0,264-1,330	0.204	**1.772**	**0,774-4,053**	**0.176**

SUPPLEMENTARY TABLE 2 Univariate Cox proportional hazards models (*continued*)

		Outcome CR-28			Outcome CR-B			Outcome 1-year mortality		
		HR	CI	p-value	HR	CI	p-value	HR	CI	p-value
Soluble markers										
Levine biomarker formula	day 0	1.177	0,553-2,506	0.673	**0.592**	**0,315-1,114**	**0.104**	**2.693**	**1,344-5,394**	**0.005**
	day 14	0.870	0,555-1,363	0.542	1.046	0,733-1,491	0.805	**1.407**	**0,981-2,017**	**0.063**
Elafin *	day 0	**1.676**	**0,918-3,060**	**0.093**	0.915	0,637-1,313	0.630	1.095	0,767-1,564	0.616
	day 14	1.217	0,624-2,373	0.564	0.913	0,523-1,594	0.749	**1.783**	**1,064-2,986**	**0.028**
HGF *	day 0	0.837	0,487-1,439	0.521	0.766	0,509-1,155	0.203	0.996	0,706-1,405	0.980
	day 14	**0.611**	**0,297-1,257**	**0.181**	0.716	0,425-1,208	0.211	**2.423**	**1,359-4,320**	**0.003**
IL2Rα *	day 0	1.099	0,453-2,665	0.835	**0.553**	**0,272-1,126**	**0.102**	1.002	0,544-1,844	0.995
	day 14	**0.600**	**0,287-1,254**	**0.174**	**0.504**	**0,280-0,910**	**0.023**	1.338	0,768-2,330	0.304
IL-8 *	day 0	0.899	0,573-1,408	0.641	0.876	0,630-1,219	0.433	**1.446**	**1,020-2,049**	**0.038**
	day 14	**0.729**	**0,543-0,978**	**0.035**	0.935	0,742-1,179	0.572	**1.262**	**0,947-1,683**	**0.113**
IL-22 *	day 0	0.915	0,621-1,349	0.654	0.900	0,693-1,168	0.429	0.974	0,763-1,245	0.835
	day 14	0.714	0,415-1,229	0.225	**0.748**	**0,506-1,106**	**0.146**	1.180	0,826-1,686	0.363
Reg3α *	day 0	0.766	0,460-1,277	0.307	**0.652**	**0,435-0,976**	**0.038**	**1.858**	**0,972-3,553**	**0.061**
	day 14	0.951	0,581-1,555	0.840	**0.781**	**0,580-1,052**	**0.104**	**1.371**	**0,869-2,165**	**0.175**
ST2 *	day 0	0.863	0,380-1,959	0.725	0.750	0,384-1,464	0.399	1.335	0,721-2,472	0.359
	day 14	0.680	0,337-1,372	0.281	0.760	0,464-1,245	0.276	**1.594**	**0,941-2,697**	**0.083**
TNFR1 *	day 0	0.782	0,360-1,697	0.533	**0.608**	**0,317-1,165**	**0.133**	**4.028**	**1,786-9,085**	**0.001**
	day 14	0.616	0,270-1,402	0.248	**0.678**	**0,382-1,205**	**0.186**	**2.862**	**1,416-5,786**	**0.003**

SUPPLEMENTARY TABLE 2 Univariate Cox proportional hazards models (*continued*)

		Outcome CR-28			Outcome CR-B			Outcome 1-year mortality		
		HR	CI	p-value	HR	CI	p-value	HR	CI	p-value
Cellular markers										
CD4+ T-cell counts *	day 0	0.954	0,666-1,365	0.796	**0.807**	**0,609-1,068**	**0.133**	**0.823**	**0,626-1,080**	**0.160**
	day 14	1.177	0,789-1,754	0.424	**1.301**	**0,949-1,782**	**0.102**	**0.553**	**0,393-0,780**	**0.001**
CD8+ T-cell counts *	day 0	1.147	0,760-1,732	0.514	**0.770**	**0,559-1,059**	**0.107**	0.922	0,699-1,216	0.567
	day 14	**1.738**	**1,050-2,878**	**0.032**	1.195	0,838-1,704	0.326	**0.764**	**0,567-1,030**	**0.077**
immature mDC1 cell counts *	day 0	1.058	0,704-1,590	0.787	0.872	0,657-1,157	0.341	**0.827**	**0,634-1,080**	**0.163**
	day 14	1.068	0,764-1,495	0.699	**1.233**	**0,977-1,557**	**0.078**	**0.623**	**0,479-0,810**	**<0,001**
mature mDC1 cell counts *	day 0	1.300	0,776-2,177	0.319	0.983	0,643-1,502	0.937	**0.711**	**0,495-1,020**	**0.064**
	day 14	1.182	0,853-1,637	0.315	**1.270**	**1,009-1,599**	**0.042**	**0.621**	**0,473-0,815**	**0.001**
mDC1 cell counts *	day 0	1.064	0,708-1,597	0.766	0.869	0,656-1,150	0.325	**0.843**	**0,650-1,094**	**0.199**
	day 14	1.110	0,801-1,538	0.531	**1.215**	**0,971-1,520**	**0.088**	**0.647**	**0,501-0,836**	**0.001**
mDC2 cell counts *	day 0	1.164	0,790-1,716	0.442	0.940	0,697-1,267	0.685	0.914	0,665-1,256	0.578
	day 14	**1.494**	**0,914-2,441**	**0.109**	**1.373**	**0,938-2,009**	**0.102**	**0.711**	**0,506-0,997**	**0.048**
pDC cell counts*	day 0	0.826	0,474-1,440	0.500	0.775	0,513-1,172	0.227	0.973	0,704-1,343	0.866
	day 14	0.943	0,591-1,505	0.806	**1.394**	**0,927-2,097**	**0.110**	**0.618**	**0,450-0,848**	**0.003**
Regulatory T-cells *	day 0	0.987	0,710-1,373	0.939	0.930	0,710-1,218	0.596	**0.771**	**0,606-0,981**	**0.034**
	day 14	0.993	0,723-1,365	0.966	1.025	0,788-1,334	0.855	**0.739**	**0,576-0,948**	**0.017**
Total B-cell counts *	day 0	0.928	0,711-1,211	0.581	0.898	0,724-1,114	0.328	**0.772**	**0,628-0,949**	**0.014**
	day 14	**1.358**	**1,017-1,812**	**0.038**	**1.137**	**0,963-1,343**	**0.131**	**0.832**	**0,697-0,994**	**0.042**
Total T-cell counts *	day 0	1.030	0,660-1,607	0.896	**0.672**	**0,468-0,965**	**0.031**	0.854	0,623-1,171	0.327
	day 14	**1.525**	**0,933-2,493**	**0.093**	**1.374**	**0,937-2,014**	**0.104**	**0.648**	**0,460-0,914**	**0.013**

SUPPLEMENTARY TABLE 2. Univariate Cox proportional hazards models from all variables. Soluble and cellular biomarkers were measured at day 0 and day 14 after first MSC infusion. In bold are shown all p-values < 0.2.
All variables with p-values <0.2 were put into multivariate analyses.
* logarithmic transformations of cytokine levels and cell counts were tested and depicted here.
CMV: Cytomegalovirus, Allo-HSCT: Allogeneic Hematopoietic Stem Cell Transplantation, GVHD: Graft versus Host Disease, MUD: Matched Unrelated Donor, MSC: Mesenchymal Stromal Cells, PBSC: Peripheral Blood Stem Cells, BM: Bone Marrow, CB: Cord Blood, HGF: Hepatocyte Growth Factor, IL2Rα: Interleukin 2 Receptor α, IL-8: Interleukin 8, IL-22: Interleukin 22, Reg3α: Regenerating islet-derived 3α , ST2: Suppression of Tumorigenicity 2 , TNFR1: Tumor Necrosis Factor Receptor 1, mDC1: myeloid Dendritic Cells 1, pDC: plasmacytoid Dendritic Cells.

	Total n (%)	CR-B n (%)	NonCR-B n (%)
SAE (%)	75 (100)	46 (61.3)	29 (30.7)
Individual patients	38	17	21
Mean CTC grade (range) *	3.59 (1-5)	3.11 (1-5)	4,36 (2-5)
Infections	36	22	14
Viral[1]	12	9	3
Bacterial[2]	10	9	1
Fungal[3]	7	1	6
viral and fungal[1,3]	1	1	0
bacterial and fungal[2,3]	3	1	2
unknown	3	1	2
Hemorrhage	6	4	2
gastro-intestinal	5	3	2
renal	1	1	0
Other severe SAEs			
cerebral infarction	2	0	2
cholecystitis	1	1	0
pulmonary embolism	1	1	0

SUPPLEMENTARY TABLE 3. Overview of Serious Adverse Events

* Common Terminology Criteria for Adverse Events (CTCAE) Version 4.0 was used.

[1] 5 infections due to CMV, 3 to norovirus, 2 to rhinovirus, 1 to EBV, 1 to BK-virus and 1 to rotavirus.

[2] 2 infections due to Pseudomonas Aeruginosa, 2 to Coagulase negative Staphylococci, 1 to E. Coli, 1 to H. Influenzae, 1 to an ESBL, 1 to Stenotrophomonas Maltophilia, 1 to Pneumocystis Jirovecii and 4 infections with unspecified culture results.

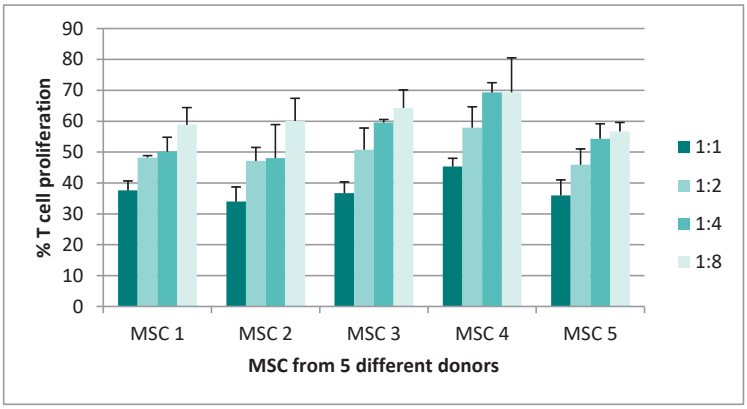
[3] all 11 fungal infections were due to Aspergillus

Soluble markers		CR-B mean		NonCR-B mean		p-value (t-test)
Levine biomarker formula	day 0	9.41		10.05		0.029
	day 14	0.45		1.62		0.025
Elafin	day 0	14.99	ng/ml	18.15	ng/ml	0.489
	day 14	5.87	ng/ml	13.56	ng/ml	0.072
HGF	day 0	247.56	pg/ml	171.15	pg/ml	0.515
	day 14	57.24	pg/ml	141.46	pg/ml	0.005
IL2Rα	day 0	1256.73	pg/ml	1482.16	pg/ml	0.407
	day 14	983.10	pg/ml	124.71	pg/ml	0.327
IL-8	day 0	448.08	pg/ml	1697.59	pg/ml	0.002
	day 14	420.67	pg/ml	1840.44	pg/ml	0.018
IL-22	day 0	29.36	pg/ml	33.25	pg/ml	0.854
	day 14	3.97	pg/ml	16.92	pg/ml	0.14
Reg3α	day 0	108.98	ng/ml	134.78	ng/ml	0.217
	day 14	114.10	ng/ml	166.40	ng/ml	0.07
ST2	day 0	287.58	ng/ml	362.56	ng/ml	0.256
	day 14	337.64	ng/ml	463.06	ng/ml	0.186
TNFR1	day 0	1525.31	pg/ml	2693.65	pg/ml	0.028
	day 14	1483.52	pg/ml	3265.02	pg/ml	0.007

Cellular markers		CR-B mean		NonCR-B mean		p-value (t-test)
CD4+ T-cell counts	day 0	69.10	cells/µl	68.10	cells/µl	0.97
	day 14	141.48	cells/µl	35.38	cells/µl	0.011
CD8+ T-cell counts	day 0	88.78	cells/µl	79.74	cells/µl	0.786
	day 14	180.88	cells/µl	63.85	cells/µl	0.157
immature mDC1 cell counts	day 0	0.51	cells/µl	0.50	cells/µl	0.99
	day 14	4.74	cells/µl	0.38	cells/µl	0.132
mature mDC1 cell counts	day 0	0.07	cells/µl	0.05	cells/µl	0.695
	day 14	1.30	cells/µl	0.08	cells/µl	0.013
mDC1 cell counts	day 0	0.58	cells/µl	0.55	cells/µl	0.954
	day 14	5.76	cells/µl	0.47	cells/µl	0.097
mDC2 cell counts	day 0	0.42	cells/µl	0.88	cells/µl	0.407
	day 14	0.89	cells/µl	0.88	cells/µl	0.983
pDC cell counts	day 0	0.55	cells/µl	0.68	cells/µl	0.647
	day 14	2.17	cells/µl	0.82	cells/µl	0.043
Regulatory T-cells	day 0	3.59	cells/µl	1.31	cells/µl	0.169
	day 14	6.21	cells/µl	1.58	cells/µl	0.275
Total B-cell counts	day 0	74.26	cells/µl	23.54	cells/µl	0.176
	day 14	97.96	cells/µl	30.00	cells/µl	0.075
Total T-cell counts	day 0	172.80	cells/µl	177.86	cells/µl	0.931
	day 14	351.42	cells/µl	122.36	cells/µl	0.05

SUPPLEMENTARY TABLE 4 Levels of measured soluble and cellular markers

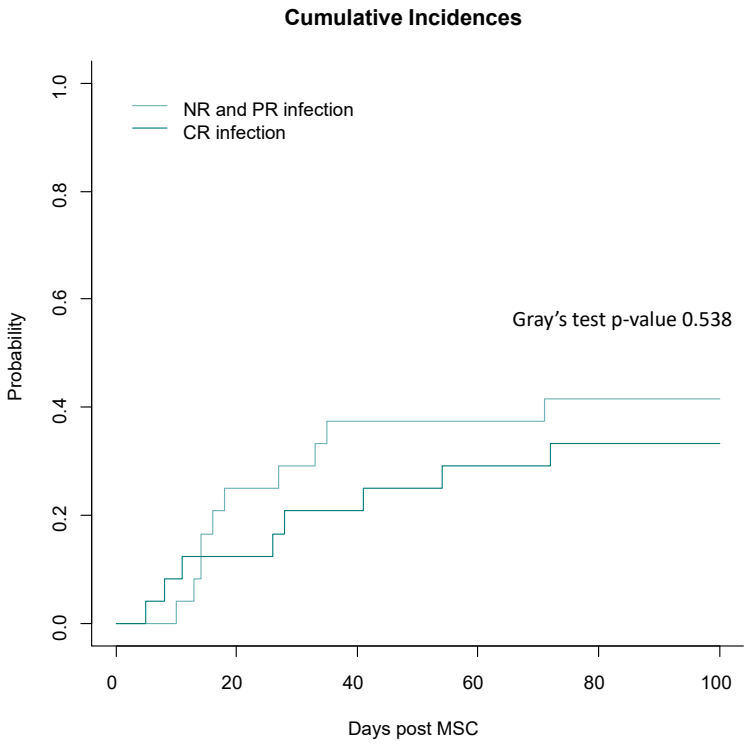
HGF: Hepatocyte Growth Factor, IL2Rα: Interleukin 2 receptor alpha, Reg3α: regenerating islet-derived protein 3 alpha, ST2: suppression of tumorigenicity 2, TNFR1: Tumor necrosis factor receptor 1, mDC: myeloid dendritic cells, pDC: plasmacytoid dendritic cells.



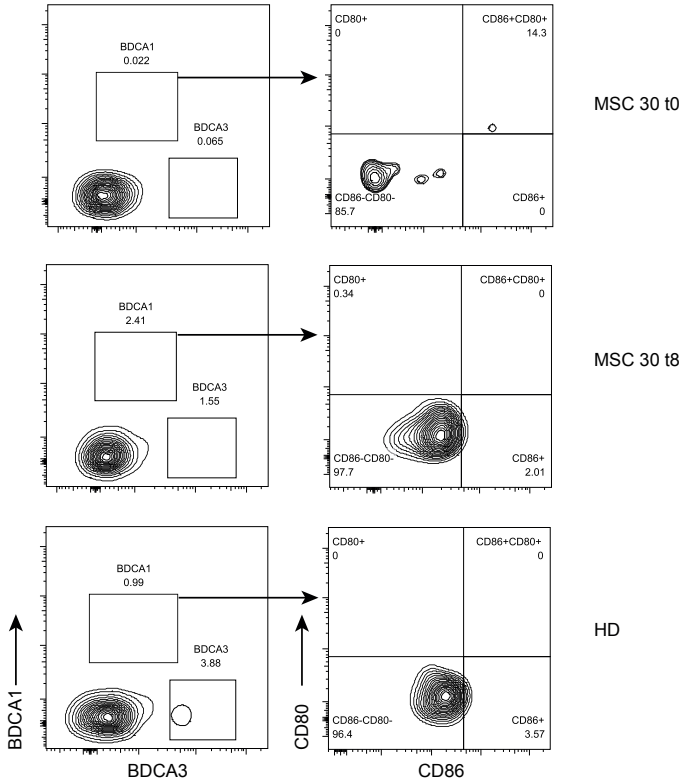
SUPPLEMENTARY FIGURE 1 T-cell proliferation

Legend: The immunosuppression profile is shown of MSC products expanded from 5 different donors. The T-cell proliferation of CD3 stimulated peripheral blood mononuclear cells (PBMC) was set at 100%. MSC were added in different MSC: PBMC ratio's varying from 1:1 to 1:8. T-cell proliferation was decreased to 38% (mean of all 5 assessed MSC donors when added in ratio MSC: PBMC of 1:1).

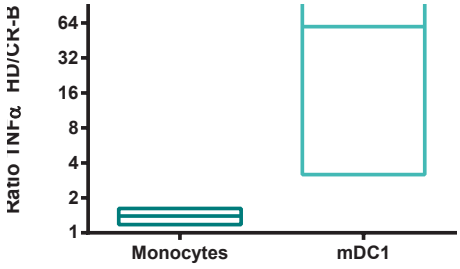
Materials and Methods: Proliferation was measured using the CellTrace™ Cell Proliferation Kit from Invitrogen (C34554) using CFSE labeling in combination with FACS analysis. Cryopreserved peripheral blood mononuclear cells (PBMC) and MSC were thawed, washed and resuspended in RPMI / 10% human serum. Viable PBMC were counted using trypan blue and diluted in warm PBS. CFSE reagent (end concentration 2µM) was added to the PBMC and incubated for 7 minutes at 37 ºC. After the addition of FCS, cells were washed in PBS/0.1% BSA. Round bottom 96 well plates were coated with 0.075 µg CD3/well and incubated for 3 hours at 37 ºC. The plates were washed twice with PBS. Then 4x 104 PBMC/well were plated in triplo and mixed with MSC in MSC : PBMC ratios varying van 1:1 to 1:8. The plates were incubated for 4-5 days at 37 ºC and 5% CO2. The cells were incubated with CD3-PE in the wells and stained with 7-AAD. Viable T-cells were analyzed using a FACS Canto (Becton Dickinson) and the results were calculated.



SUPPLEMENTARY FIGURE 2. Competing risk analysis of risk of infection with competing event death in first 100 days after first MSC infusion between CR-B and NonCR-B patients represented by the cumulative incidence of infections. No difference could be observed (Gray's test p-value 0.538).



SUPPLEMENTARY FIGURE 3. Gating strategy.
mDC1 from patients and healthy donors have similar phenotypes. Dendritic cells were identied from the live gate based on scatter, followed by excluding lineage cells (CD3, CD14, CD19 and CD20). mDC1 were identied by BDCA1 positivity. Immature and mature mDCs were accordinly discriminated by expression of CD80/86 as shown in representative plots above. Panels of antibodies used are described in more detail in the Methods section. MSC: mesenchymal stromal cells, HD: healthy donor, t:time point.



SUPPLEMENTARY FIGURE 4
TNFα secretion in monocytes and mDC1 from 2 CR-B patients and 2 healthy controls was measured by multiplex immunoassays after LPS stimulation overnight. The ratio of TNFα levels of the 2 healthy donors compared to the TNFα levels of the 2 CR-B patients was compared. Monocytes from both healthy donors and CR-B patients secreted almost equal amounts of TNFα upon stimulation. However TNFα secretion in mDC1 from CR-B patients was markedly decreased compared to healthy donors, reflected in a higher ratio.

2.2

Standardizing assessment of cellular biomarkers in GVHD cohorts by ECLIPSE and DAMACY

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Manuscript in preparation

ABSTRACT

Standardizing measurements of cellular biomarkers and confirmation of predictive biomarkers remains a major challenge in clinical practice in particular when treating patients with severe acute GVHD, a life-threatening complication after allogeneic stem cell transplantation (allo-HSCT). Therefore we first assessed whether recently developed unbiased flow cytometry analysis platforms can be used for a more standardized analysis of cellular biomarkers. We used a combination of "Elimination of Cells Lying in Patterns Similar to Endogeneity" (ECLIPSE) followed by "Discriminant Analysis of MultiAspect CYtometry" (DAMACY). This approach allowed indeed a more investigator independent analysis and a reproduction of predictive cellular biomarkers in a prospective clinical trial cohort of patients suffering from steroid-refractory GVHD who were uniformly treated with mesenchymal stromal cells (MSC). However, testing whether such biomarkers were also predictive in a second real-world cohort treated within a hospital exemption program could not confirm these results. Our data support the registration of hospital exemption programs and additional linkage to immune monitoring and emphasize the importance of introducing standardized measurements for testing biomarkers in independent clinical cohorts before implementation.

INTRODUCTION

Acute graft versus host disease (GVHD) remains a life-threatening complication and substantially reduces efficacy of allogeneic hematopoietic stem cell transplantation (allo-HSCT). In particular, the outcome of patients with severe steroid-refractory (SR) acute GVHD continues to be poor. Multiple new treatment options for SR-aGVHD entered the field such as the Jak 1/2 inhibitor ruxolitinib, Bruton's tyrosine kinase (BTK) inhibitor ibrutinib and mesenchymal stromal cells (MSC) (1-7). MSC are multipotent cells capable of differentiating into various cell types such as osteoblasts, chondrocytes and adipocytes. Moreover, MSC are able to modulate immune responses through different mechanisms such as T-cell inhibition via indolamine 2,3 dioxygenase (IDO) as a paracrine effect (8, 9). Administration of MSC has been reported by others and us (4-7, 10) as an interesting treatment option that significantly improves overall survival (OS) in responding patients. Therefore insurance and fast track approval has recently been granted to MSC products in Korea, Canada, Japan and the USA (11, 12). However, GVHD is a very heterogeneous disease and to date it is under debate which agent is most suitable for which patient (13). To allow for a more personalized GVHD treatment, tools for early determination of the subset of patients responsive to a specific therapy are important to avoid losing valuable time and allow for optimal usage of precious financial resources.

An attempt to define predictive markers of response to steroid-refractory GVHD treatment with MSC was performed by our center during a non-randomized phase II study between 2009-2012 (www.clinicaltrials.gov #NCT00827398) (4). This study defined clinical but also biological predictors of response. However, since then it has become more clear that analyses of cellular biomarkers are very difficult to standardize across centers (14). Outcome of conventional manual flow cytometry analysis depends substantially on the individual person analyzing data. Therefore, we report on using a new potentially non-subjective method to analyze multicolor flow cytometry data named ECLIPSE (Elimination of Cells Lying in Patterns Similar to Endogeneity). ECLIPSE can discover disease-specific cell populations, as has been shown in asthmatic patients (15), and allows to focus on small cellular subpopulations. In addition, we tested whether the previously reported Discriminant Analysis of MultiAspect CYtometry (DAMACY) on the cell populations identified by ECLIPSE, can be used to objectively classify patients as responders or non-responders (16). Furthermore, we took advantage of an ongoing hospital exemption program treating patients suffering from SR-aGVHD with MSC in line with current Dutch guidelines (17). Immune monitoring was performed within an extensive biobanking protocol. This allowed assessing

whether previously defined predictive cellular biomarkers remain valid in the real-world setting.

METHODS

Hospital exemption cohort and biobanking

Patients in the hospital exemption cohort were treated according to the study protocol that is extensively described in Te Boome *et al.* (4). All patients gave a written informed consent for being treated with a non-registered Advanced Therapy Medicinal Product (ATMP) and agreed to immune monitoring through a biobank protocol at regular intervals approved by the local ethical committee (University Medical Center Utrecht, HSCT biobank protocol 11-063).

Statistics

Comparability between the two patient cohorts was retrospectively assessed (Table 1) using independent samples t test or Anova in case of >2 groups. Primary outcome measures were a complete resolution of all GVHD related symptoms lasting for at least 1 month (CR_{GVHD}) and one-year overall survival (OS). Cox proportional hazards models and Kaplan Meier estimates were used for analyzing OS. Gray's test was used for analyzing response status in competing risk analysis including relapse and death as competing risks in R (for Windows, R version 3.4.4, R Project, http://www.r-project.org). All reported p-values are two-sided.

MSC production and administration

MSC were produced in the GMP grade Cell Therapy Facility as described earlier (supplemental methods (4)). Briefly MSC were derived from third party non-HLA matched healthy donors as agreed to by the Dutch Central Committee on Research Involving Human Subjects (CCMO, protocol NL41015.041.12, "Biobanking bone marrow for MSC expansion"). MSC are isolated by plastic adherence, cultured in platelet lysate and seeded in CellStacks. When 80-100% confluency was reached, cells were harvested using trypsin. All MSC used for clinical application were passage 3, were tested for sterility and were >95% positive for CD73/CD90 and CD105. MSC were administered at day 1, 8, and 21 as reported at a median dose of 1,8 x 10^6 per kg bodyweight (4).

Flow cytometry and plasma analyses

Blood samples were processed as described in Methods and Supplementary Methods in Te Boome *et al.* (4). In short we measured absolute lymphocyte numbers by

TruCount (manufacturer's protocol, BD Biosciences, Breda, The Netherlands). Peripheral blood mononuclear cells (PBMCs) were isolated and cryopreserved until further analysis. Plasma was stored at −80 °C. Plasma samples were analyzed for multiple cytokines using multiplex immunoassays and ELISA. Flow cytometry samples were analyzed with an LSR-II and LSRFortessa flow cytometer (both BD Biosciences). Acquired data were analyzed using FACS Diva software (BD Biosciences).

Data pre-processing for ECLIPSE analysis

The flow cytometry standard (fcs) files of the study cohort were preprocessed by gating lymphocytes based on FSC/SSC and subsequently gating the CD3-CD19-CD14-CD20- lymphocytes. The cells within this gate were exported as fcs files and used for subsequent multi-dimensional analysis. From the fcs files of the hospital exemption cohort lymphocytes were gated based on FSC/SSC and the CD3-CD19-CD14-CD56- lymphocytes were exported for multi-dimensional analysis. Prior to ECLIPSE analysis fcs files were transformed, mean centered and scaled based on the one-year OS group.

ECLIPSE

For detailed information about the ECLIPSE method and algorithm we refer to the recent paper of Folcarelli *et al.* (15). In short, the patient samples were divided in 2 groups based on one-year OS status. The Control group consisted of patients with an OS of less than 1 year (n=25), the one-year mortality group. The Response group contained patients with an OS of 1 year or more (n=18), the one-year survival group. In the first step of ECLIPSE a Control Model was generated by describing variability of cell phenotypes of the one-year mortality group in a Simultaneous Component Analysis (SCA) model. Secondly, the cells of the one-year OS group were projected in the Control Model. The Control Model focusses on the marker expressions of the most abundant cell subsets within the one-year mortality group. To focus on the cell subsets specific for the one-year OS group, all cells similar to cells present in the one-year mortality group were removed from the dataset. Finally, a new PCA model was generated based on the cellular properties of the cells specific for the one-year OS group. This model is called the ECLIPSE model and describes the one-year OS-specific cells best in a 2-dimensional plot. Also donor-specific cells from the one-year mortality group, which are not represented by every sample in the group, are left after ECLIPSE elimination of cells, and these were projected in the ECLIPSE model. For the hospital exemption cohort a separate ECLIPSE model was generated following the above mentioned steps.

DAMACY

The multi-dimensional classification method DAMACY is extensively described by Tinnevelt *et al.* (16). After ECLIPSE elimination, the remaining cells per sample were exported as fcs files containing the original fluorescent intensities and imported in the DAMACY algorithm. The dataset was transformed and samples were mean centered and scaled based on the whole dataset. Samples were grouped based on the one-year OS as described for ECLIPSE. First a PCA-model, the base model, was generated based on the data within both groups. To objectively predict which samples belong to the one-year OS group and the one-year mortality group, DAMACY repeatedly removed 1 sample from the dataset and, based on the cell phenotypes of the remaining samples per group, classified the removed sample as belonging to one of the groups. The accuracy, sensitivity and specificity of the cross-validated classification were calculated.

RESULTS

Clinical outcome

Between January 2009 and July 2014 102 patients with severe SR-aGVHD received a total of 299 MSC infusions derived from twelve different BM donors. Median number of infusions was 3 (range 1-4). All MSC infusions were tolerated well without any acute infusional toxicity. Baseline characteristics of patients are described in Table 1. No significant clinical differences could be detected between the study cohort and hospital exemption cohort except the mean MSC dose per infusion as the dose of MSC infused/kg bodyweight was decreased from 2.0 $x10^6$ to 1.0 $x10^6$ cells/kg in the hospital exemption cohort. 49% of patients reached a complete resolution of all GVHD related symptoms lasting for at least 1 month (CR_{GVHD}). One-year OS for the entire cohort was 41,6% with a significantly improved one-year OS for responding patients (83,7%) vs. non-responding patients (1,9%, log rank test $p<0,001$, Figure 1A,B). Causes of death were relapse of primary malignancy (9,7%), GVHD (43,5%), infection (32,3%) and other (14,5%) as reported in more detail in van der Wagen *et al.* (chapter 3 of this thesis, manuscript in preparation).

Automated multi-dimensional analysis methods confirms predictive cellular biomarker

An increase in immature myeloid dendritic cells (mDCs) measured 2 weeks after initiation of MSC infusion was an independent predictor of one-year OS in our previously published prospective study cohort (4) when measured by conven-

	All patients		Study cohort		exemption program		p-value
Patients - n (%)	102	(100)	48	47,1	54	52,9	
Age - years (range)	44,5	(1,3-68,9)	44,3	1-68	44,6	2-67	0,938
Child - n (%)	15	(14,7)	7	14,6	8	14,8	0,974
Male - n (%)	71	(69,6)	31	64,6	40	74,1	0,306
Time from allo-HSCT to enrollment - mean days (median)	112	(74)	112	85	112	63	0,997
Time from diagnosis aGvHD to enrollment - mean days (median)	24	(11)	24	14,5	24	10,5	0,936
Mean number of MSC per infusion - n x 10^6/kg (range)	1,5	(0,8-2,7)	1,6	1,7	1,3	1,3	**<0,001**
Primary disease - n (%)							0,638
Myeloid neoplasms	58	(56,9)	27	56,3	31	57,4	
Lymphoid neoplasms	36	(35,3)	16	33,3	20	37	
Non malignant disorders	8	(7,8)	5	10,4	3	5,6	
Stemcell source - n (%)							0,701
PBSC	77	(75,5)	37	77,1	40	74,1	
BM	7	(6,9)	4	8,3	3	5,6	
CB	17	(16,7)	7	14,6	10	18,5	
Type of donor - n (%)							0,955
Sibling	21	(20,6)	10	20,8	11	20,4	
MUD	81	(79,4)	38	79,2	43	79,6	
Conditioning regimen - n (%)							0,306
Myeloablative	31	(30,4)	17	35,4	14	25,9	
Overall GVHD grade - n (%)							0,557
grade 2	29	(28,4)	12	25	15	27,8	
grade 3	65	(63,7)	33	68,8	32	59,3	
grade 4	8	(7,8)	3	6,3	5	9,3	
Skin GVHD - n (%)	50	(49)	25	52,1	25	46,3	0,564
Gut GVHD - n (%)	88	(86,3)	42	87,5	46	85,2	0,738
Liver GVHD - n (%)	40	(39,2)	17	35,4	23	42,6	0,464
Patients pretreated with 2nd line GVHD agents - n (%)	4	(3,9)	1	2,1	3	5,6	0,220
Complete response GVHD	50	(49)	24	50	26	48,1	0,854
One year overall survival	42	(41,2)	20	41,7	22	40,7	0,987

TABLE 1: Baseline characteristics and results comparing patients treated in clinical trial with patients treated in hospital exemption cohort.
Legend: Possible differences in baseline characteristics were tested retrospectively in patients treated with MSC in the original clinical trial cohort versus patients treated with MSC in the hospital exemption program. Complete response was defined as a complete absence of any GVHD related symptoms lasting at least 1 month. Groups were compared using independent samples t test or Anova in case of >2 groups. No significant differences could be detected except the mean MSC dose per infusion as the dose of MSC infused/kg bodyweight was decreased from 2.0 to 1.0 x10^6 cells/kg in the hospital exemption cohort.

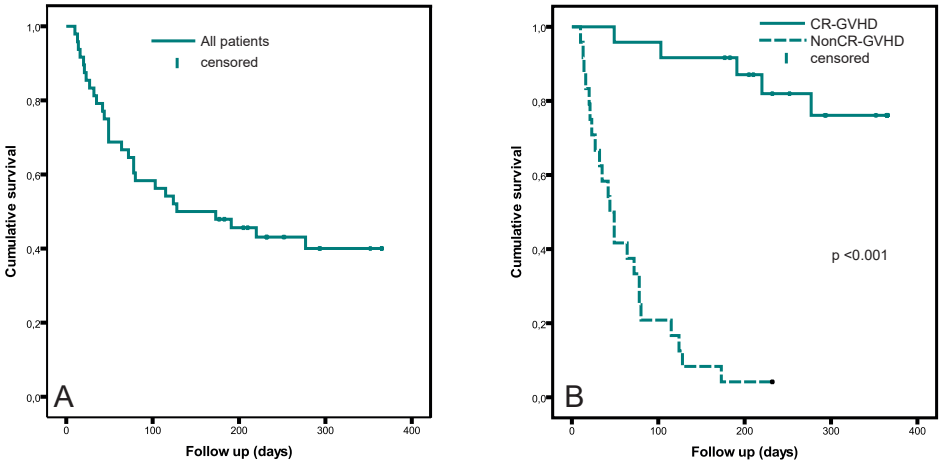

FIGURE 1.
Kaplan Meier curves plotting overall survival for all patients in the cohort (A), between CR_{GVHD} patients and $NonCR_{GVHD}$ patients (B). Log-rank test was used to test for significant differences.

tional flow cytometry. However since flow cytometry analyses, especially of small cell subsets, depend largely on manual gating we explored a novel non-subjective automated analysis system to overcome these challenges, recently suggested by others for a more investigator independent analysis (15, 16). Therefore, we analyzed the flow cytometry data of the prospective study cohort using a combination of two automated multi-dimensional analysis methods. We hypothesized, based on our previous findings that a response to therapy, defined by a one-year OS, would lead to the rise of response-specific cell populations, while these cell populations would not be present in the one-year mortality group. Cell counts are generally low after transplantation and in particular the expected most predictive subset of dendritic cells (DCs) are very low in numbers (4). Also the outcome of analyses could be influenced by minor differences in personal gating preferences of individuals, therefore flow cytometry files were analyzed using the unsupervised multidimensional analysis method ECLIPSE. The key feature of ECLIPSE is the automated removal of similar cells between 2 groups, which enables to focus on sparse response-specific (or in this case OS-specific) cells. First the study cohort was examined with ECLIPSE. In line with our previous findings, in the ECLIPSE model of patients with a one-year OS, a cell population was found expressing Blood Dendritic Cell Antigen (BDCA) 1, which were hardly present in the one-year mortality group (patients with an OS <1 year) (Figure 2A+B). These BDCA-1+ cells were identified as immature mDCs and gating this cell subset in the ECLIPSE space showed a significant difference in percentage of

immature mDCs between patients from the one-year mortality group vs. patients from the one-year OS group (Fig 2C+D, p=0.013). Subsequently DAMACY, a multi-dimensional classification method, was performed on the fcs files containing the cells which were left after ECLIPSE elimination. Classification of patient samples as belonging to the one-year mortality group or to the one-year OS group was performed with a classification accuracy of 73%, which is compared to the percentage of samples with increased mDCs identified by manual gating (specificity 0.70 and sensitivity 0.75, figure 2E). The vectors per marker can be used as a compass describing the expression of the cells. In the DAMACY cell map the long BDCA-1 loading pointing in the direction of response-specific cells (blue) indicates immature mDCs are most important for the variance between both groups, and thus for the classification. Hereby confirming in an unbiased manner the role of mDCs in aGVHD in a prospective study cohort. In conclusion, ECLIPSE and DAMACY analyses of flow cytometry analysis of GVHD cohorts provide powerful tools to objectively identify small cellular subsets in a heterogeneous disease and subsequently classify patients accordingly.

Hospital exemption cohort does not confirm cellular biomarkers

It is imperative to confirm outcomes of clinical trials in 'the real-world'. Not only because clinical trial results often prove difficult to replicate in daily clinical practice, also because regulatory authorities can use real-world data to re-evaluate the impact of novel therapies. As the treatment of our hospital exemption cohort was linked to a biobanking initiative we could not only address clinical potency of MSC outside the setting of a clinical trial, but also investigate the cellular biomarker to predict one-year OS. Firstly we used our new unbiased flow cytometry algorithms in an independent blinded operator fashion. ECLIPSE elimination of matching cells between the one-year mortality and one-year OS group was performed (Figure 2F+G), however no significant differences in distribution of cell populations were identified. Accordingly, the DAMACY classification results for this cohort were poor, with an accuracy of only 40% (data not shown). Thus we could not validate immature mDCs as a prognostic biomarker for OS in this independent cohort with the new investigator independent analysis method.

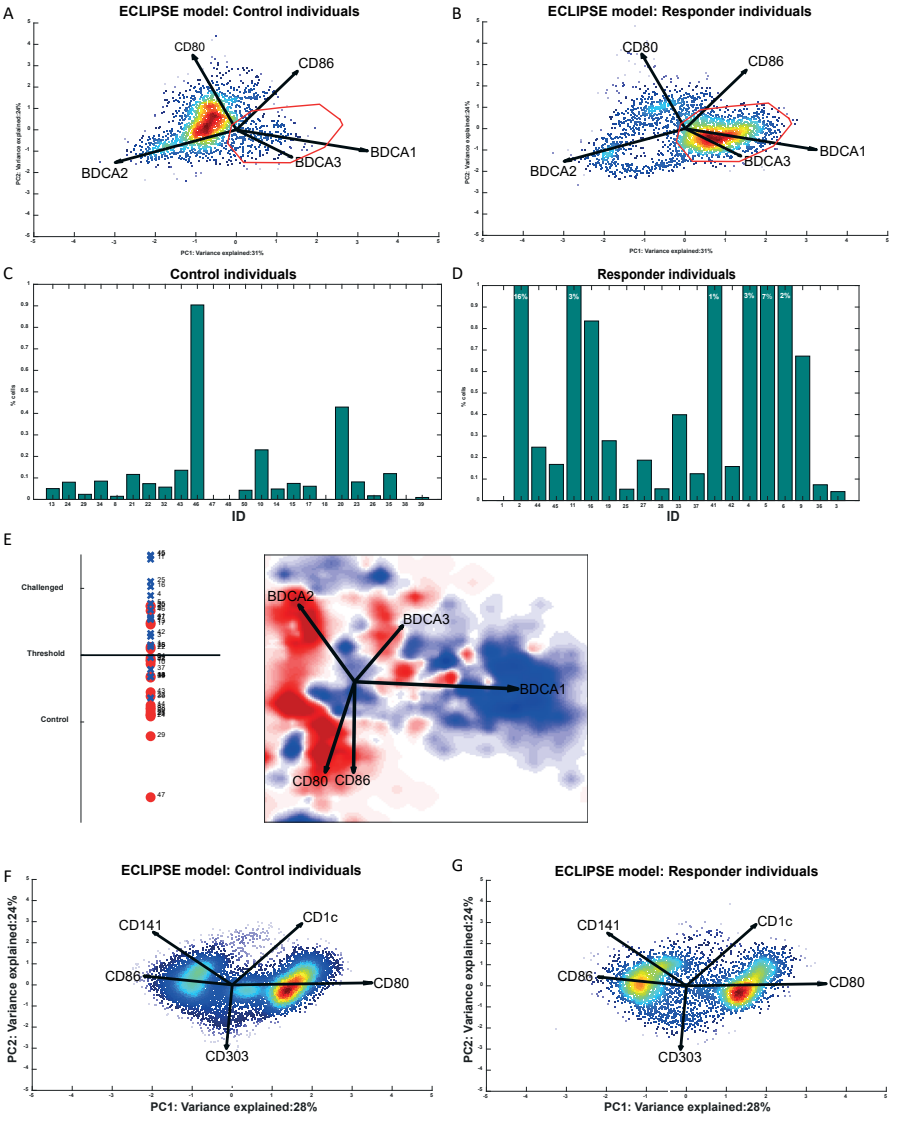

FIGURE 2. ECLIPSE and DAMACY confirm the presence of immature mDCs in the 1-year OS group in the study cohort, but this could not be validated in the hospital exemption cohort.
A+B) ECLIPSE model of the study cohort showing the donor-specific cells of all samples of the one-year mortality group (A) and the response-specific cells of the one-year OS group (B). The BDCA-1 positive cell subset was gated (A+B in red) and the percentage of cells within this gate per sample for the one-year mortality group (C) and the one-year OS group (D). A significant difference was found between both groups (p=0.013). E) DAMACY map of the cells left after ECLIPSE elimination of the one-year mortality group vs. one-year OS group. The left panel shows the DAMACY classification of the samples from the mortality group (red) and the one-year OS group (blue). In the right panel the DAMACY base model shows the areas which contain more cells of the one-year mortality group (red), or the one-year OS group (blue). Areas in which cells are equally represented by both groups, or which do not contain any cells are white. F+G) ECLIPSE model of the hospital exemption cohort showing the donor-specific cells of all samples of the one-year mortality group (F) and the response-specific cells of the one-year OS group (G)

DISCUSSION

To our knowledge we hereby present the clinical data of the largest SR-aGVHD patient cohort treated with MSC worldwide linked to a comprehensive cellular biobanking protocol. First we show that the new flow cytometry analysis platforms ECLIPSE and DAMACY are able to confirm previously defined biomarkers of clinical response to MSC treatment in a defined study cohort. This approach is therefore an intriguing investigator independent method for assessing cellular biomarkers. Secondly we provide evidence that previously reported biomarkers are not useful in the real-world setting.

Major challenge in the analyses for cellular subsets remains the unbiased analysis of small subpopulations. Different methods have been provided in the past to standardize methods such as the EuroFlow initiative (18). However, major limitations remain the dependency on defined machines such as BD FACSCanto II (BD Biosciences, San Jose, CA) BD LSRII (BD Biosciences) and Cyan ADP (Dako-Cytomation).

In addition, manual gating is currently performed on mostly a binary combination of cell characteristics. However, with the current increasing numbers of cellular markers that can be measured, this is very labour intensive and highly operator dependent. Also, as manual gating is performed hierarchically, smaller cell populations not yet clearly defined in standard protocols can be missed. GVHD cohorts, due to the many different clinical variables and in general low cell numbers are notoriously difficult in terms of reproducibility (19). To overcome these problems, we tested whether the recently reported method ECLIPSE is a useful tool for this difficult cohort as it removes control-like cells from the analysis, thereby increasing the focus on response-specific cells (15). In this case ECLIPSE eliminated cells matching with the cell phenotypes present in the one-year mortality group, leaving survival-specific cells for further analysis. By removing the matching cells between the 2 groups, rare cell subsets are more emphasized. In other dimension reduction methods, like viSNE (20) and HSNE (21), or in cluster methods like SPADE (22) and flowSOM (23), rare disease-specific cells or cell clusters may be overshadowed by the more redundant non-response-specific cell populations. Therefore we tested its suitability for both our clinical trial and real-world settings. Using ECLIPSE we found an increase in immature mDCs in the survival group. However, ECLIPSE is mostly a visualization method and does not automatically quantify the distribution of cell populations, neither does it classify samples into groups. To test whether the response-specific cells which were found by ECLIPSE, were truly predictive for one-year OS, we applied the unsupervised classification model DAMACY, which confirmed this with an

accuracy of 73%. Importantly, also here the cell map shows that the BDCA1+ immature mDCs are most predictive for this classification.

Secondly, we addressed whether by utilizing this platform of unbiased analysis we can confirm previously identified biomarkers in a second real-world hospital exemption cohort. However, the previously identified predictive marker for OS at day 14, namely immature mDCs (4) could not be reproduced. Possible explanations for failing to validate this cellular biomarker could be due to technical differences in flow cytometry measurements between the two cohorts, e.g. different FACS machines used (BD LSR-II versus BD LSRFortessa), which is also an obstacle for EuroFlow (18). This underlines the sensitivity of flow cytometry measurements and the importance of the validation of clinical biomarkers before putting them to clinical use. This also reflects the difficulty for the clinical application of cellular biomarkers across different patient cohorts and different centers. A proteomics approach might be more robust as analyses are more automated and standardized as was recently shown for ST2 and Reg3α in the MAGIC consortium (24). However immune monitoring of cellular subsets in GVHD patients will remain a valuable tool to provide biological insights and new techniques in data processing such as ECLIPSE and DAMACY algorithms could prove vital for providing an unbiased analysis method.

In summary, the combination of ECLIPSE and DAMACY flow cytometry analysis platforms can be used to analyze cellular biomarkers in an unbiased way. We show the technique is applicable to GVHD cohorts and confirmed our earlier reported biomarker immature mDCs in a prospective cohort. However, these findings could not be validated in an independent cohort showing these analysis platforms are not yet ready for clinical implementation.

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3

Efficacy of bone marrow derived MSC for steroid refractory acute GVHD associates with age and a defined molecular profile of MSC donors

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ABSTRACT

Mesenchymal Stromal Cells (MSC) are a promising treatment option for patients with severe steroid-refractory acute graft versus host disease (aGVHD) after allogeneic stem cell transplantation (SCT). We evaluated clinical outcome of patients with grade II-IV steroid-refractory aGVHD treated with bone marrow (BM) derived MSC in a hospital exemption cohort (n=54) and compared this to clinical outcome in our previously reported prospective clinical study (n=48). Favorable clinical outcome in this real-world setting did not differ from clinical outcome in the prospective clinical cohort, complete resolution of GVHD symptoms (CR_{GVHD}) was observed in 48% versus 50% and one-year overall survival (OS) 41% versus 41.7% of patients. While we could confirm for both cohorts that patient age and severity of GVHD are predictive marker of OS, pooling data of the cohorts allowed assessment of additional factors such as MSC properties. Surprisingly a significant survival benefit was observed for patients treated with MSC derived from young BM donors (i.e. age <10 years: later referred to as young MSC). Young MSC showed increased T-cell suppression in vitro, however ex vivo immune suppressive capacity of MSC alone was not predictive for OS. Therefore we searched for additional beneficial properties of MSC through a transcriptome analysis. MHCII protein complexes such as HLA-DPA1 and HLA-DRB1 were strongly downregulated in MSC derived from young BM donors compared to the older counterparts. WGCNA and STRING analysis revealed several cell cycle related processes, such as RNA splicing, cytoskeleton reorganization and pro-apoptotic pathways to be upregulated in MSC derived from young BM donors, which is in line with the previous assumption that apoptosis is crucial for MSC immunosuppressive activity. We suggest that age should be taken into account when selecting MSC donors.

INTRODUCTION

Acute graft versus host disease (aGVHD) is a life-threatening complication and substantially reduces efficacy of allogeneic hematopoietic stem cell transplantation (allo-HSCT). In particular, the outcome of patients with steroid-refractory aGVHD continues to be poor. Mesenchymal stromal cells (MSC) are multipotent cells capable of differentiating into various cell types such as osteoblasts, chondrocytes and adipocytes. MSC are able to modulate immune responses through different mechanisms such as T-cell inhibition via indolamine 2,3 dioxygenase (IDO) as a paracrine effect (1, 2). Administration of MSC has been reported by others and us (3-7) as a promising treatment option that significantly improves overall survival (OS) in responding patients. However, studies are not consistent and varying outcomes have been reported which probably relates to differences in MSC products and dosing though there are release criteria for MSC regarding phenotype, sterility and differentiation potential, no in vitro markers of MSC function are known to correlate with clinical outcome (8, 9). Immunomodulatory effects of MSC can be affected by several factors such as the tissue of origin, culture method (e.g. FCS vs. platelet lysate), dosing and passage number. Also intrinsic donor properties such as gender and age have been shown to possibly affect T-cell immunosuppressive effects in vitro (10), therefore MSC products with normalized immunosuppressive capacities are propagated (11). However, standardized tests to measure the immunosuppressive capacities of MSC batches from different donors or produced via different culturing procedures are lacking. Although insurance and fast track approval has recently been granted to MSC products in Korea, Canada, Japan and the USA, differences arising from donor variability and the inability to link surrogate markers of the product to clinical efficacy could prove to be a major obstacle in clinical trials (12-16) and in daily clinical practice.

We performed an open-label, non-randomized phase II study between 2009-2012 (www.clinicaltrials.gov #NCT00827398) in which we treated 48 patients suffering from steroid-refractory aGVHD with MSC infusions (3). After completing this study, we continued the study protocol in a hospital exemption program, treating another 54 patients. We here present the cumulative data of 102 patients with steroid-refractory aGVHD with bone marrow (BM) derived MSC infusions from whom we prospectively gathered clinical data and blood samples. Analyzing the hospital exemption cohort allowed us not only to test whether efficacy is equivalent outside the strict inclusion criteria of a clinical trial setting, but also to assess clinical predictors of response in a real-world setting. In addition

the pooled large cohort provided the unique opportunity to investigate whether specific MSC product or MSC donor characteristics influence clinical outcome.

METHODS

Patients

All patients provided written informed consent. In- and exclusion criteria for the clinical study were previously described (3). We evaluated the impact of individual donors as well as donor properties such as age, T-cell suppression potency, cell viability and culture characteristics on clinical response and overall survival in our cohort of 102 patients with grade II-IV steroid refractory aGVHD treated with BM derived MSC. Primary outcome measures were one-year OS and response of GVHD. CR_{GVHD} is defined as a complete resolution of GVHD-related symptoms in all involved organs (overall grade 0) for at least 1 consecutive month.

MSC production and administration

MSC were produced in the GMP grade Cell Therapy Facility as described earlier (supplemental methods (3)). Briefly MSC were derived from third party non-HLA matched healthy donors (CCMO, protocol NL41015.041.12). MSC were isolated by plastic adherence, cultured in platelet lysate and seeded in double layer Cell-STACK (Corning). When 80-100% confluency was reached, cells were harvested using trypsin (ThermoFisher Scientific). All MSC used for clinical application were passaged 3 times, tested for sterility and were >95% positive for CD73/CD90 and CD105. Culture characteristics that were compared between donors were: population doubling time, T-cell suppressive capacity and cell viability. MSC were administered at day 1, 8, and 22 as reported (3) at a median dose of 1.8 x 10^6 per kg bodyweight.

MSC assays

T-cell suppressive capacity was tested by means of co-incubation of MSC with T-cells stained with Cell Trace Violet (LifeTechnologies, dilution 1:2500). T-cells from 4 different healthy PBMC donors were isolated by means of anti-human CD3 magnetic particles (BD) and then stimulated with PMA (10 ng/ml). T-cells of each PBMC donor were seeded in 1:1 ratio to MSC in 96 wells plates and fluorescent signal of Cell Trace Violet labelled T-cells was measured by flow cytometry (FACS-CantoII, BD) after 4 days. All experiments were performed in duplicate.

Cell viability was tested by PrestoBlue stainings according to the manufacturers protocol (Invitrogen by Life Technologies, MAN0003232). MSC were seeded into

96 wells plates at a density of 500, 1000, 2000 and 4000 cells/well and absorbance was measured (emission at 600 nm – excitation at 570 nm, Versamax Microplate reader, SofMax Pro 6.2.2). All experiments were performed in triplicate.

RNA Isolation and sequencing:

RNA isolation was performed using the RNeasy Mini kit (Qiagen) according to manufacturer's instructions. Quantity of RNA was analyzed with Qubit (Invitrogen) and the quality of RNA was checked using the Bioanalyzer (Agilent). RNA samples with a minimal RNA integrity number (RIN) of 8 were prepared for sequencing using TruSeq Stranded mRNA Library Prep for NeoPrep (Illumina) and sequenced with the 1x75bp High-Ouput kit (Illumina) on the Illumina NextSeq 500 sequencer (USEQ, Utrecht, The Netherlands).

Differential Expression Analysis:

Raw sequencing reads were aligned to the Homo Sapiens Genome build GRCh38.79 using STAR aligner (17) and read counts per gene were generated using Subread FeatureCounts (18, 19). Full quartile normalization (EDAseqR package (20)) on filtered data (genes with more than 1 count per million in at least half of the samples) was performed to homogenize the data distribution. Batch effects and unknown surrogate variables were further corrected by using the residuals of a fitted general linear model, considering sex and batch as random effect, as an input to the RUVSeq R package (21). Differential expression analysis was performed using DeSeq2 (22) and *edgeR* (23) R packages employing the batch corrected data as input. In both cases, sex was accounted in the negative binomial model. Differentially-expressed genes were defined as those with Bonferroni-Hochberg adjusted p-value <0.01 and a Fold Change >1.5.

Weighed Gene Correlation Network Analysis (WGCNA)

WGCNA R studio package was used for the analysis (24). Briefly, soft-thresholding was applied in the adjacency matrix of the top 4000 most variant genes in order to obtain a scale-free topology. Six modules of genes were then retrieved after average clustering the derived Topological Overlapping Matrix (TOM) distance (Supplementary figure 1). The *eigen-vector* from every module was correlated (Pearson Correlation) with the increase of age, in order to highlight the most important group of genes (modules) associated with it.

Pathway analysis

To investigate the major differential metabolic processes between MSC derived from young and old BM donors, gene ontology (GO) enrichment analysis was per-

formed on the WGCNA modules (ClusterProfiler R package) (25). P-value cutoff and q-value cutoff were set at 0.05 and 0.01 respectively. Genes with a module Gene Significance (GS) >0.5 were used as input in order to reduce noise.

Statistics

Cox proportional hazards models and Kaplan Meier estimates were used for analyzing OS. Gray's test was used for analyzing response status in competing risk analysis including relapse and death as competing risks in R (for Windows, R version 3.4.4, R Project, http://www.r-project.org). Parameters with a probability level <10% in univariate analysis were considered for multivariate models. In all other cases a probability level <5% was found to be significant. All reported p-values are two-sided. Two R statistical packages for RNA-seq analysis were used for calculating differentially expressed genes (DEGs) (DeSeq & EdgeR). DEGs were considered if they overlapped across the two methods and a False Discovery Rate/ adjusted p-value<0.01.

RESULTS

Comparable response rate and one-year OS in real-world cohort when compared to prospective study cohort

First we assessed whether the real-world cohort participating in the hospital exemption program differed from the prospective clinical trial cohort in terms of baseline characteristics. No differences were observed except for the mean MSC dose per infusion as the dose of MSC infused/kg bodyweight was decreased from 2.0 to 1.0 x10^6 cells/kg in the hospital exemption cohort (Table 1). We then compared clinical outcomes between the cohorts. Despite the lower MSC dose per infusion, no differences in CR_{GVHD} (48% versus 50%, p-value 0.854) or one-year OS (41.7% versus 41%, p-value 0.987) was observed between the hospital exemption and the clinical trial cohort (Table 1 and figure 1A). As the two clinical cohorts showed equal outcomes, we pooled the two cohorts to investigate predictive factors for outcome such as MSC properties. The pooled cohort covers a time period between January 2009 and July 2014 wherein 102 patients received 299 MSC infusions derived from 12 different BM donors. Median number of infusions was 3 (range 1-4). The majority (75.5%) of patients received all MSC infusions from the same donor, 20.6% received MSC from 2 donors and 3.9% received MSC from 3 different donors. Two donors were used to treat 28.4% and 43.1% of patients respectively. All MSC infusions were tolerated well without any acute infusional toxicity. When the two cohorts were taken together, 49% of patients achieved

Baseline characteristics	exemption program		Study cohort		p-value	All patients	
Patients - n (%)	54	52,9	48	47,1		102	(100)
Age - years (range)	44,6	2-67	44,3	1-68	0,938	44,5	(1,3-68,9)
Child - n (%)	8	14,8	7	14,6	0,974	15	(14,7)
Male - n (%)	40	74,1	31	64,6	0,306	71	(69,6)
Time from allo-HSCT to enrollment - mean days (median)	112	63	112	85	0,997	112	(74)
Time from diagnosis aGvHD to enrollment - mean days (median)	24	10,5	24	14,5	0,936	24	(11)
Mean number of MSC per infusion - n x 10^6/kg (range)	1,3	1,3	1,6	1,7	**<0,001**	1,5	(0,8-2,7)
Primary disease - n (%)					0,638		
Myeloid neoplasms	31	57,4	27	56,3		58	(56,9)
Lymphoid neoplasms	20	37	16	33,3		36	(35,3)
Non malignant disorders	3	5,6	5	10,4		8	(7,8)
Stemcell source - n (%)					0,701		
PBSC	40	74,1	37	77,1		77	(75,5)
BM	3	5,6	4	8,3		7	(6,9)
CB	10	18,5	7	14,6		17	(16,7)
Type of donor - n (%)					0,955		
Sibling	11	20,4	10	20,8		21	(20,6)
MUD	43	79,6	38	79,2		81	(79,4)
Conditioning regimen - n (%)					0,306		
Myeloablative	14	25,9	17	35,4		31	(30,4)
Overall GVHD grade - n (%)					0,557		
grade 2	15	27,8	12	25		29	(28,4)
grade 3	32	59,3	33	68,8		65	(63,7)
grade 4	5	9,3	3	6,3		8	(7,8)
Skin GVHD - n (%)	25	46,3	25	52,1	0,564	50	(49)
Gut GVHD - n (%)	46	85,2	42	87,5	0,738	88	(86,3)
Liver GVHD - n (%)	23	42,6	17	35,4	0,464	40	(39,2)
Patients pretreated with 2nd line GVHD agents - n (%)	3	5,6	1	2,1	0,22	4	(3,9)
Results							
CR_{GVHD}- n (%)	26	48	24	50	0,854	50	**(49)**
1-year OS- n (%)	22	41	20	41,7	0,987	42	**(41,6)**

TABLE 1: Baseline characteristics and outcome comparing patients treated in clinical trial with patients treated in hospital exemption cohort.
Legend: Possible differences in baseline characteristics were tested retrospectively in patients treated with MSC in the original clinical trial cohort versus patients treated with MSC in the hospital exemption program. Groups were compared using independent samples t test or Anova in case of >2 groups. No significant differences could be detected except the mean MSC dose per infusion as the dose of MSC infused/kg bodyweight was decreased from 2.0 to 1.0 x10^6 cells/kg in the hospital exemption cohort. aGVHD: acute Graft versus Host Disease, MSC: mesenchymal stromal cells, PBSC: Peripheral Blood Stem Cells, BM: bone marrow, CB: cord blood, MUD: matched unrelated donor.

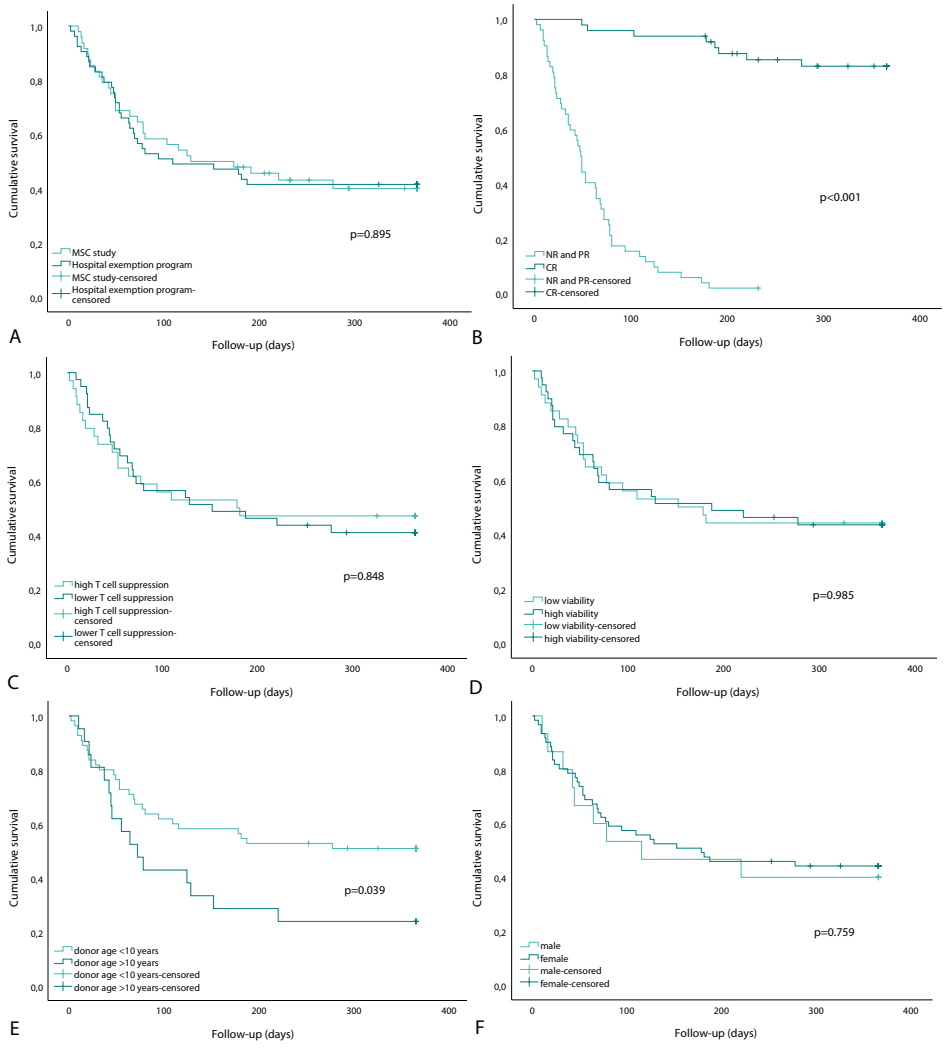

FIGURE 1. Kaplan Meier curves were constructed to plot one-year OS. A and B show data from all patients (n=102). C-F show selected data for patients treated with only 1 MSC donor (n=77). Log-rank test was used to test for significant differences. NR: non responder, PR: partial responder, CR: CR_{GVHD}, complete resolution of all GVHD related symptoms lasting at least one month.

A: No difference in one-year OS in patients treated in the study cohort or hospital exemption program (41.7% vs 41%, p-value 0.895).

B: Significant survival benefit for CR_{GVHD} vs. $NonCR_{GVHD}$ patients (83.7% vs. 1.9%, p-value <0.001).

C/D/F: No difference in one-year OS in patients treated with MSC derived from donors with high vs. low T-cell suppression, high vs. low cell viability or different gender.

E: Significant survival benefit for patients treated with MSC donors <10 years of age vs. >10 years of age (50.9% vs. 23.8%, p-value 0.039).

CR_{GVHD}. One-year OS for the entire cohort was 41.6% with a significantly improved one-year OS for responding patients (83.7%) vs. non-responding patients (1.9%, log rank test p-value <0.001, Figure 1). Causes of death were relapse of primary malignancy (9.7%), GVHD (43.5%), infection (32.3%) and other (14.5%).

Table 2: Baseline characteristics MSC donors

Donors - n (%)	**12**	**(100)**
Median age in years (range)	9,5	(2-33)
Male - n (%)	6	(50)
Median number of MSC infusions (range)	7	(1-46)

TABLE 2. Baseline characteristics of MSC donors used in the reported patient cohort.
MSC: Mesenchymal Stromal Cells

Young age of MSC donors associates with improved overall survival

Donor age of MSC has been reported to associate with different molecular properties (26). When we clustered donors into cohorts below or above 10 years (median MSC donor age at time of donation) we observed a significant survival benefit for patients treated with MSC derived from young (<10 years of age) compared to older (>10 years) MSC donors (Figure 1, log rank p-value 0.039). We performed all analyses for both the entire cohort of 102 patients and for the subgroup of 77 patients that received MSC from a single MSC donor. In a multivariate Cox proportional hazards model (Table 3), including all univariate tested parameters with a probability level <10% (Supp. table 1) showed no good predictors for CR_{GVHD}. However, in the pooled cohort we identified, in line with our previous cohort, patient age, severity of GVHD/liver GVHD as important predictive variables for OS for both the entire cohort and the subgroup of patients treated with only one donor. Intriguingly MSC donor age remained a significant predictive variable for OS in a multivariate analysis, suggesting that MSC derived from younger donors might have favorable properties for the treatment of acute GVHD.

MSC specific properties of younger BM donors and clinical outcome

Several factors could contribute to the age-related effects of MSC. Cell viability of cultured MSC as a surrogate marker for fitness was tested by PrestoBlue staining measured daily during 1 week of culturing. We did observe some interdonor variations in cell viability from day 5 on (Figure 2C), however these differences did not impact one-year OS (Figure 1) or CR_{GVHD}. No difference in cell viability was

detected at any day during 1 week of culturing between MSC derived from donors < or > 10 years of age (Figure 2D).

Direct suppression of T-cells in mixed lymphocyte cultures has been correlated with the efficacy of MSC to reduce aGVHD (11). We investigated the effect of age on the immune suppressive properties of MSC. We used passage 2 MSC samples

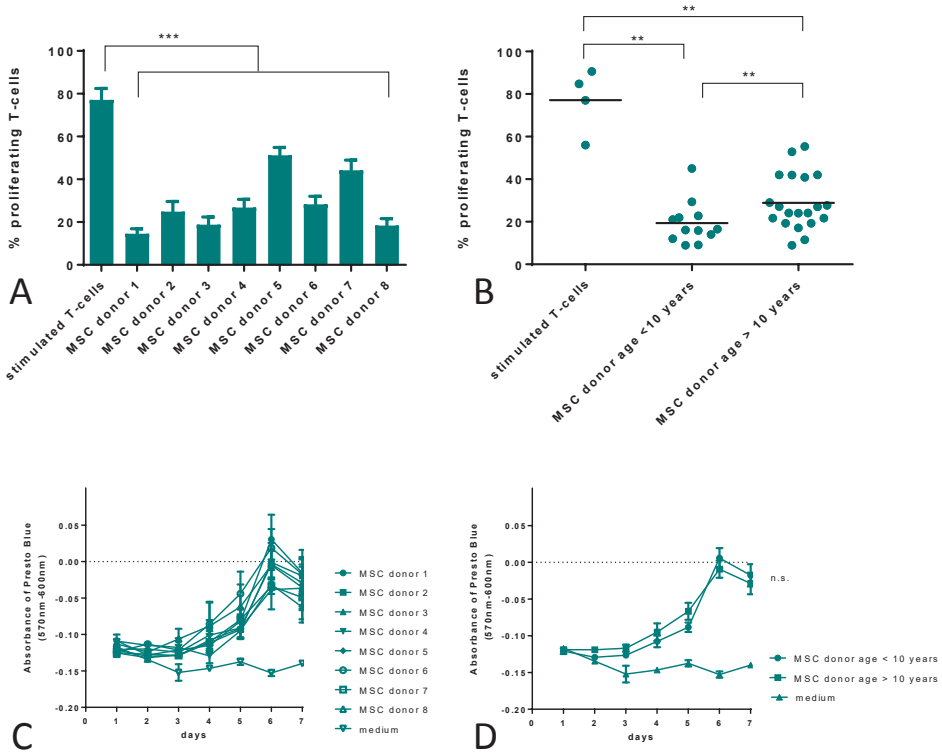

FIGURE 2. A/B: MSC suppressive capacity was tested by means of co-incubation with stimulated T-cells stained with Cell Trace Violet. T-cells from 4 different PBMC donors were isolated using anti CD3 magnetic beads and stimulated with PMA (10 ng/ml). T-cells of each donor were seeded in 1:1 ratio to MSC and fluorescent signal from Cell Trace Violet labeled T-cells was measured by flow cytometry after 4 days. All experiments were performed in duplicate.

A: All MSC donors gave significant suppression of T-cell proliferation compared to stimulated T-cells. Mean and s.e.m. are depicted.

B: Younger donors give a more profound T-cell suppression than MSC derived from older donors (>10 years of age, p-value 0.007). Means of duplicate experiments are depicted.

C/D: Cell viability is expressed by means of PrestoBlue absorbance (570nm-600nm, Life technologies). Data shown are from 2 experiments with 1000 MSC seeded per well (96 wells plate) in triplo. MSC were seeded in 48 wells plates (1000 MSC/well) and cultured under normal conditions. PrestoBlue was added according to the manufacturers protocol, incubated for 20 minutes at 37 C° and 1 plate was measured every day during 1 week. Similar results were obtained with MSC density 500, 2000 and 4000 per well. Mean and s.e.m. are depicted. All experiments were performed in triplicate.

C: Cell viability per MSC donor.

D: No difference in cell viability was detected at any day during 1 week of culturing between MSC derived from donors < or > 10 years of age.

from 8 of 12 MSC donors that together were used for 92.4% of all MSC infusions in the cohort of 102 patients. As expected, co-cultivation of PMA activated T-cells with MSC derived from these 8 donors led to significant suppression of T-cell activation (Figure 2A). MSC derived from younger donors showed an enhanced

		Outcome CR-GVHD	
	HR	**CI**	**p-value**
		ANALYSES ON ALL PATIENTS (n=102)	
Stemcell source	0.526	0.232-1.192	0.12
Overall GVHD grade (grade 2 vs. grade 3 and 4)	0.681	0.423-1.097	0.11
		ANALYSES ON PATIENTS TREATED WITH ONLY 1 MSC DONOR (n=77)	
Overall GVHD grade (grade 2 vs. grade 3 and 4)	0.63	0.386-1.03	0.06
MSC donor age (grouped > or < 10 years)	0.516	0.226-1.176	0.12
		Outcome 1-year mortality	
	HR	**CI**	**p-value**
		ANALYSES ON ALL PATIENTS (n=102)	
Overall GVHD grade (grade 2 vs. grade 3 and 4)	**1.708**	**1.037-2.812**	**0.036**
Patient age	**1.023**	**1.006-1.04**	**0.0063**
		ANALYSES ON PATIENTS TREATED WITH ONLY 1 MSC DONOR (n=77)	
Liver GVHD	**1.356**	**1.069-1.719**	**0.012**
Patient age	**1.022**	**1.006-1.039**	**0.0087**
MSC donor age (grouped > or < 10 years)	**2.006**	**1.091-3.687**	**0.025**

TABLE 3. Multivariate Cox proportional hazards models and Kaplan Meier estimates were used for analyzing outcome overall survival.
Gray's test was used for analyzing response status in competing risk analysis including relapse and death as competing risks in R (for Windows, R version 3.4.4). Parameters with a probability level <10% in univariate analyses were considered for multivariate models. In bold are shown all statistically significant p-values. CI: Confidence Interval, CMV: Cytomegalovirus, GVHD: Graft versus Host Disease, HR: Hazard Ratio,MSC: Mesenchymal Stromal Cells

capacity to suppress T-cell activation (Figure 2B). However, ex vivo T-cell suppressive capacity of MSC in mixed lymphocyte reaction did not associates with one-year OS (Figure 1) and CR_{GVHD} (competing risks death and relapse).

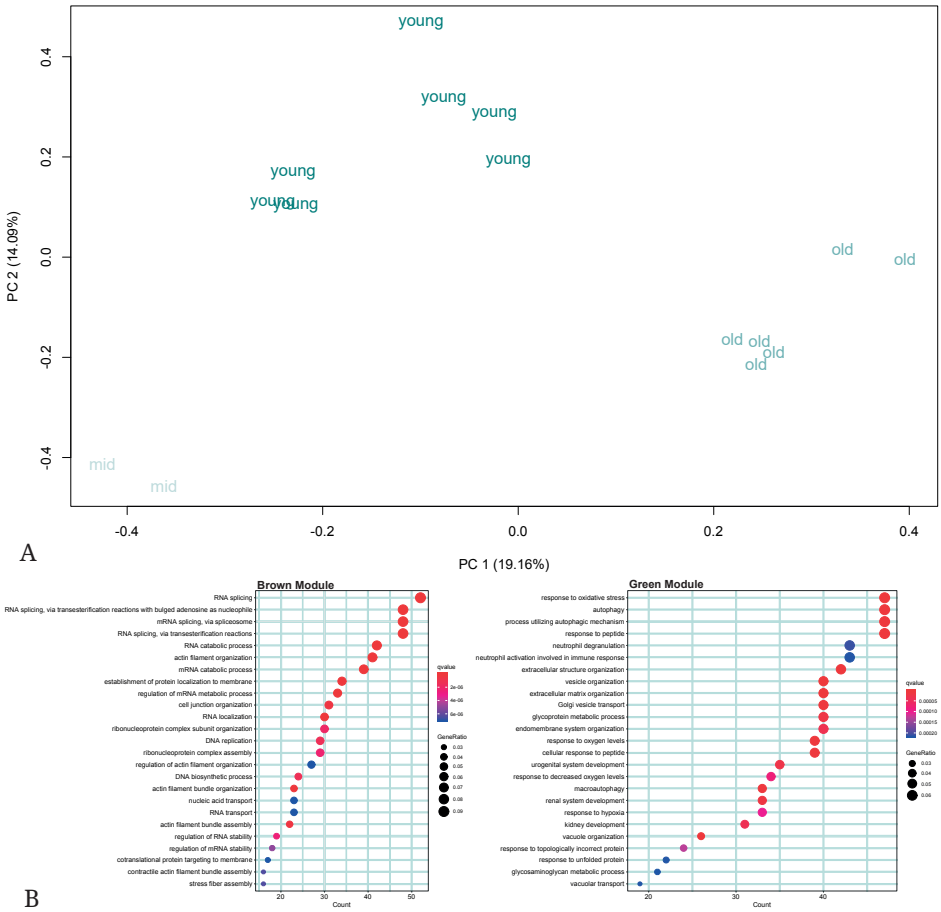

FIGURE 3.
A: Principal component analysis (PCA) from RNA-seq data of young BM donors (age <10 years) versus old BM donors (age>20 years) and mid-age BM donors (age 10-20 years) shows a clear segregation of donors based on age.
B: GO enrichment terms of the brown and green modules from the WGCNA. Genes with a GS>0.5 were used as input. Count, number of genes found in the input that belong to the corresponding enriched term; Gene ratio, Count divided by the total amount of genes configuring the enriched term; q-value, Bonferroni-Hochberg p-value correction.

Differential expression profiles are observed in MSC derived from young versus old BM donors

To identify other potential beneficial assets of MSC derived from young BM donors we performed transcriptome analysis. In order to increase the power of the analysis we extended the sample size of MSC of young (<10 years) and old (>20 years) MSC donors, to 8 donors from the clinical cohort and 8 additional donors. Principal component analysis (PCA) on the batch corrected and normalized RNA sequencing data from these 16 MSC donors showed a clear segregation of young and old donors (Figure 3A). The comparison of the young versus old donors revealed 104 differentially expressed (DE) genes having an absolute fold change (FC) >1.5. From those DE genes, 73 genes were downregulated and 31 genes were upregulated. As we hypothesized that membrane associated proteins might be involved, we performed a GO enrichment analysis for membrane located processes. This revealed that MHCII protein complexes with genes such as HLA-DPA1 and HLA-DRB1 were strongly downregulated in MSC derived from young BM donors compared with the older counterparts (Supplementary figure 1).

To highlight genes and pathways correlated with MSC age, we performed a Weighed Gene Correlation Network Analysis (WGCNA (27-30). Two modules showed a correlation with the increase of age, the green (Pearson correlation (PC) = 0.85; p-value <0.001) and the brown modules (PC = -0.85; p-value <0.001), composed of 952 and 832 genes respectively (Supplementary figure 2). This implied that the genes included in those modules are highly representative of ageing of the MSC donor (green module) or young BM MSC (brown module). Thirty-two DEG were present in the green module, while 10 DEG were present in the brown module (Supplementary table 2), suggesting that these genes could me involved in the age-related differences in MSC function that we observed.

In order to identify putative gene networks preferred in younger donors, GO enrichment analysis of genes with absolute values of GS>0.5 in both modules was performed. These analyses retrieved various autophagy, extracellular matrix (ECM) reorganization and oxidative stress processes in the green module (Figure 3B) providing the most important features that may describe ageing in MSC. In the same way, several RNA splicing and cytoskeleton reorganization processes, together with some pro-apoptotic GO terms (Table 4A), were found to be enriched in the brown module (Figure 3B), thus describing a potential functional landscape in MSC from young donors.

In a recent article from Hennrich *et al.* a proteomic approach was used to define ageing in different cell types, including MSC (26). Interestingly, in the case of young MSC, several cell cycle related pathways (including RNA splicing and cytoskeleton reorganization) and a pro-apoptotic process (GO term *Apoptosis*

induced DNA fragmentation), were highlighted to be significant, corresponding to several of the enriched terms obtained for the brown module in our work. In order to see which of those genes are indeed expressed into protein, we further look at the genes included in the GO enriched terms from our WGCNA modules, and the differentially expressed proteins included in the Kyoto Encyclopedia of

A

Pathway ID	Pathway description	Count	p-value	q-value	Genes
GO: 1900119	Positive regulation of execution phase of apoptosis	4	0.0005	0.0074	BAX/BOK/DLC1/ TP53
GO: 0097194	Execution phase of apoptosis	8	0.0039	0.0327	AKT1/BAX/BOK/ DLC1/ FEN1/HMGB1/ HMGB2/TP53
GO: 0006921	Cellular component disassembly involved in execution phase of apoptosis	5	0.0048	0.0376	BAX/BOK/FEN1/ HMGB1/ HMGB2

B

Gene symbol	Ensembl	Base mean counts	Gene fold change
BAX	ENSG00000087088	2353.126	1.1708
AKT1	ENSG00000142208	5079.15	1.1920
DLC1	ENSG00000164741	11121.432	1.2644
TP53	ENSG00000141510	2523.378	1.4078
BOK	ENSG00000176720	1582.419	1.4166
HMGB1	ENSG00000189403	5546.639	4.4272
FEN1	ENSG00000168496	1456.711	1.6531
HMGB2	ENSG00000164104	3739.549	1.7381

TABLE 4.
A: Enriched apoptotic GO terms in the Brown module (related to MSC derived from young BM donors). GO pathway identification (Pathway *ID*), *pathway description*, number of genes present in the corresponding pathway from our data set (*Count*), *p-value* and *q-value* (Bonferroni-Hochberg p-value correction) of the enrichment, and hgnc gene symbol notation (*Genes*) of the genes found in our data set from the enriched GO term, are represented.
B: Gene Fold Change found in the apoptotic GO terms from the comparison young vs old MSCs. These genes belong to the brown module of the WGCNA (young MSC related). *Gene Symbol,* gene *ensembl* notation, *Base mean counts* (mean counts of the respective gene through all the MSC samples in the RNA seq analysis), *Gene Fold Change* (fold change obtained from Deseq2 RNA seq analysis), are represented.

Genes and Genomes (KEGG) enriched terms from the work of Hennrich *et al.* (26). Of the upregulated (green module) and of the downregulated genes (brown module) from the WGCNA, 72% (31/43) and 86% (24/28) respectively are found to be regulated in the same way when cross-checked by protein expression. This shows the reliability of the current gene-based analysis. From the brown module, related with young MSC, we found a total of 39 proteins analyzed in both works, 9 corresponding to RNA splicing (HNRNPA1, HNRNPM, SRSF3, HNRNPH1, U2AF1, PRPF19, POLR2E, NUDT21, CCAR2), 2 to cell cycle (PCNA, PRMT1) and 1 to apoptosis (FEN1) (Supplementary table 4). Regardless of the biological impact of such proteins, the expression levels either determined by RNA or protein analyses might serve as surrogate markers for the potency of MSC in vivo and may shed more light on their working mechanisms.

DISCUSSION

Here we present the clinical data of a large steroid-refractory aGVHD patient cohort treated with MSC. Our data confirm outcomes of clinical trials in a real-world cohort. In addition we confirmed previous reports suggesting that patient age, GVHD severity and age of MSC donor affect overall survival.

A major limitation of many studies investigating Advanced Therapy Medicinal Products (ATMP) such as MSC, is the sample size. This can result in approval of ATMP with relatively small single arm studies if the effect size is large enough such as recently been done for Chimeric Antigen Receptor (CAR) T-cell products (31). We provide an alternative strategy to improve lessons learned from academic phase II studies by introducing a close clinical and biological monitoring of consecutive hospital exemption programs. This effort is in line with the European Medicines Agency (EMA) registry initiative exploring the usage of registries for post market approval. However, such registries could also be a valuable tool to monitor the success and quality of hospital exemption programs as suggested most recently by the League of European Research Universities (LERU), an association of 23 leading research-intensive universities in Europe (www.leru.org). We support this assumption by demonstrating equivalent outcomes from a prospective phase 2 clinical trial (3) and the retrospective analysis of now 54 patients treated within a hospital exemption program. We feel this underlines the importance of registering patient data (e.g. in the European society for Blood and Marrow Transplantation (EBMT) registry) of patients treated with cellular therapies (8).

With the huge variation in production processes and sources of MSC, as evidenced by a recent survey of EBMT (8), it is almost impossible to judge if certain production or donor properties affect clinical outcome. We tried to overcome this limitation by expanding the phase II cohort with the hospital exemption cohort treated with MSC, which have been uniformly produced for all patients. This resulted in the observation that not only previously reported clinical factors such as patient age and severity of GVHD correlate with clinical outcome, but also the age of the MSC donor (3, 32). This unexpected observation created the unique opportunity to further investigate potential molecular mechanisms in order to develop a set of MSC characteristics that can be used as a potential potency assay for MSC prior to clinical application.

Ex vivo measurements of the capacity of MSC to suppress other immune cells, in particular T-cells, has been suggested to serve as surrogate marker for MSC functionality (11). However, standardization of these assays is difficult across institutions and T-cell suppression itself did not correlate with overall survival. MSC of younger donors had a higher capacity to suppress T-cell proliferation, suggesting that this mechanism can be part of the effective mode of action, but is most likely not the only one. Our extensive RNA sequencing analysis revealed clear clustering of MSC based on age of the BM donor. This is striking as patients were treated with generally regarded 'young' MSC donors with a maximum age of 33 years at time of BM donation. Apparently even in this relatively small age range from the current work differences exist that contribute to the difference in clinical response. Differential expression analysis revealed 73 downregulated and 31 upregulated genes in the MSC derived from young (<10 years of age) versus old (>20 years of age) MSC donors, being important MHCII proteins.

Recent findings from Galleu *et al.* suggested that the ability of MSC to undergo apoptosis after encountering cytotoxic cells is essential for their immunomodulation effect through recipient phagocytes and secretion of IDO (33). Coincidental to this observation some GO pro-apoptotic terms, together with their respective genes, are enriched (Table 4A) or tend to be upregulated respectively at a younger age (Table 4B). Moreover, the list of candidate genes identified through RNA sequencing are also supported by recent data from Hennrich *et al.* which highlights, by means of proteome analyses, age-effected pathways in MSC related to apoptosis (26). Critically appraising our GO enrichment we show that 72% of upregulated and 86% of downregulated genes are regulated in the same way as the protein expression tested by Hennrich *et al.* It furthermore highlights age-effected pathways in MSC related, between others, to apoptosis, reinforcing the notion that this is one of the major processes attributing to MSC immunosuppression in GVHD treatment. An important difference in the datasets is however

the age range of MSC donors as our BM donors range from 2-33 years whereas the BM donors used in the Hennrich paper range from 20-60 years of age. This different age range could explain the variation between the findings, although striking information about MSC ageing obtained by the proteomic approach can still be retrieved as well by the current transcriptomic analysis. Moreover, not all the genes found to be relevant in the present work were measured in the proteomic analysis, thus leaving open the possibility that more gene targets from our list are translated as well into proteins. As a recommendation, we encourage to further explore pro-apoptotic related proteins as potential surrogate markers of MSC immunosuppressive activity.

In conclusion we confirm clinical outcome of a prospective clinical study in a real-world setting. In addition, we identified MSC donor age to be associated with a significant improved one-year OS. MSC derived from donors <10 years show a distinct molecular profile with differences in pro-apoptotic genes. Since age of the MSC BM donor is a useful variable for donor selection we would like to argue to preferably make use of MSC derived from very young BM donors, <10 years of age, to treat patients with steroid-refractory aGVHD. In addition, the identified pathways could assist in characterizing MSC and further elucidate their working mechanisms.

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SUPPLEMENTAL MATERIALS

	Outcome CRGVHD			Outcome 1-year mortality		
	HR	**CI**	**p-value**	**HR**	**CI**	**p-value**
ANALYSES ON ALL PATIENTS (n=102)						
Patients: adult vs. children	1.38	0.614-3.104	0.435	0.929	0.441-1.958	0.847
Patient age	0.999	0.983-1.015	0.873	1.01	0.996-1.025	0.173
Patient sex	1.21	0.650-2.250	0.548	1.131	0.660-1.940	0.654
Primary disease						
myeloid neoplasms vs. lymphoid neoplams	1182	0.624-2.237	0.608	1.316	0.775-2.236	0.309
myeloid neoplasms vs. non-malignant disorders	2.209	0.840-5.812	0.108	0.578	0.177-1.888	0.364
Days from allogeneic SCT to enrollment	1	0.999-1.002	0.775	1	0.998-1.002	0.773
Days from diagnosis GVHD to enrollment	1.001	0.994-1.007	0.856	1.005	0.997-1.013	0.216
SCT donor type (sibling vs. MUD)	0.823	0.423-1.601	0.566	1.452	0.754-2.798	0.265
Stemcell source						
PBSC vs. BM	0.513	0.123-2.140	0.36	1.175	0.467-2.954	0.732
PBSC vs. CB	1.157	0.586-2.283	0.674	**0.511**	**0.231-1.132**	**0.098**
CMV mismatch	0.827	0.329-2.078	0.687	0.867	0.396-1.898	0.722
Conditioning regimen (myeloablative vs non-myeloablative)	1.128	0.624-2.037	0.69	**2.211**	**1.192-4.100**	**0.012**
Total body irradiation	1.053	0.592-1.872	0.86	1.14	0.675-1.923	0.625
Overall GVHD grade (grade 2 vs. grade 3 and 4)	**0.572**	**0.323-1.015**	**0.056**	**1.717**	**0.927-3.180**	**0.086**
Number of GVHD organs involved at enrollment	**0.626**	**0.403-0.974**	**0.038**	1.361	0.937-1.977	0.106
Skin GVHD	1.087	0.618-1.912	0.773	0.788	0.472-1.315	0.362
Gut GVHD	0.77	0.369-1.609	0.488	1.931	0.829-4.495	0.127
Liver GVHD	**0.388**	**0.195-0.773**	**0.007**	**1.659**	**0.995-2.768**	**0.053**
Skin GVHD	1.087	0.618-1.912	0.773	0.788	0.472-1.315	0.362
Mean MSC dose per infusion	1.503	0.795-2.842	0.21	0.681	0.377-1.228	0.201
Number of MSC donors						
1 donor vs 2 donors	1.25	0.576-2.714	0.572	1.434	0.794-2.590	0.232
1 donor vs 3 donors	2.147	0.648-7.109	0.211	0.361	0.050-2.624	0.314
ANALYSES ON PATIENTS TREATED WITH ONLY 1 MSC DONOR (n=77)						
MSC donor viability (low vs high)	1.006	0.544-1.858	0.986	1.006	0.544-1.858	0.986
MSC T-cell suppression (high vs. low)	1.062	0.573-1.969	0.848	1.062	0.573-1.969	0.848
MSC donor age (grouped > or < 10 years)	**1.904**	**1.022-3.547**	**0.043**	**1.904**	**1.022-3.547**	**0.043**
MSC donor sex	0.891	0.427-1.859	0.759	0.891	0.427-1.859	0.759

SUPPLEMENTARY TABLE 1. Univariate Cox proportional hazards models from all variables. In bold are shown all p-values < 0.1 which were used for multivariate analysis. BM: Bone Marrow, CB: Cord Blood, CI: Confidence Interval, CMV: Cytomegalovirus, GVHD: Graft versus Host Disease, HR: Hazard Ratio, MUD: Matched Unrelated Donor, MSC: Mesenchymal Stromal Cells, PBSC: Peripheral Blood Stem Cells, SCT: Stem Cell Transplantation.

Gene_Symbol	ensembl	Fold Change	MM	GS	Module
DCHS1	ENSG00000166341	4.706	0.688	0.547	Brown
EPHB2	ENSG00000133216	2.091	0.848	0.823	Brown
EZR	ENSG00000092820	1.879	0.849	0.842	Brown
GLIPR2	ENSG00000122694	1.856	0.933	0.814	Brown
MEX3D	ENSG00000181588	1.834	0.816	0.754	Brown
RPS28P7	ENSG00000227097	5.910	0.579	0.506	Brown
SOX11	ENSG00000176887	8.850	0.828	0.717	Brown
STMN3	ENSG00000197457	5.711	0.806	0.728	Brown
TRIM59	ENSG00000213186	1.740	0.883	0.847	Brown
WEE1	ENSG00000166483	1.684	0.938	0.893	Brown
ACSS3	ENSG00000111058	-2.511	0.927	0.737	Green
ADGRL2	ENSG00000117114	-2.048	0.977	0.79	Green
AOX1	ENSG00000138356	-2.637	0.975	0.843	Green
BMP6	ENSG00000153162	-5.467	0.861	0.846	Green
CACNA2D1	ENSG00000153956	-2.618	0.962	0.86	Green
CD74	ENSG00000019582	-9.386	0.792	0.661	Green
CDH6	ENSG00000113361	-10.863	0.969	0.763	Green
CHRDL1	ENSG00000101938	-6.888	0.988	0.825	Green
DEPTOR	ENSG00000155792	-4.244	0.932	0.746	Green
EPDR1	ENSG00000086289	-3.398	0.882	0.785	Green
FGF7	ENSG00000140285	-5.185	0.943	0.73	Green
GHR	ENSG00000112964	-4.108	0.967	0.85	Green
HEPH	ENSG00000089472	-3.399	0.892	0.741	Green
HLA-DPA1	ENSG00000231389	-28.272	0.65	0.499	Green
HLA-DRB1	ENSG00000196126	-116.187	0.821	0.567	Green
INKA2	ENSG00000197852	-2.961	0.949	0.826	Green
NIPAL2	ENSG00000104361	-1.886	0.957	0.8	Green
PDE1C	ENSG00000154678	-4.038	0.707	0.585	Green
PGF	ENSG00000119630	-9.118	0.864	0.809	Green
PLCB1	ENSG00000182621	-2.366	0.927	0.847	Green
PPP1R3C	ENSG00000119938	-2.460	0.977	0.874	Green
SAT1	ENSG00000130066	-2.580	0.84	0.818	Green
SEL1L3	ENSG00000091490	-2.085	0.923	0.816	Green
SESN3	ENSG00000149212	-4.034	0.895	0.785	Green
SLC1A1	ENSG00000106688	-2.107	0.883	0.77	Green
SORBS2	ENSG00000154556	-2.650	0.949	0.741	Green
STS	ENSG00000101846	-1.745	0.732	0.675	Green
TBX18	ENSG00000112837	-6.887	0.898	0.734	Green
TINAGL1	ENSG00000142910	-6.759	0.883	0.665	Green
UST	ENSG00000111962	-1.775	0.923	0.875	Green
XG	ENSG00000124343	-7.890	0.875	0.773	Green
ZNF423	ENSG00000102935	-3.254	0.963	0.757	Green

SUPPLEMENTARY TABLE 2. Differentially expressed genes present in the green (related with ageing) and brown (related with youth) modules.
Columns represent *gene symbol*, gene *ensembl* notation, *Fold Change* of the corresponding gene in the comparison young versus old MSC, Module Membership (*MM*), Gene Significance (*GS*) and WGCNA *module*.

	All patients treated with 1 MSC donor		donor <10 years		donor >10 years		p-value
Patients - n (%)	77	100	56	72,7	21	27,3	
Age - years (range)	44	1-68	42,8	1-67	42,3	1-68	0,359
Child - n (%)	12	15,6	9	16,1	3	14,3	0,85
Male - n (%)	55	71,4	42	75	13	61,9	0,263
Time from allo-SCT to enrollment - mean days (median)	108,1	72	115,6	71	88,5	74	0,393
Time from diagnosis aGvHD to enrollment - mean days (median)	21,7	10,5	24,4	13	14,1	10	0,252
Mean number of MSC per infusion - n x 10^6/kg (range)	1,51	1,41	1,43	1,36	1,73	1,9	**0,021**
Primary disease - n (%)							0,54
Myeloid neoplasms	41	53,2	31	55,4	10	47,6	
Lymphoid neoplasms	30	39	21	37,5	9	42,9	
Non malignant disorders	6	8,8	4	7,1	2	9,5	
Stemcell source - n (%)							0,713
PBSC	56	72,7	40	71,4	16	76,2	
BM	7	9,1	5	8,9	2	9,5	
CB	13	16,9	10	17,9	3	14,3	
Type of donor - n (%)							0,157
Sibling	19	24,7	16	28,6	3	14,3	
MUD	58	75,3	40	71,4	18	85,7	
Conditioning regimen - n (%)							0,341
Myeloablative	23	29,9	15	26,8	8	38,1	
Overall GVHD grade - n (%)							0,806
grade 2	22	28,6	17	30,4	5	23,8	
grade 3	47	61	31	55,4	16	76,2	
grade 4	8	10,4	8	14,3	0	0	
Skin GVHD - n (%)	40	51,9	30	53,6	10	47,6	0,647
Gut GVHD - n (%)	66	85,7	47	83,9	19	90,5	0,471
Liver GVHD - n (%)	31	40,3	22	39,3	9	42,9	0,779
Patients pretreated with 2nd line GVHD agents - n (%)	3	3,9	3	5,4	0	0	0,103

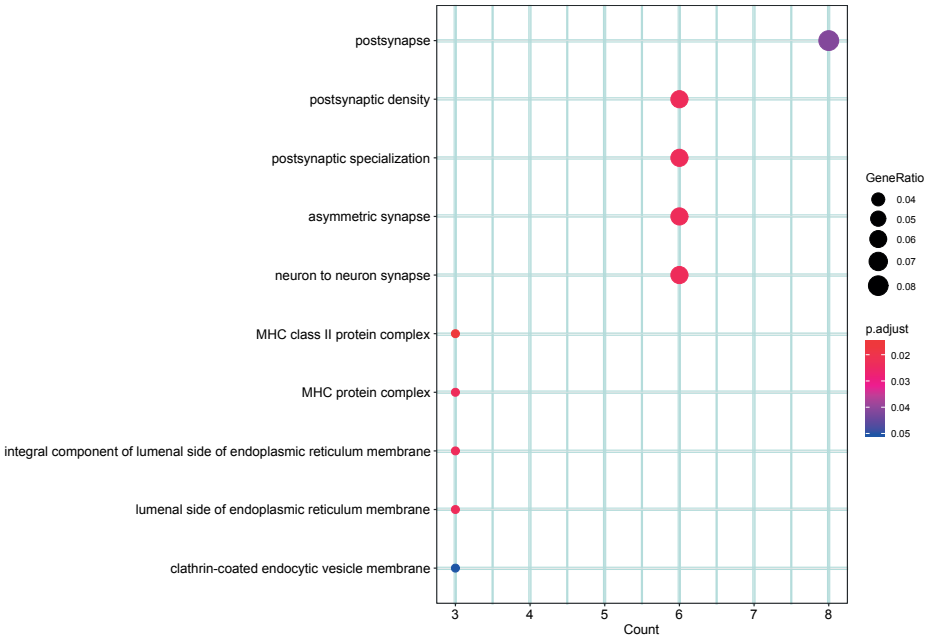
SUPPLEMENTARY TABLE 3. To retrospectively test if the difference in one-year OS between patients treated only with MSC derived from very young BM donors (<10 years) was not due to differences in baseline characteristics between the groups we tested clinical variables. Groups were compared using independent samples t test or Anova in case of >2 groups. aGVHD: acute Graft versus Host Disease, MSC: mesenchymal stromal cells, PBSC: Peripheral Blood Stem Cells, BM: bone marrow, CB: cord blood, MUD: matched unrelated donor.

Gene Symbol	ensembl	Gene Significance	WGCNA module	Gene Fold Change	Protein Regulation	GO pathway
CCAR2	ENSG00000158941	0.842	brown	1.248	Up	RNA splicing
CDC73	ENSG00000134371	0.772	green	-1.185	Up	----------
COL1A2	ENSG00000164692	0.436	brown	NA	Up	----------
ERCC2	ENSG00000104884	0.507	brown	1.158	Up	----------
FEN1	ENSG00000168496	0.831	brown	1.653	Up	Apoptosis
FN1	ENSG00000115414	0.336	green	NA	Up	----------
GNG5	ENSG00000174021	0.512	brown	NA	Up	----------
HNRNPA1	ENSG00000135486	0.849	brown	1.356	Up	RNA splicing
HNRNPC	ENSG00000092199	0.394	brown	1.097	Up	----------
HNRNPH1	ENSG00000169045	0.738	brown	1.196	Up	RNA splicing
HNRNPM	ENSG00000099783	0.838	brown	1.490	Up	RNA splicing
LBR	ENSG00000143815	0.827	brown	1.348	Up	----------
LIN7A	ENSG00000111052	0.747	green	-1.576	Up	----------
LMNB1	ENSG00000113368	0.827	brown	2.037	Up	----------
MED16	ENSG00000175221	0.738	brown	1.339	Up	----------
MYH10	ENSG00000133026	0.191	brown	NA	Up	----------
NUDT21	ENSG00000167005	0.690	brown	1.122	Up	RNA splicing
NUP107	ENSG00000111581	0.850	brown	1.292	Up	----------
PAPOLA	ENSG00000090060	0.408	green	-1.093	Up	----------
PARP1	ENSG00000143799	0.788	brown	1.309	Up	----------
PCNA	ENSG00000132646	0.775	brown	1.352	Up	Cell cycle
PCOLCE	ENSG00000106333	0.819	brown	1.428	Up	----------
POLR2E	ENSG00000099817	0.706	brown	1.187	Up	RNA splicing
PRMT1	ENSG00000126457	0.629	brown	1.189	Up	Cell cycle
PRPF19	ENSG00000110107	0.784	brown	1.279	Up	RNA splicing
RPL21	ENSG00000122026	0.370	green	-1.295	Up	----------
RPL23A	ENSG00000198242	0.398	brown	NA	Up	----------
RPL8	ENSG00000161016	0.785	brown	1.165	Up	----------
RPS15	ENSG00000115268	0.519	brown	1.128	Up	----------
RPS19	ENSG00000105372	0.736	brown	1.231	Up	----------
SLC1A3	ENSG00000079215	0.339	brown	NA	Up	----------
SNRPD2	ENSG00000125743	0.485	brown	1.108	Up	----------
SRSF3	ENSG00000112081	0.743	brown	1.256	Up	RNA splicing
SRSF5	ENSG00000100650	0.368	green	-1.089	Up	----------
SSRP1	ENSG00000149136	0.796	brown	1.246	Up	----------
TBL1XR1	ENSG00000177565	0.466	brown	NA	Up	----------
TOP2B	ENSG00000077097	0.775	brown	1.074	Up	----------
U2AF1	ENSG00000160201	0.635	brown	1.262	Up	RNA splicing
UBE2E1	ENSG00000170142	0.616	green	-1.211	Up	----------
UBTF	ENSG00000108312	0.812	brown	1.214	Up	----------
VCP	ENSG00000165280	0.435	brown	1.102	Up	----------
XRCC5	ENSG00000079246	0.593	brown	1.064	Up	----------
XRCC6	ENSG00000196419	0.611	brown	1.098	Up	----------
AK4	ENSG00000162433	0.696	green	-1.682	Down	Purine metabolism

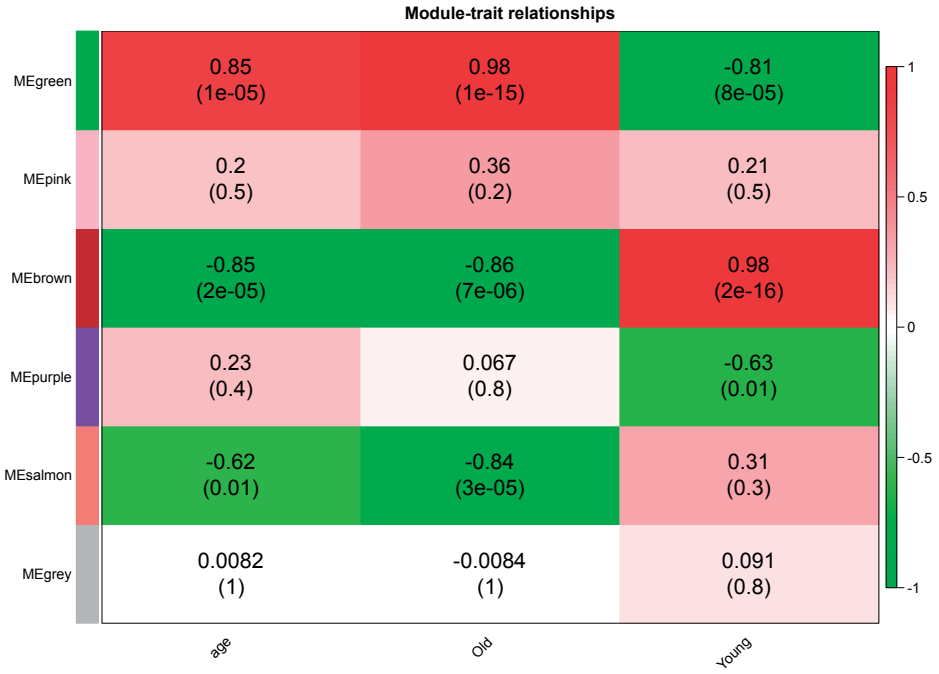
SUPPLEMENTARY TABLE 4. Genes from the green and brown WGCNA modules analyzed with a proteomic approach by Hennrich *et al*. (*continued*)

Gene Symbol	ensembl	Gene Significance	WGCNA module	Gene Fold Change	Protein Regulation	GO pathway
ATP6V1A	ENSG00000114573	0.818	green	-1.367	Down	Autophagy, Purine metabolism
ATP6V1B2	ENSG00000147416	0.458	brown	NA	Down	----------
ATP6V1E1	ENSG00000131100	0.632	green	-1.182	Down	Autophagy
ATP6V1G1	ENSG00000136888	0.758	green	-1.347	Down	Autophagy
BNIP2	ENSG00000140299	0.683	green	-1.196	Down	----------
CASP3	ENSG00000164305	0.600	green	-1.359	Down	Autophagy
CD47	ENSG00000196776	0.543	green	-1.137	Down	ECM reorganization
CDKN1A	ENSG00000124762	0.392	green	-1.195	Down	----------
CHMP5	ENSG00000086065	0.641	green	-1.195	Down	----------
COL4A2	ENSG00000134871	0.193	green	NA	Down	----------
CTSB	ENSG00000164733	0.496	green	-1.153	Down	----------
CTSL	ENSG00000135047	0.601	green	-1.271	Down	ECM reorganization
DYNC1LI2	ENSG00000135720	0.688	green	-1.195	Down	----------
ERLIN2	ENSG00000147475	0.760	green	-1.195	Down	----------
FAS	ENSG00000026103	0.836	green	-1.404	Down	----------
FGF2	ENSG00000138685	0.746	green	-1.395	Down	ECM reorganization
GNAS	ENSG00000087460	0.062	brown	NA	Down	----------
HSD17B12	ENSG00000149084	0.732	green	-1.322	Down	ECM reorganization
RHOC	ENSG00000155366	0.769	brown	1.183	Down	----------
RRM2B	ENSG00000048392	0.821	green	-1.551	Down	Oxidative stress
SH3KBP1	ENSG00000147010	0.736	green	-1.309	Down	----------
SLC1A1	ENSG00000106688	0.770	green	-1.962	Down	----------
SLC44A2	ENSG00000129353	0.258	green	-1.049	Down	----------
STAT1	ENSG00000115415	0.717	green	-1.368	Down	Oxidative stress
STXBP1	ENSG00000136854	0.829	green	-1.491	Down	----------
UGGT2	ENSG00000102595	0.611	green	-1.224	Down	----------
VPS37A	ENSG00000155975	0.668	green	-1.281	Down	Autophagy

SUPPLEMENTARY TABLE 4. Genes from the green and brown WGCNA modules analyzed with a proteomic approach by Hennrich et al. (*continued*)
The direction (up- or down- regulation) of the gene expression from our RNAseq analysis and the proteomic dysregulation are highly coincident. *Gene Symbol,* gene *ensembl* notation, *Gene Fold Change* (fold change from young versus old MSC obtained from Deseq2 RNA seq analysis), *Protein Regulation* (direction of the protein expression -up, upregulated; down, downregulated- obtained in the work of Hennrich *et al.* of young versus old MSC), *Gene Significance* of WGCNA , *WGCNA* assigned *module* and *GO* enrichment *pathway*, were represented.



SUPPLEMENTAL FIGURE 1.
GO enrichment terms of the differentially expressed genes from the *Cell Compartment* module. MHC terms were found to be enriched; Gene ratio, Count divided by the total amount of genes configuring the enriched term; q-value, Bonferroni-Hochberg p-value correction.



SUPPLEMENTAL FIGURE 2. Module-trait correlations from WGCNA results.
In "Y" axes, the different modules, stated by a colour. In "X" axes, the clinical trait studied in this work as a continuous variable (age) or as a binary variable (young, MSC donors <10 years of age; old, MSC donors >20 years of age). Numbers represent the Pearson Correlation (also labelled with a color scale from red – highly correlated- to green -highly uncorrelated-), and the p-value of the correlation between brackets. Green and Brown gene modules show the highest absolute correlation values for age in the binary fashion (old/young) and in the continuous scale (age).

4.1

Prospective evaluation of sequential treatment of sclerotic chronic Graft versus Host Disease with rituximab and nilotinib

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ABSTRACT

Sclerotic chronic Graft Versus Host Disease (cGVHD) still has a large impact on morbidity and mortality after allogeneic hematopoietic stem cell transplantation (HSCT). We performed the first prospective study to test whether sequential therapy of the anti-CD20 antibody rituximab followed by 6 months treatment with tyrosine kinase inhibitor nilotinib is a favorable treatment strategy for patients with sclerotic cGVHD. Twenty-nine patients were included, 24 were available for analysis. We observed objective responses in 71% of patients (2 patients CR, 15 patients PR). Moreover, 2 out of 5 patients suffering from severe ulcerations showed complete resolution of ulcers. Observed responses lasted until the end of study follow up. The majority of responding patients could reduce daily corticosteroid dose with more than 50%. Furthermore, CD5+ B-cells are significantly lower (p=0.007) in responding patients at baseline, proposing a new biomarker predictive for response. In conclusion, sequential treatment of rituximab followed by nilotinib associates with a very high response rate in this difficult to treat patient population. CD5+ B-cells could assist in guiding treatment choices and might be a first step towards more personalized cGVHD treatment. This trial was registered at the Dutch clinical trial registry as NTR1222.

INTRODUCTION

Chronic Graft versus Host Disease (cGVHD) is a devastating complication affecting approximately 60% of patients after allogeneic hematopoietic stem cell transplantation (allo-HSCT). Despite novel low GVHD platforms (1-5) and more individualized ATG applications (6) the number of patients suffering from cGVHD is still rising. Major reasons are increased use of allo-HSCT in older recipients, improvements made in treatments post allo-HSCT prolonging survival and the increased administration of pre-emptive or prophylactic donor lymphocyte infusions(7). A major hurdle for the treatment of cGVHD remains the poor understanding of the diverse pathophysiology of cGVHD complicating treatment decisions and the development of new therapeutic strategies. Generally recommended first-line therapy consists of glucocorticoid therapy combined with calcineurin inhibition. Affected patients require long term use of these immunosuppressive drugs associated with the development of severe side effects and hampered quality of life. Options for second-line therapy are numerous but consensus on the most favorable choice of agent(s) has not been reached. B-cell depletion has shown to have a beneficial effect on fibrosis with reported response rates between 66-86%(8-12). Tyrosine kinase inhibitoion (TKI) with imatinib also showed response rates ranging from 22-79% (13-16). In patients with cGVHD with cutaneous sclerosis response rates to both monotherapy with rituximab and monotherapy with imatinib seem to be worse, 27% and 26% respectively (17). Also, monotherapy with either B-cell depletion or TKI almost never leads to a complete resolution of cGVHD symptoms. We speculate that combining B-cell depletion with rituximab and tyrosine kinase inhibition with nilotinib would result in more profound and durable responses in patients with sclerotic cGVHD. We chose nilotinib due to higher cellular uptake and possible decreased cellular efflux compared to imatinib (18, 19). Both drugs block the intracellular tyrosine kinase c-abl, a downstream target of pro-fibrotic TGF-β signaling, and both drugs are inhibitors of the PDGF receptor that can directly and indirectly, through activation of Src kinases and c-abl, promote extracellular matrix formation (20). In this light we performed a prospective phase II clinical study in which we treated 29 patients with the combination of rituximab and nilotinib. Also patients with ulcerative skin lesions, known to barely respond to monotherapies, were included. In addition, we tested whether our previously identified biomarker (10), which suggest responsiveness to rituximab treatment, can also predict responsiveness to the combination therapy, as many published biomarkers for responsiveness to cGVHD treatment could often not be confirmed in independent prospective cohorts (21).

METHODS

Patients

The clinical trial (amendment to NTR1222, trial registry The Netherlands) with treatment with rituximab and nilotinib was approved by the Institutional Review Board of the University Medical Center Utrecht and is in accordance with the Declaration of Helsinki. All patients gave written informed consent before enrollment.

Patients were eligible when diagnosed with cGVHD with skin localization refractory to or dependent on first-line treatment with steroids (SR-cGVHD) and/or calcineurin inhibitors and ≥18 years old. Patients were ineligible to participate with WHO performance score >2, life expectancy of <6 months, active systemic (viral) infections, treatment with rituximab or TKI in the previous year, inadequate renal or liver function, low neutrophil counts (<1.5x10^9/l), low hemoglobin (<6,2 mmol/l) or low platelets (<75 x10^9/l), a history of pancreatitis, a known impaired cardiac function or were pregnant or breastfeeding.

Study visits were scheduled monthly for the duration of 13 months. At each study visit patients were evaluated according to 2005 NIH cGVHD consensus response criteria working group recommendations (22) as the study was set up before the updated response criteria from 2014 (23). Serial blood sampling was performed every other month. Peripheral Blood Mononuclear Cells (PBMCs) were isolated and lymphocyte numbers were directly measured by Trucount (manufacturers protocol, BD Biosciences). PBMCs were frozen and stored in liquid nitrogen until further analysis. Plasma and serum samples were stored at -80°C.

Evaluation of response

Complete overall response (CR) was defined as resolution of all reversible manifestations in each organ or site. Partial response (PR) was defined as an improvement of more than 50% in at least one cGVHD manifestation without progression in any other organ. Stable disease (SD) was defined by no change in the extent or severity of the disease in any organ. Deterioration of symptoms in a specific organ by a 25% increase in the scale used to measure disease manifestations related to cGVHD was termed progressive disease (PD). Patients were evaluated monthly however final response scoring took place at 13 months after start of the study. Response evaluation was based on NIH response criteria working group report from 2006 (24).

Treatment with rituximab and nilotinib

Rituximab (MabThera®, F. Hoffmann-La Roche Ltd, Basel, Switzerland) was administered intravenously with 4 weekly infusions at a dose of 375mg/m^2. Approximately 1 week after the last rituximab infusion, nilotinib (Tasigna®, Novartis Pharmaceuticals Corporation) was started b.i.d. 300mg. When patients experienced side effects, nilotinib could be reduced to either b.i.d. 200mg or q.d. 400mg or stopped if dose reduction was not sufficient. No dose adjustments were made for grade 1 or 2 toxicity. Any toxicity should be resolved within 28 days in order to resume study drug at the reduced dose.

Flow cytometry

All flow cytometry was performed on an LSR Fortessa (BD, 4 lasers) flow cytometer and data analysis was performed using FACS Diva Software (BD Biosciences). Fluorescent labeled beads (CS&T beads, Becton Dickinson) were used to check the performance and verify optical path and stream flow of the flow cytometer. This procedure enables controlled standardized results and allows the determination of long-term drifts and incidental changes within the flow cytometer. No changes were observed which could affect the results during the study period. Antibodies used for flow cytometry: CD38-PerCP-Cy5.5, CD27-APC, IgM-BrilliantViolet421, CD1d-BrilliantViolet510, CD21-BrilliantViolet711, CD24-PE-CF594, CD10-PE-Cy7, CD70-FITC, CD43-APC, CD27-BrilliantViolet510, CD86-PE-Cy7, CD4-PerCP-Cy5.5, CD28-BrilliantViolet421, TCRγδ-PE, CD8-PE-Cy7, CD127-BrilliantViolet421, CD25-PE, CD16-BrilliantViolet510, CD56-PE-Cy7 (BD), CD3-AlexaFluor700, CD5-PE, HLA-DR-PerCP-Cy5.5, CD20-BrilliantViolet421, CD45RO-BrilliantViolet711 (BioLegend), CD19-eFluor780, FoxP3-APC (eBioscience), CCR7-APC (R&D Systems) and TCRVβ11-FITC, TCRVα24(Beckman Coultier).

Statistical analysis

Power calculation was performed with an alpha of 0.05 and a power of 80% to show a response rate of 30% in 24 patients. Statistical analyses of patient data, construction of Receiver Operating Curves (ROC) and probability of survival by means of Kaplan-Meier estimates were performed using SPSS (IBM Statistics, version 21, Amsterdam, The Netherlands). Data from flow cytometry experiments and multiplex immune assays were performed using GraphPad Prism 6 for Windows (GraphPad Software, La Jolla, CA, USA). Gaussian-distributed groups were compared using Student's t-test. Data not normally distributed were compared using Mann-Whitney U test. Paired comparisons were calculated using Wilcoxon matched-pairs signed rank test. In all cases, a probability level of 5% ($P<0.05$) was considered significant.

RESULTS

Study cohort and clinical response rates

We prospectively tested clinical efficacy of anti CD20 treatment in combination with TKI in SR-cGVHD patients with skin involvement. We hypothesized this combination would have an additive beneficial effect in this patient category. Between January 2012 and November 2015, 29 adult patients with moderate or severe cGVHD were enrolled. We screened 35 patients and 6 patients could not be enrolled due to mainly prolonged QT interval (Figure 1), suggesting we included a representative cohort of heavily pretreated patients (Supp. Figure 1) seen during daily clinical practice. Patient baseline characteristics are depicted in table 1. In short, the majority of patients were male and the median age was 49 years. All patients had received several lines of treatment (range 1-5, supp. Figure 1) before enrollment and were found to be steroid refractory. Two patients had received rituximab prior to study inclusion, their last rituximab doses were administered 27 and 31 months before study entry. Five patients went off study for different

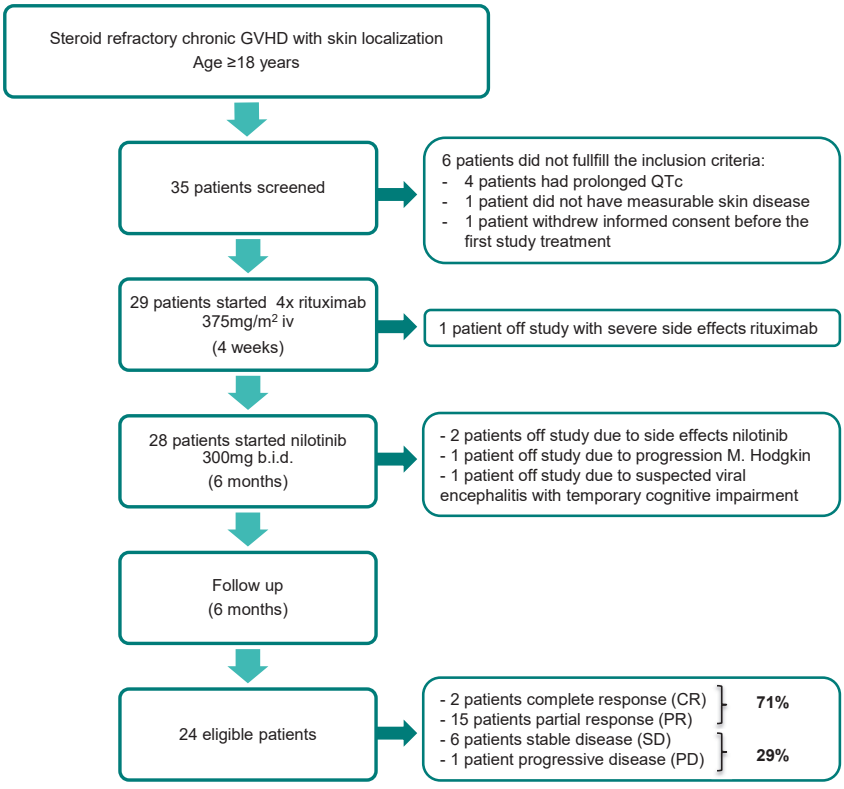

FIGURE 1: Flow chart of study setup and results.

Patients		29	100%
Male sex		22	75,9%
Mean age in years	(range)	48,9	21-70
cGVHD characteristics			
Organ involvement	(%)		
Skin (sclerosis)		29	100%
Joints-fascia		26	89,7%
Mouth		26	89,7%
Eyes		22	75,9%
GI		15	51,7%
Liver		12	41,4%
Lungs		11	37,9%
Genital		11	37,9%
Median number prior regimens	(range)	2	1-5
Median prednisolone dose at start	(mg/kg/day)	0,35	0,1-1,0
Ulcerative chronic GvHD at start		5	17,2%
NIH grade severity cGVHD at start			
severe		22	75,9%
moderate		7	24,1%
Transplantation characteristics			
Donortype			
sibling		13	44,8%
MUD		15	51,7%
haploidentical		1	3,4%
Myeloablative conditioning regimen		7	24,1%
Mean days after allo SCT	(range)	1354	411-3820
Primary diagnosis			
AML		7	
Multiple myeloma		6	
ALL		5	
Lymphoma		4	
MDS		2	
CLL		2	
Myeloproliferative disorder		2	
Aplastic anemia		1	

TABLE 1: Baseline characteristics.
MUD: matched unrelated donor; AML: Acute Myeloid Leukemia; ALL: Acute Lymphoblastic Leukemia; MDS: Myelodysplastic Syndrome; CLL: Chronic Lymphocytic Leukemia.

reasons (Figure 1), therefore 24 patients were eligible for analysis. We observed an overall response in 71% of patients (2 patients CR, 15 patients PR, 95% CI interval for ORR 51-90%). From the two patients who have been treated previously with rituximab, one patient did not tolerate nilotinib and went off study before any response could be evaluated. The other had a stable disease after treatment with rituximab and nilotinib. In order to avoid overestimation of response by early dropouts, we calculated also total response rate including patients that went off study after receiving rituximab and nilotinib. Then response rate remains 69%. Only 1 patient showed progressive disease (PD) and the remaining 6 patients showed stable disease (SD). Moreover, 2 out of 5 patients suffering from severe ulcerations at baseline had a complete resolution of ulcers at the end of the treatment period. Responding patients show a significant decrease (Figure 2, p=0.0012) in cGVHD affected body surface area (BSA) at month 13 of the study compared to baseline. This is mostly explained by a significant reduction in non-moveable sclerosis. From month 7 onwards there was a significant difference between affected BSA in responders vs. non-responders (Figure 2). Not only skin manifestations of cGVHD improved but we also observed responses in other affected organs, mainly oral and gastrointestinal tract, less in genital cGVHD (Figure 3). Differences between baseline and end of study follow up at 13 months are depicted. Interestingly we also observed an improvement in lung cGVHD in 33% of affected patients, however this was not our primary outcome measure and therefore lung function tests were only performed by discretion of the treating physician. No patients fulfilled response criteria at evaluation at 1 month after only receiving rituximab, all showed stable disease.

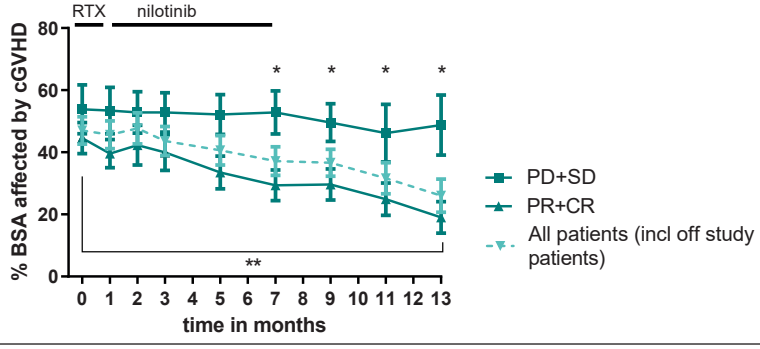

FIGURE 2: Response of total body surface area affected.
BSA: Body Surface Area; RTX: rituximab; PD: progressive disease; SD: stable disease; PR: partial response; CR: complete response. Mean with SEM shown. *: p-value<0.05, **: p-value<0.01

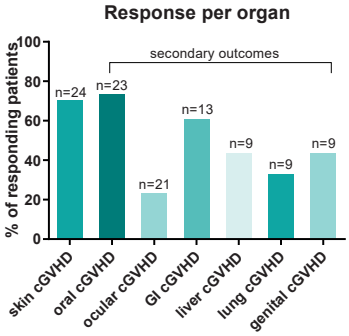

FIGURE 3: Response per organ.
N=total number of patients with cGVHD in the affected organ at the start of the study. Liver and lung cGVHD was not determined by biopsies but by laboratory evaluation of liver enzymes and FEV1 studies respectively.

Impact on immune suppressive drugs

We assessed impact of objective clinical responses on usage of immune suppressive drugs. There was no significant difference in corticosteroid use between PR+CR patients versus SD+PD patients at baseline (data not shown). Corticosteroid dose could be tapered in responding patients (PR and CR, p=0.0131) but not in non-responding patients (Figure 4A). The majority (57%) of responding patients could reduce their daily prednisolone dose with >50%. Also other immunosuppressive drugs (ciclosporin and mycophenolate) could be tapered in the majority of responding patients and to a lesser extent in some patients with stable disease (Figure 4B).

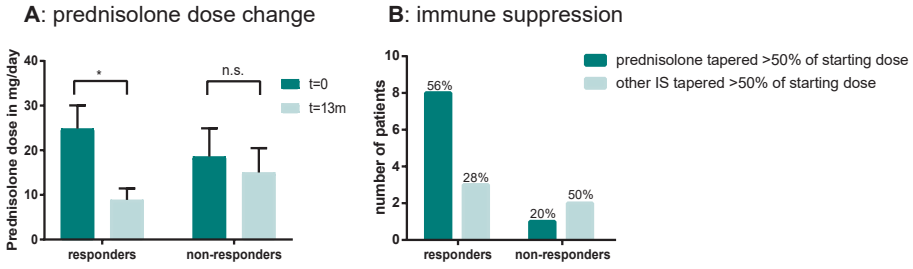

FIGURE 4: Reduction in immune suppression in responding patients.
A: a significant reduction in total use of prednisolone in mg/kg/day during the study period in responding patients (PR+CR patients, n=17, Mann Whitney test, p=0.0131), mean with SEM shown. No significant reduction in prednisolone use in non-responding patients (SD+PD patients, n=7, Mann Whitney test, p=0.7273). B: Number of patients that could taper their immunosuppressive drugs. Percentages above the black bars are calculated on total number of patients that used prednisolone at the start of study.

Side effects and dose reductions

Rituximab was well tolerated except for one patient who showed a neurological syndrome resembling Guillain Barre after 2 infusions and therefore went off study. The patient recovered completely without sequelae. Nilotinib in a starting dose of 300 mg b.i.d., was only tolerated by 10 patients without side effects (Supp. figure 2). Remaining patients needed dose reductions to 200mg b.i.d. which was well tolerated by the majority. Side effects included fatigue, nausea, pain in extremities and prolonged QT-interval on standard ECG monitoring. 38% of patients experienced a serious adverse event (SAE) during the study (Figure 6). We observed no neutropenia, only leukopenia grade 3 in one patient within the context of a viral infection. One patient died due to progressive osteomyelitis with multiresistant bacteria (1-year OS entire cohort 96,5%).

Prospective analysis of biomarker and prediction model

In our earlier study (10) on SR-cGVHD patients treated with monotherapy rituximab we identified a B-cell subset that seemed predictive of clinical response. By analyzing immune subsets (indicated panels in Methods), lower absolute number of CD20+CD5+ B-cells was the only significant predictive parameter (p-value 0.007) at baseline in responding patients (Figure 5). This finding is consistent with the results from our earlier study (10). In particular we observed no significant differences at baseline in either total B-cells, memory or naïve B-cell subsets as opposed to other reports (10, 17). Absolute T-cell numbers in cGVHD patients were lower compared to healthy donors (n=5) and no GVHD controls (n=5), however this was not significant. Responding cGVHD patients did not have significantly different T-cell numbers either at baseline or during follow up. Also regulatory T-cells did not differ between the groups at baseline or during follow up.

To allow upfront decision-making with our cellular biomarker on which patients might benefit most from treatment containing rituximab, we constructed an ROC curve for the absolute number of CD20+CD5+ B-cells with an area under the curve (AUC) of 84,9% (p-value 0,008). With a threshold at 11 cells/µl the positive predictive value is 93,8%. In univariate logistic regression analysis on outcome response, the number of CD5+ B-cells was the only significant variable (p-value 0,004, Supp. table 1).

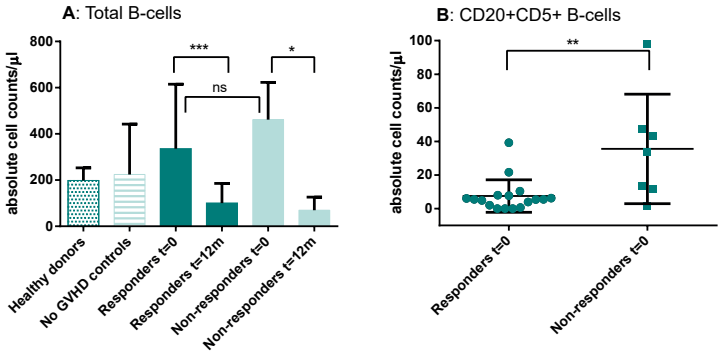

FIGURE 5. Subset of CD5+ B-cells differ at baseline between responding and non-responding patients.

A: no significant differences in total B-cells between healthy donors, no GVHD controls, responders (CR+PR patients) and non-responders (SD+PD patients) at the start of the study. A significant decrease in total B-cells in the patients after receiving rituximab, showing B-cells had not recovered to baseline levels after 1 year. Mean and SD are depicted.

B: Significant difference (p=0,007) in CD5+ B-cells at baseline between responders and non-responders.

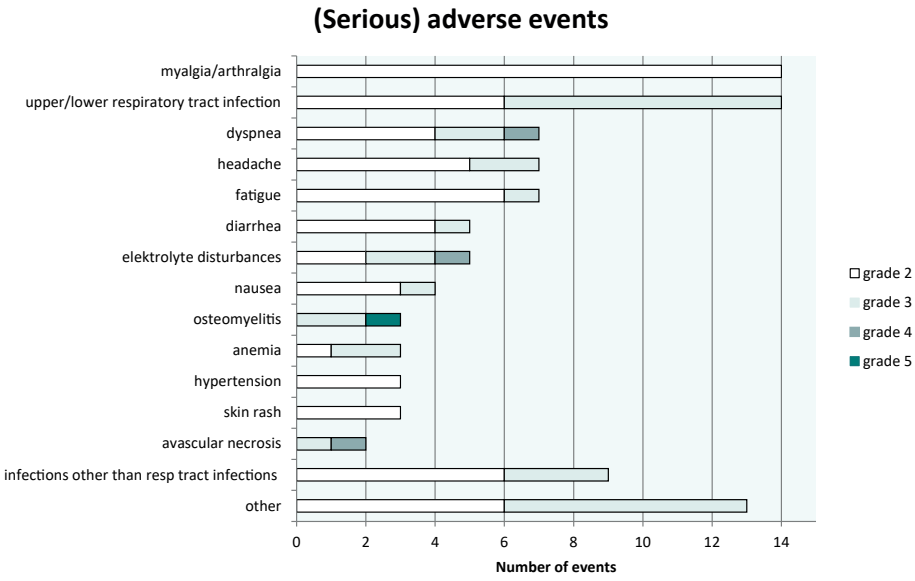

FIGURE 6: (Serious) adverse events during the study. 38% of patients experienced an SAE during the study period.

Most frequently encountered adverse events were myalgia and arthralgia and respiratory tract infections (both with bacterial and viral pathogens). Infections other than respiratory tract infections were grade 3: abdominal sepsis, fever of unknown origin and encephalitis however no pathogens found upon extensive investigations. Grade 2 events were 2 CMV reactivations, 2 sinusitis, 1 conjunctivitis and 1 urinary tract infection. Category 'other' contains as grade 3 events: anorexia, leucopenia, 2 increased GGT at laboratory evaluation, 1 progression of underlying multiple myeloma, 1 pancreatitis and 1 Guillain Barre like syndrome upon infusion with rituximab. Grade 2 events in category 'other' contain: 2 abdominal pain, 1 anorexia, 1 tinnitus, 1 constipation and 1 malaise.

DISCUSSION

We performed a prospective phase II clinical trial in SR-cGVHD patients whom we treated with a combination of rituximab and nilotinib. We observed a clinical response in 71% of patients with 2 patients even reaching a CR. Immune suppression could be tapered in this heavily pretreated patient group. The observed responses were durable and lasted until the end of study follow up when patients had already discontinued the study medication for 6 months. Toxicity was manageable but dose reductions for nilotinib were needed regularly. The majority of responding patients could reduce their daily corticosteroid dose with more than 50%. Interestingly, also in patients with SD immune suppression could be tapered without causing deterioration of cGVHD symptoms. Intriguingly we found a low number of CD5+ B-cells as positive predictor for therapeutic responses in this study, as we also identified in our previous RTX-only based study (10). Validation of this cellular biomarker in other patient cohorts could show if this B-cell subset could identify patients with a high likelihood of response and thus provide a possibility for personalized cGVHD therapy.

Our data suggest combining rituximab with nilotinib might have two major advantages when compared to other treatments. Firstly, responses of patients with ulcerative cGVHD can be expected, as indicated by the response observed in two patients with severe ulcers. This is important since ulcerative cGVHD is considered very difficult to treat. Secondly, we found clinical responses were durable throughout the study follow up period and often even improving further during this period. Response rates up to 70% have also been reported with RTX monotherapy, questioning the true additive value of nilotinib in our study (8-11). However, these favorable outcomes might be a consequence of mainly including good risk patients as evidenced by the recent observation that first line therapy of cGVHD associates with an overall response rate of 83% (25). Outcomes in cGVHD with cutaneous sclerosis response rates to both monotherapy with rituximab and monotherapy with imatinib have been reported to be substantially lower (17). In this light our data demonstrate that also severely affected patient cohorts (76% NIH score severe) with sclerotic skin disease at a mean of 45 months after allogeneic HSCT, which is substantially longer than many other studies (8, 12) including a recent cohort reported to receive ibrutinib (26), benefit. Thus patients even at this late stage of disease benefit most likely substantially from combination therapy with B-cell depletion and TKI. Side effects caused by nilotinib may be a possible limitation and showed not to be less than earlier reported with the use of imatinib in cGVHD (13, 15). The number of patients in our study did not allow for subgroup analysis and therefore we cannot comment on the possible differences

in clinical outcome between patients that were treated according to the study protocol and patients that had a dose reduction because of side effects. As several patients still showed improvement of symptoms after 6 months of treatment, maintenance therapy with a TKI seems appealing. However maintenance therapy in these patients might come at the cost of more severe side effects. Prospective clinical data are needed to obtain more information regarding optimal treatment duration.

Reproducibility of biomarkers in cGVHD is a challenge (21) and therefore biomarkers are currently not used in daily practice to help decide which treatment to choose. In this light, it is intriguing that we could confirm our earlier identified cellular biomarker in an independent cohort of SR-cGVHD patients. However as the number of patients in our trial was limited, these results should be further explored in larger patient cohorts. We found the subset of B-cells that is identified by CD5 positivity to be significantly higher among non-responders to rituximab and nilotinib. The optimal cutoff value was slightly different in the current cohort possibly explained by fully normalized and accredited flow cytometry measurements. The biological role of these CD5+ B-cells is still largely unknown although they are known to also produce autoreactive antibodies (27). In auto-immune diseases such as rheumatoid arthritis (RA) CD5 production is elevated and in ANCA+ vasculitis the percentage of CD5+ B-cells can predict relapse (28). As cGVHD resembles auto-immune diseases in many aspects it seems reasonable this subset is also involved in the complex pathophysiology of cGVHD.

One limitation of our study is that NIH 2005 response criteria were used as the study was initiated before 2014 and CRFs from the study did not include all necessary parameters for recalculations in line with current NIH guidelines. However, considering the rapid developments with several new promising treatment options for SR-cGVHD patients such as ruxolitinib (29, 30), and ibrutinib (26, 31) guidance on which patients might benefit from the one or other compound will be essential in order to wisely use restricted resources. Considering that prospective clinical studies comparing different compounds will be scarce and many compounds never enter phase III trials as they are no longer of great market value, utilizing a rational approach with biomarkers validated in different cohorts might provide a first rational for a personalized cGVHD therapy. We therefore propose to take the number of circulating CD20+CD5+ B-cells along in future clinical trials. Corticosteroid refractory patients with low circulating CD20+CD5+ B-cells could then be considered to be first treated with rituximab and nilotinib or another TKI. In contrast, patients with high CD20+CD5+ B-cells could then immediately proceed to other possible treatment options. Unfortunately, no biomarkers have yet been identified that can predict clinical response with

either ruxolitinib or ibrutinib. However, our observation that several patients not responding to rituximab and nilotinib still responded to ruxolitinib (29) suggests that different modes of action are active during cGVHD. Future trials would benefit from an intensive biomarker monitoring, to better dissect responders from non-responders.

In summary, we provide evidence that combining rituximab and nilotinib can generate long lasting responses in severely affected patients and even allow complete resolution of ulcers as well as improvement of lung GVHD. We also show low circulating CD20+CD5+ B-cells to be a possible new biomarker to predict response. Including biomarkers into the decision on which compound is used for cGVHD treatment could be a first step towards a personalized and cost effective cGVHD therapy.
Supplementary information is available at *Bone Marrow Transplantation's* website.

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CONFLICT OF INTEREST

JK is cofounder and chief scientific officer of GADETA. His work is partly supported by a grant from Novartis however Novartis had no part in the design, analysis or interpretation of the data or the writing of the manuscript. This work was further supported in part by grants from the Dutch Cancer Society to LvdW: KWF-UU 2011-5250 and to JK KWF-UU 2010-4669, 2013-6426, 2014-6790 and 2015-7601.

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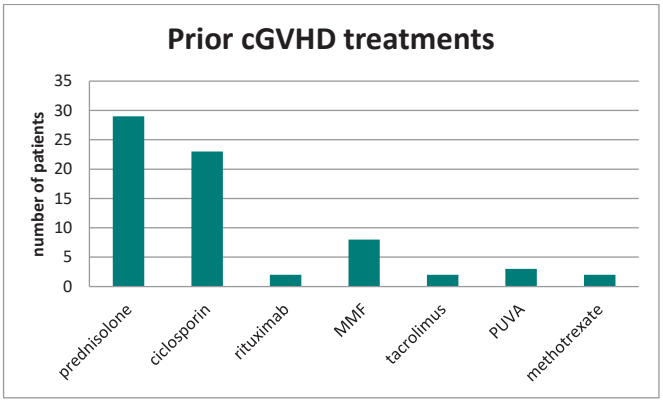
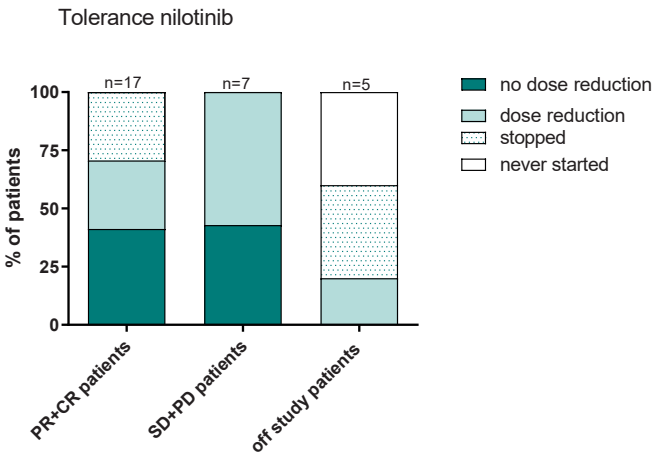
SUPPLEMENTAL MATERIALS



SUPPLEMENTARY FIGURE 1. Number of patients that received this specific cGVHD treatment before entering the study.
Oral agents were only included when they had at least been administered for three months. Rituximab treatment consisted in both patients of 4 cycles of 375 mg/m^2 intravenously.
MMF: mycophenolate mofetil
PUVA: Psoralen plus UltraViolet A



SUPPLEMENTARY FIGURE 2: the majority of both responding and non-responding patients needed a dose reduction of nilotinib due to side effects.
The reduced dose of 200 mg b.i.d. was mostly well tolerated. Five patients in the PR+CR group stopped nilotinib due to side effects.

Factor	OR	95% CI	P value
Age	1,032	0,975-1,092	0,283
Sex	942360338	0,00-	0,999
Type of HSCT donor (sib vs MUD)	0,311	0,046-2,110	0,232
CD5+ B-cells < 11/µl blood	0,022	0,002-0,293	**0,004**
prior number of cGVHD therapies	0,201	0,037-1,084	0,062

SUPPLEMENTARY TABLE 1: Logistic regression analysis on outcome response.
OR: Odds Ratio; CI: Confidence Interval

4.2

Tyrosine Kinase Inhibitor levels matter in treating chronic GVHD

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TYROSINE KINASE INHIBITOR LEVELS MATTER IN TREATING CHRONIC GVHD

Graft versus Host Disease (GVHD) remains the major complication of allogeneic stem cell transplantation (allo-SCT) thereby limiting its widespread use despite many recent advances in transplantation techniques and management (1). In particular, chronic GVHD (cGVHD) results in substantial mortality and reduced quality of life. Tyrosine kinase inhibition (TKI) using imatinib has shown clinical efficacy in cGVHD (2-6). Other TKI's such as nilotinib as monotherapy or in combination with rituximab have been investigated (7, 8). Observed overall response rates of 25% (partial response after six months monotherapy nilotinib) and 71% (complete and partial response after sequential treatment of rituximab and nilotinib) were found (7, 8). Many kinase inhibitors show both exposure-response and exposure-toxicity relationships (9) and consequently therapeutic drug monitoring is widely accepted and already applied in Chronic Myeloid Leukemia (CML) treatment. Here we performed a retrospective analysis of samples obtained from our prospective clinical trial (8) to study this relationship.

In our prospective phase II clinical trial in patients with steroid refractory (SR) sclerotic cGVHD (8), patients were sequentially treated with 4 cycles of rituximab followed by six months of nilotinib 300 mg b.i.d. treatment. A dose reduction to 200 mg b.i.d. was allowed when patients experienced side effects. The study was conducted in accordance with the Declaration of Helsinki and the protocol was reviewed by the local ethics committee. All patients provided written informed consent. This trial was registered at the Dutch clinical trial registry as NTR1222. During the study blood sampling at regular intervals was performed. Plasma samples were stored at -80˚C. Response was assessed after 13 months of follow-up. Nilotinib concentrations in plasma were determined using a validated high-performance liquid chromatography tandem mass spectrometry assay (10).

Trough levels were calculated using the algorithm proposed and validated by Wang *et al.*(11).:

$$C_{\min} = \mathrm{Conc}_{\mathrm{measured}} * 0.5^{\left(\frac{\tau - \mathrm{TAD}}{t1/2}\right)},$$

Where tau is the dosing interval (i.e. 12 hours for nilotinib), TAD is the time since last dose and $t_{1/2}$ is the nilotinib half-life (i.e. 17 hours). Samples drawn before the time point at which the maximal concentration is reached (i.e. 3 hours) or samples drawn more than 12 hours after the last dose were excluded from the analysis. Number of samples ranged from 1-4 per patient and were obtained at sampling times 2, 3, 5 or 7 months. For statistical analysis, for each patient the mean of the

available trough levels were used. Twenty-nine patients were included in the clinical trial of which 24 were eligible for response evaluation (Supplementary table 1). In total, 17 patients (71%) showed a response (15 patients achieving a partial response and two patients a complete response). The remaining 7 patients were termed non-responders (6 patients had stable disease and 1 patient had progressive disease). The reference therapeutic range for nilotinib was 829-1500 µg/L, as used as reference in CML.

Nilotinib trough concentrations were available for 18 patients of 24 that completed the study (11 responders and 7 non-responders). Samples from remaining patients were unavailable due to practical reasons, 1 patient forgot to withhold his medication at time of sampling and from 1 patient the wrong tubes were sampled. Four remaining missing patients all used nilotinib less than one month due to side effects as described earlier (8) and were therefor not sampled for trough concentrations. Patients of whom trough concentrations were available all used nilotinib for a duration of 4,5-6 months (median 6 months). Nilotinib concentration in one patient was not measurable which indicated non-compliance. This patient was omitted from further analysis as he was already during the trial suspected of non-compliance due to erroneous medication administration. The number of samples did not differ between responders (median 2,5 and mean 2,5) and non-responders (median 2 and mean 2,14). Nilotinib trough concentrations in responders were significantly higher than in non-responders (mean nilotinib trough level 1071 µg/L versus 782 µg/L, p=0.03, figure 1A). Interestingly, no significant difference in trough concentration was observed between patients treated with 300 mg b.i.d. and 200 mg b.i.d. (1067 µg/L vs 974 µg/L, p=0.66, figure 1B). We could not relate this to drug interactions e.g. by use of azoles. Remarkably, no significant difference in nilotinib trough concentration was found between patients with and without reported drug toxicity (Figure 1C). Nilotinib 300 mg b.i.d. was tolerated by 10 patients whereas the remaining patients needed a dose reduction to 200 mg b.i.d.. Duration of nilotinib use did not impact trough concentrations (data not shown). Most frequently observed side effects were fatigue, myalgia, nausea, respiratory tract infections and prolonged QT interval as described before (8). In the 4 patients with nilotinib trough concentrations available before and after dose reduction from 300 to 200 mg b.i.d. we observed a decrease in mean trough concentrations from 1163 µg/L to 928 µg/L (p 0.33, Figure 1D). Prednisolone doses did not differ between responding and non-responding patients at any time point throughout the study period (data not shown) however due to the limited sample size these date are purely descriptive and should be interpreted with caution.

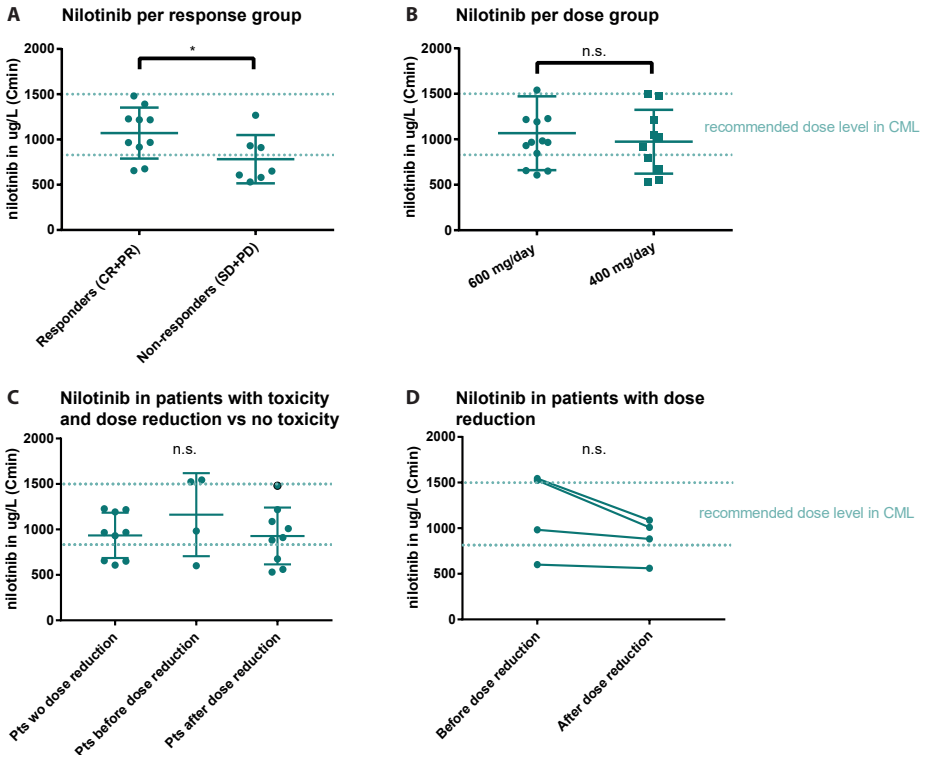

FIGURE 1. Nilotinib trough levels.
A: Responding patients (1 CR and 10 PR) show significantly higher mean nilotinib trough concentrations compared to non-responders (1071 µg/L vs 782 µg/L, p=0.03). B: Mean nilotinib trough concentrations did not differ between the different dose groups of 300 vs 200 mg b.i.d. (1067 µg/L vs 974 µg/L, p=0.66). C: Mean trough concentrations did not differ between patients not experiencing toxicity (935 µg/L) versus patients that experienced toxicity on nilotinib 300 mg b.i.d. (1163 µg/L) and accordingly received a dose reduction to 200 mg b.i.d. (928 µg/L). D: Nilotinib trough concentrations before and after dose reduction in 4 patients show a non-significant (1163 µg/L vs 885 µg/L, p=0.125) decrease.

In our prospective cohort of cGVHD patients we showed that trough concentrations of nilotinib correlate with clinical outcome and the optimal concentration range seems to overlap with the recommended concentration range in CML. As these are results of a posthoc analysis from a phase 2 study not primarily set up to study this relationship results are exploratory. We conclude that dose monitoring of nilotinib should be considered for patients treated for cGVHD, especially in non-responding patients. Intriguingly our study might also explain the lower response rates of only 25% reported by Chen *et al.* (7), as median trough serum nilotinib concentration reached in this study were only 407 µg/L which is more than 50% lower than the observed trough levels in our study.

We could not clearly link trough levels of nilotinib with observed toxicity. This might be due to the fact that the initial doses administered in our study are lower as compared to dosing in CML patients. Alternatively the lack of correlation is a consequence of the small sample size, therefor we cannot make strong conclusions regarding toxicity.

In conclusion, a possible concentration-effect relationship for nilotinib on response of cGVHD was observed, indicating that therapeutic drug monitoring of nilotinib might improve response rates for patients receiving TKI and rituximab for the treatment of cGVHD.

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DISCLOSURE OF CONFLICTS OF INTEREST

JK is cofounder and scientific advisor of GADETA. His work is partly supported by a grant from Novartis; however, Novartis had no part in the design, analysis, or interpretation of the data or the writing of the manuscript.

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SUPPLEMENTAL MATERIALS

Baseline characteristics			
Patients		17	100%
Male sex		15	88%
Mean age in years	(range)	49.3	22-68
Responders		10	59%
cGVHD characteristics			
Organ involvement	(%)		
Skin (sclerosis)		17	100%
Joints-fascia		16	94%
Mouth		13	76%
Eyes		16	94%
GI		7	41%
Liver		7	41%
Lungs		12	71%
Genital		5	29%
Severe cGVHD (NIH criteria)		14	78%
Transplantation characteristics			
Donortype			
sibling		9	50%
MUD		8	44%
haploidentical		1	6%
Myeloablative conditioning regimen		3	17%
Mean days after allo SCT	(range)	1432	538-3820
Primary diagnosis			
AML		3	18%
Multiple myeloma		5	29%
ALL		2	12%
Lymphoma		1	6%
MDS		2	12%
CLL		1	6%
Myeloproliferative disorder		2	12%
Aplastic anemia		1	6%
Nilotinib characteristics			
Patients with mean nilotinib Cmin within CML range		11	65%
Patients with mean nilotinib Cmin below CML range			
in responding patients		2 out of 10	
in nonresponding patients		4 out of 7	

SUPPLEMENTARY TABLE 1. Baseline characteristics
MUD: Matched Unrelated Donor; AML: Acute Myeloid Leukemia; ALL: Acute Lymphoblastic Leukemia; MDS: Myelodysplastic Syndrome; CLL: Chronic Lymphocytic Leukemia; CML: Chronic Myeloid Leukemia.

5

Current options for treatment of Graft versus Host Disease: a new guideline

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SAMENVATTING:

Allogene stamceltransplantatie (SCT) is een belangrijke behandelingsmodaliteit met een curatieve potentie voor een verscheidenheid aan (voornamelijk hematologische) aandoeningen. Ondanks voortschrijdende ontwikkelingen in het veld van allogene SCT blijft graft-versus-host ziekte (GVHD) na SCT een belangrijk klinisch probleem, wat gepaard gaat met een significante morbiditeit en mortaliteit. Voornamelijk door het ontbreken van gedegen vergelijkende studies is de behandeling van GVHD weinig uniform. Daarnaast is het adequaat scoren van de ernst van de aandoening niet eenvoudig. We beogen met dit artikel een handleiding te geven voor een meer uniforme en zoveel mogelijk op wetenschappelijk bewijs gebaseerde behandeling van zowel acute als chronische GVHD.

ABSTRACT:

Allogeneic stem cell transplantation (SCT) is an important treatment modality with curative potential for a diversity of (mainly hematological) disorders. Despite continuous development in the field of SCT, graft-versus-host disease (GVHD) remains an important clinical problem that causes significant morbidity and mortality. Due to a paucity of thorough comparative studies, treatment of GVHD is not uniform. We aim to provide a guideline for a more uniform and evidence-based treatment of both acute and chronic GVHD.

INLEIDING:

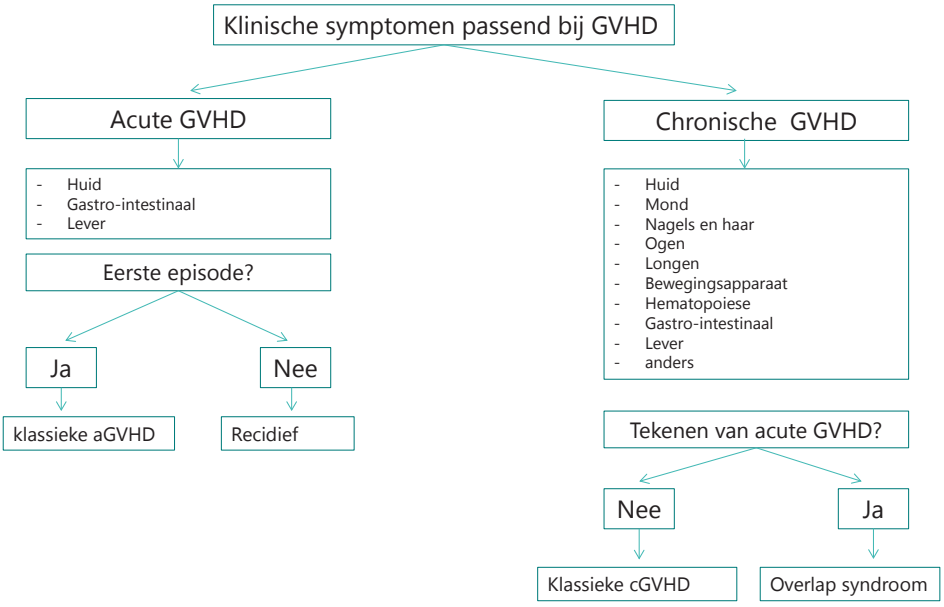
Allogene stamceltransplantatie (SCT) is een belangrijke behandelingsmodaliteit met curatieve potentie voor verschillende (voornamelijk hematologische) aandoeningen. Het transplantaat vervangt niet alleen de hematopoëse van de donor in het beenmerg, maar is ook de bron van een nieuw immuunsysteem. De werking van de SCT vloeit voort uit de reactie van effectorcellen van dit donor-immuunsysteem tegen de maligne cellen van de patiënt (graft-versus-tumor effect, GvT). Deze van de donor afkomstige immuuncellen kunnen zich echter ook keren tegen gezonde weefsels van de patiënt, wanneer zij die als 'non-self' herkennen. Dit kan tot grote schade leiden aan gezonde weefsels (graft-versus-host disease, GVHD). Idealiter zou er na SCT een sterk GvT-effect optreden, zonder de ongewenste GVHD. Helaas is het tot op heden niet goed mogelijk gebleken deze effecten van elkaar te scheiden. Door beïnvloeding van de activiteit van het immuunsysteem middels immuunsuppressiva wordt gepoogd een balans te vinden tussen het optreden van gunstige en ongewenste immunologische effecten. Desondanks ontstaat bij 15-60% van de patiënten GVHD. Ondanks voortschrijdende ontwikkelingen in het veld van allogene SCT gaat GVHD gepaard met aanzienlijke morbiditeit en mortaliteit. Voorheen werd het onderscheid tussen acute GVHD en chronische GVHD gemaakt o.b.v. het moment van optreden (respectievelijk vóór of na de 100^{e} dag post-SCT). In de praktijk blijkt deze strikte scheiding op grond van enkel een tijdscriterium kunstmatig. De huidige National Institutes of Health (NIH)-consensus onderscheidt twee hoofdvormen van GVHD, namelijk acute GVHD en chronische GVHD, maar ook een overlapsyndroom (zie ook Figuur 1). Het onderscheid wordt gemaakt op grond van tijdstip van optreden in combinatie met de symptomatologie.

Algemeen is duidelijk dat corticosteroïden de hoeksteen zijn van de behandeling van zowel acute als chronische GVHD. Over de vraagstukken welke andere interventies met corticosteroïden gecombineerd zouden moeten worden, of wat een volgende therapie zou moeten zijn bij falen van corticosteroïden is veel minder duidelijkheid. Behandelstrategieën zijn bij gebrek aan gerandomiseerde studies soms gebaseerd op retrospectieve cohort studies, maar vaak ook op 'expert opinion' en ervaringen binnen het eigen centrum. Behandelstrategieën kunnen daardoor uiteenlopen, mede afhankelijk van lokale ervaringen met en beschikbaarheid van verschillende middelen. Om de zorg voor Nederlandse patiënten post-SCT te waarborgen is er behoefte aan een meer uniforme en zoveel mogelijk op wetenschappelijk bewijs gebaseerde landelijke richtlijn.

Dit artikel heeft als doel de op de HOVON-website gepubliceerde 'HOVON behandelrichtlijn GVHD' onder de aandacht te brengen. Het is onzes inziens ook voor niet-transplanterende, maar wel verwijzende hematologen belangrijk om

hiervan up-to-date kennis te hebben. Achtereenvolgens zullen de behandeling van acute GVHD en chronische GVHD besproken worden. De pathofysiologie van GVHD, conditioneringschema's, transplantaatbewerkingen en regimes voor GVHD-profylaxe vallen buiten de scope van deze behandelrichtlijn en worden hier dan ook niet besproken.

Daar de ontwikkelingen in de behandeling van GVHD snel verlopen, is de meest actuele versie van deze behandelrichtlijn te vinden op de HOVON-website: http://www.hovon.nl/upload/File/werkgr_stamcel/GvHD richtlijn definitief.pdf.



FIGUUR 1. Nomenclatuur GVHD. Aangepast naar figuur 1 uit 'Current issues in cGVHD' Socie & Ritz, Blood 2014.
NB: conform de richtlijn is besloten de indeling naar progressief (actieve of acute GVHD overgaand in chronische GVHD), quiescent (chronische GVHD gevolgd op eerdere episode van acute GVHD) en de novo chronische GVHD achterwege te laten.

ACUTE GVHD

Acute GVHD presenteert zich typisch in de eerste maanden na SCT en kan optreden bij patiënten die na hun SCT profylactisch immuunsuppressiva gebruiken, alsook bij patiënten die na hun SCT geen immuunsuppressiva (meer) gebruiken.

Acute GVHD uit zich in één of meer van de volgende drie orgaansystemen: huid (maculopapulaire rash), lever (stijging van bilirubine en/of transaminasen) en tractus digestivus (diarree). Uiteraard is er bij het optreden van deze verschijnse-

len een differentiaaldiagnose, die onder andere ook infectieuze en medicamenteuze oorzaken omvat. Waar mogelijk dient de diagnose GVHD daarom middels histologie bevestigd te worden. Na vaststelling van acute GVHD, wordt de betrokkenheid per orgaan gestadieerd en de algehele ernst van de GVHD gegradeerd volgens de Glucksbergcriteria, zie Tabel 1 en 2 (1).

Stadium	Huid	Lever	Darm
0	Geen rash	Bilirubine <33 µmol/l	<500 mg diarree/dag of misselijkheid
1	Maculopapulaire rash < 25% totale huid oppervlak	Bilirubine 34-50 µmol/l	500 - 999 ml diarree /dag
2	Maculopapulaire rash 25-50% totale huid oppervlak	Bilirubine 51-102 µmol/l	1000 - 1499 ml diarree /dag
3	Maculopapulaire rash >50% totale huid oppervlak	Bilirubine 103-254 µmol/l	1500 - 1999 ml diarree /dag
4	Gegeneraliseerde erythrodermie + bullae (evt. desquamatie)	Bilirubine > 255 µmol/l	> 2000 ml diarree /dag; of ernstige buikpijn met of zonder ileus

TABEL 1 Stadiëringvan aGvHD per target orgaan

Graad	Huid		Lever		Darm
I	stadium 1-2		stadium 0		stadium 0
II	stadium 3	*of*	stadium 1	*of*	stadium 1
III	stadium 0-3	*en*	stadium 2-3	*of*	stadium 2-4
IV	stadium 4	*of*	stadium 4		-

TABEL 2 Gradering van acute GVHD op grond van de orgaanstadia

BEHANDELING BIJ ONTSTAAN ACUTE GVHD TIJDENS IMMUUNSUPPRESSIE

Box 1 toont het algoritme voor behandeling van acute GVHD in hoofdlijnen, welke hier wordt toegelicht.

Behandeling graad I

Bij de behandeling van GVHD graad I, die per definitie enkel de huid betreft, is in principe het beleid dit eerst met lokale immuunsuppressiva te behandelen.

In geval van acute GVHD graad I met meerdere risicofactoren voor een ernstig verloop (>= 3 uit Box 2), valt pre-emptieve systemische behandeling als bij graad II te overwegen.

Behandeling graad II

Hoewel er in de formele gradering geen onderscheid wordt gemaakt, wordt er in de dagelijkse praktijk soms voor gekozen om graad II te splitsen in graad IIa (stadium 1 van de darm i.c.m. lever stadium 0 en huid stadium 0-2) en graad IIb (huid stadium 3 of lever stadium 1 i.c.m. darm stadium 0-1).

Behandeling graad III en IV

Volgens Box 1.

Graad I
- continueren lopende immuunsuppressiva, evt. hervatten reeds gestaakte ciclosporine
- topicale steroïden, bijv. triamcinolon 0.1% (klasse 2) of mometason 0.1% (klasse 3) crème

Graad IIa
- start prednison 1 mg/kg/dag (SORT (Strength of Recommendation Taxonomy [2]) level C), naast de lopende immuunsuppressiva.
- zo mogelijk inclusie in studie (HOVON 112; http://www.hovon.nl/studies/studies-per-ziektebeeld/sct.html?action=showstudie&studie_id=96&categorie_id=11)

Graad IIb
- start prednison 2 mg/kg/dag + calcineurineremmer op therapeutische spiegel OF
- zo mogelijk inclusie in studie (HOVON 112; http://www.hovon.nl/studies/studies-per-ziektebeeld/sct.html?action=showstudie&studie_id=96&categorie_id=11)

Graad III-IV
- start prednison 2 mg/kg/dag ([3] SORT level B) + calcineurineremmer op therapeutische spiegel OF
- zo mogelijk inclusie in studie (HOVON 112; http://www.hovon.nl/studies/studies-per-ziektebeeld/sct.html?action=showstudie&studie_id=96&categorie_id=11)

BOX 1: Hoofdlijn van de behandeling van acute GVHD, indien ontstaan tijdens gebruik van immuunsuppressie

Algemene opmerkingen

Bij patiënten met een relatieve contra-indicatie voor hoge dosis steroïden, zoals bij actieve infecties, kan worden overwogen met de helft van de aanbevolen doses steroïden te starten. In een gerandomiseerde studie leek lager gedoseerd starten geen negatieve invloed te hebben op overleving, maar bij de ernstigere acute GVHD was in dat geval wel vaker additionele immuunsuppressie nodig (2).

Buiten studieverband is er op basis van de huidige literatuur geen indicatie voor tripeltherapie in de eerstelijnsbehandeling van acute GVHD, hetgeen betekent dat myfortic of MMF wordt gestaakt indien dit nog als profylaxe werd gegeven.

- donor = matched unrelated donor (MUD)
- MUD met een minder dan 10/10 HLA-match
- vrouwelijke donor voor een mannelijke patiënt
- GVHD profylaxe met alleen calcineurineremmer/mycofenolaat mofetil (MMF)
- hoog CD3-getal in het transplantaat (> 250x10^6/kg)
- optreden van GVHD vroeg na transplantatie (vóór herstel van neutrofielen > 1.0x10^9/L)
- subtherapeutische ciclosporine spiegels na transplantatie (< 150 µg/L op 2 opeenvolgende metingen)

BOX 2: Risicofactoren voor een ernstig verloop van acute GVHD

BEHANDELING BIJ ONTSTAAN ACUTE GVHD ZONDER IMMUUNSUPPRESSIE

Wanneer acute GVHD optreedt tijdens immuunsuppressie is dit doorgaans een ongewenst effect en wordt er ingegrepen zoals boven beschreven. Er zijn ook situaties waarin alloreactiviteit is gewenst en er geen immuunsuppressie wordt gegeven, zoals bijvoorbeeld na therapeutische donorlymfocyteninfusie (DLI; een infusie van donor T-cellen om extra immuuntherapeutisch effect te bewerkstelligen, bijvoorbeeld wegens recidief ziekte of gemengd chimerisme met hoge kans op ziekterecidief). In dat geval kan men enige mate van GVHD accepteren alvorens die volledig te behandelen en kan men een getrapt starten van de GVHD-behandeling overwegen. Dit vergt een gepersonaliseerde aanpak. In de overweging dienen onder andere te worden meegenomen: de ernst van de GVHD, de aangedane organen, de snelheid van progressie van de GVHD en het doel van de alloreactiviteit. De genoemde tijdsintervallen zijn derhalve slechts een leidraad. Box 3 vat de hoofdlijn voor dit beleid samen.

Graad I:
- triamcinolon 0.1% 1-2 dd, bij onvoldoende effect clobetasol 0.05% (Dermovate), eventueel prednisolon 30-60 mg 1dd p.o.

Graad II:
- prednisolon 1 dd 1 mg/kg. Indien bij snelle respons van GVHD het taperen van prednisolon snel mogelijk is, geen ciclosporine bijgeven. Indien na 7-10 dagen geen effect van hoge dosis prednison: ciclosporine 2 dd 1.5 mg/kg i.v. toevoegen en prednisolon afbouwen (bij nierproblemen MMF 2 dd 15 mg/kg p.o. of i.v.)

Graad III-IV:
- prednisolon 2 dd 1 mg/kg i.v. Indien bij snelle respons van GVHD het taperen van prednisolon snel mogelijk is, geen ciclosporine bijgeven. Indien na 7-10 dagen geen effect van hoge dosis prednison: ciclosporine 2 dd 1.5 mg/kg i.v. toevoegen (bij nierproblemen MMF 2 dd 15 mg/kg p.o. of i.v.)

BOX 3: Hoofdlijn van de behandeling van acute GVHD, indien ontstaan zonder gebruik van immuunsuppressie

REFRACTAIRE ACUTE GVHD

Er kan op grond van de literatuur geen sluitende definitie gegeven worden van steroïd-refractoriteit; ook internationaal bestaat hierover geen duidelijke consensus (3). Bij het besluit om over te gaan tot een volgende lijn van therapie dienen tenminste de volgende aspecten overwogen te worden:

- welke organen zijn betrokken: huid (minder bedreigend) versus lever versus darm (potentieel levensbedreigend)
- de ernst van de GVHD-verschijnselen
- de mate van gebrek aan respons (persisterende partiële respons versus fulminante progressie)
- de in- en exclusiecriteria van klinische studies voor deze patiëntengroep

NB: indien sprake is van recidief of progressie van GVHD tijdens of na het afbouwen van de eerstelijnsbehandeling met corticosteroïden en een calcineurineremmer, dan wordt dit niet als refractaire ziekte beschouwd. De eerstelijnsbehandeling dient dan opnieuw volgens het protocol gegeven te worden.

BEHANDELING REFRACTAIRE ACUTE GVHD

- bij voorkeur behandeling in studieverband:
  - o HOVON 113 (http://www.hovon.nl/studies/studies-per-ziektebeeld/sct.html?action=showstudie&studie_id=97&categorie_id=11)
  - o JAK-2 remmer studies (nog te openen)
- indien er geen lopende studie is of geen inclusie mogelijk is, dan gelden mesenchymale stromale cellen (MSC), anti-thymocytenglobuline (ATG), MMF of ruxolitinib als meest aangewezen behandelmogelijkheden, waarbij de werkgroep vooralsnog geen voorkeur kan uitspreken op basis van de huidige beschikbare literatuur. In de literatuur beschreven opties voor tweedelijns behandeling worden beschreven in Tabel 3.

Behandeling	dosering	% respons	overleving	SORT level	Referenties
MSC	1-2 x 10^6 cellen/kg op dag 1, dag 8, (dag 22)	72% overall respons (39% complete respons)	50% (6 mnd)	B	(4)
		50% complete respons	44% (1 jaar)	B	(5)
ATG (ATGAM, paard)	zesmaal om de dag 30 mg/kg/dag *of* 12 dagen 15 mg/kg/dag	57%	45% (6 mnd)	B	(6)*
ATG (Thymoglobuline, konijn)	5 achtereenvolgende dagen 3mg/kg/dag	56%	55% (6 mnd)	B	(7)**
MMF	2dd 20mg/kg	60% (CR op dag 28)	64% (9 mnd)	B	(8)
		55%	30% (2 jaar)	C	(9)
Etanercept	2x per week 25 mg sc. gedurende maximaal 8 weken	46%	Niet afdoende beschreven	C	(10, 11)
Ruxolitinib	start 2 dd 5 mg p.o., indien geen toxiciteit ≥ graad 2 na 3 dagen verhogen naar 2 dd 10 mg p.o.	82%	79% (6 mnd)	C	(12, 13)
Psoraleen-UV-A-bestraling (PUVA) bij geïsoleerde GVHD van de huid	Via dermatoloog	x	X	C	(14)
Infliximab	1x/week 10mg/kg	59%	Niet afdoende beschreven	C	(15)

TABEL 3
* Enkele oudere, retrospectieve studies met slechtere respons en overlevingsdata buiten beschouwing gelaten.
** Konijn-ATG gecombineerd met methylprednisolon geeft geen betere uitkomst dan methylprednisolon alleen.

ONDERSTEUNENDE THERAPIE

Het gebruik van immuunsuppressiva verhoogt het risico op infecties en virus-reactivaties. Er wordt profylaxe geadviseerd tegen bacteriële, virale, fungale en protozoale infecties. Aangezien lokale omstandigheden en behandelprotocollen kunnen verschillen is het onderstaande een voorbeeld:

- cotrimoxazol 1dd 480mg
- valaciclovir 2dd 500mg
- voriconazol 2dd 200mg

Indien patiënt en/of donor voor de transplantatie positief waren, moet de patiënt regelmatig gecontroleerd worden op EBV- en CMV- reactivatie, volgens lokaal protocol.

Met de cumulatieve dosis steroïden neemt het risico op osteoporose toe. Er wordt profylaxe geadviseerd volgens lokaal protocol, middels bijvoorbeeld:

- alendroninezuur 1x/week 70mg (of equivalent)
- calcium- en vitamine D-suppletie

CHRONISCHE GVHD

Diagnose en ernst

De diagnose van chronische GVHD wordt gesteld o.b.v. opgestelde NIH criteria die in 2014 zijn gereviseerd (16). De diagnose wordt gesteld o.b.v. tenminste 1 diagnostisch criterium of tenminste 1 onderscheidend criterium (Box 4) met daarbij een biopt dan wel specifieke functietest (bijv. longfunctie, Schirmertest) en evaluatie door een specialist welke chronische GVHD aantoont in hetzelfde, dan wel een ander orgaan. Infecties en andere aandoeningen kunnen klachten geven die overeenkomen met de symptomen van chronische GVHD. Deze dienen te worden uitgesloten.

Orgaan of lokalisatie	Diagnostisch (Voldoende om de diagnose cGVHD te stellen)	Onderscheidend[1] (Passend bij cGVHD, maar onvoldoende om de diagnose te mogen stellen)	Andere verschijnselen of onclassificeerbare entiteiten [2]	Algemeen (Passend bij acute en chronische GVHD)[3]
Huid	Poikiloderma Lichen planus-achtige kenmerken Sclerotische kenmerken Morphea-achtige kenmerken Lichen sclerosus-achtige kenmerken	Depigmentatie Papulosquameuze laesies	Dis-/anhydrose Ichthyose Keratosis pilaris Hypopigmentatie Hyperpigmentatie	Erytheem Maculopapuleuze rash Pruritus
Nagels		Dystrofie Longitudinale lijnvorming, splitten of broosheid Onycholyse Pterygium unguis Verlies van nagels (meestal symmetrisch; waarbij alle nagels zijn aangedaan)		
Hoofdhuid en lichaamsbeharing		Spontane ontwikkeling van littekenweefsel of alopecia van de hoofdhuid (na herstel van chemotherapie) Verlies van lichaamsbeharing	Dunner wordend hoofdhaar, meestal ongelijk verdeeld, met vergroving van het haar (niet verklaard door endocriene of andere oorzaak) Premature vergrijzing van het haar	
Mond	Lichen planus-achtige kenmerken	Xerostomie Mucoceles Mucosale atrofie Ulcera Pseudomembranen		Gingivitis Mucositis Erytheem Pijn

TABEL 4 (vertaald vanuit (16)) (*continued*)

Orgaan of lokalisatie	Diagnostisch (Voldoende om de diagnose cGVHD te stellen)	Onderscheidend[1] (Passend bij cGVHD, maar onvoldoende om de diagnose te mogen stellen)	Andere verschijnselen of onclassificeerbare entiteiten [2]	Algemeen (Passend bij acute en chronische GVHD)[3]
Ogen		Nieuw ontstane droogheid, zanderigheid of pijnlijkheid van de ogen Cicatriciale conjunctivitis Keratoconjunctivitis sicca Confluerende gebieden van punctaat keratopathie	Fotofobie Periorbitale hyperpigmentatie Blefaritis (erytheem van de oogleden met oedeem)	
Genitalia	Lichen planus-achtige kenmerken Lichen sclerosus-achtige kenmerken Vrouwen: vaginale verlittekening of clitorale/labiale verkleving Mannen: Phimosis of urethrale/ meatus verlittekening of stenose	Erosies Fissuren Ulcera		
Tractus digestivus	Oesophagusweb Stricturen of stenose in het bovenste 2/3 deel van de oesophagus		Exocriene pancreasinsufficiëntie	Anorexie Misselijkheid Braken Diarree Gewichtsverlies *Failure to thrive* (kinderen)
Lever				Totaal bilirubine, AF, ALAT >2 × ULN
Luchtwegen	Bronchiolitis obliterans gediagnosticeerd middels longbiopsie BOS[4]	Air trapping en bronchiectasieën op CT-thorax	Cryptogene organiserende pneumonie Restrictieve longziekte	

TABEL 4 (vertaald vanuit (16)) *(continued)*

Orgaan of lokalisatie	Diagnostisch (Voldoende om de diagnose cGVHD te stellen)	Onderscheidend[1] (Passend bij cGVHD, maar onvoldoende om de diagnose te mogen stellen)	Andere verschijnselen of onclassificeerbare entiteiten [2]	Algemeen (Passend bij acute en chronische GVHD)[3]
Spieren, fascia, gewrichten	Fasciitis Stijfheid van gewrichten of contracturen secundair aan fasciitis of sclerose	Myositis of polymyositis[5]	Oedeem Spierkrampen Arthralgie of arthritis	
Hematopoietisch en immunologisch			Thrombocytopenie Eosinofilie Lymfopenie Hypo- of hypergammaglobulinemie Auto-antistofvorming (AIHA en ITP)	
Anders			Fenomeen van Raynaud Pericard- of pleuravocht Ascites Perifere neuropathie Nefrotisch syndroom Myasthenia gravis Cardiale geleidingsstoornis of cardiomyopathie	

TABEL 4 (vertaald vanuit (16))
AF = alkalische fosfatase; ALAT= alanine aminotransferase; AIHA= autoimmuun hemolytische anemie; ITP= idiopathische thrombocytopenische purpura. ULN= Upper Limit of Normal
1 In alle gevallen dienen een infectie, medicamenteus effect, maligniteit of andere oorzaak te worden uitgesloten.
2 Kan worden beschouwd als passend bij cGVHD als de diagnose is bevestigd.
3 Algemeen wordt beschouwd als verschijnselen passend bij zowel acute als chronische GVHD.
4 BOS kan als een diagnostisch verschijnsel van cGVHD worden beschouwd mits er ook sprake is van een karakteristiek verschijnsel of symptoom in een ander orgaan.
5 De diagnose cGVHD kan alleen worden gesteld middels biopt.

De ernst van de ziekte wordt vervolgens bepaald op basis van een scoringssysteem dat tevens is opgesteld door de NIH diagnosis and staging working party (16), welke score formulieren volledig zijn opgenomen in Appendix A. De score bestaat uit de categorieën 'mild', 'moderate' en 'severe' en is gebaseerd op het aantal betrokken organen en de ernst van de chronische GVHD in die organen. Belangrijk om te vermelden is dat in het algemeen een score van 3 in een orgaan al leidt tot de classificatie 'severe' echter al bij een longscore van 2 is dit ook het geval. Het exact vastleggen en goed classificeren van de ernst van chronische GVHD kan lastig en tijdrovend zijn. Idealiter wordt dit dan ook gedaan door ervaren artsen of verpleegkundig specialisten en ook volgens het NIH scoringssysteem vastgelegd in het (digitale) dossier.

De diagnose chronische GVHD wordt gesteld o.b.v. tenminste 1 diagnostisch criterium OF tenminste 1 onderscheidend criterium (zie tabel 4) met daarbij een biopt of specifieke functietest.

- 'Mild':
  - 1 of 2 organen betrokken met score ≤ 1 EN
  - Longscore 0
- 'Moderate':
  - 3 of meer organen aangedaan met score ≤ 1 OF
  - Tenminste 1 orgaan (uitgezonderd longen) met een score 2 OF
  - Longscore 1
- 'Severe':
  - Tenminste 1 orgaan met score 3 OF
  - Longscore 2 of 3

Opmerkingen:

- De hoogste van de twee huid scores wordt gebruikt voor de totale score
- Bij het scoren van de long wordt de FEV1 gebruikt i.p.v. de klinische ernst van de symptomen
- Indien de volledige afwijking in een orgaan wordt veroorzaakt door een niet chronische GVHD ziekte, wordt de score van dit orgaan NIET meegenomen in de totale score
- Indien de afwijking in een orgaan multifactorieel bepaald is (GVHD + andere ziekte) wordt de score van dit orgaan WEL meegenomen in de totale score

BOX 4: diagnose en gradering chronische GVHD

De zogenaamde 'Seattle criteria' waarbij enkel onderscheid wordt gemaakt tussen 'limited' en 'extensive disease' worden niet meer gebruikt.

BEHANDELING

Systemische behandeling is alleen geïndiceerd bij patiënten met 'moderate' of 'severe' chronische GVHD. Bij milde chronische GVHD kan meestal worden

volstaan met lokale therapieën, deze behandelingen vallen buiten de scope van de richtlijn. Wel is multidisciplinaire behandeling vaak noodzakelijk, waarbij de meest geconsulteerde specialismen de dermatologie, longgeneeskunde, oogheelkunde en gynaecologie zijn.

Eerstelijns behandeling bestaat uit prednisolon 0,5-1 mg/kg per dag, gevolgd door een afbouwschema met of zonder calcineurineremmer (ciclosporine of tacrolimus) (17) (18). Deze medicatie kan gradueel worden afgebouwd zodra de chronische GVHD is hersteld (17). In het algemeen wordt prednisolon het eerst afgebouwd vanwege de te verwachten toxiciteit op de lange termijn. Afbouwschema's van prednisolon in de setting van chronische GVHD kunnen verschillen, maar zijn allemaal gebaseerd op het feit dat prednisolon gedoseerd wordt op de laagst mogelijke dosis waarbij de chronische GVHD nog onder controle is. Indien er tijdens het afbouwen weer een exacerbatie optreedt van de chronische GVHD dan dient de dosering prednisolon weer te worden verhoogd met 2 dosisniveaus, zie tabel 4.

Week	Dosis (mg/kg lichaamsgewicht)
0	1,0
2	1,0 / 0,5* (starten 2 weken na objectiveerbare verbetering)
4	1,0 / 0,25*
6	1,0 / 0* (voortzetten totdat alle klinische symptomen zijn verdwenen)
8	0,7 / 0* (starten na resolutie van alle klinische symptomen)
10	0,55 / 0*
12	0,45 / 0*
14	0,35 / 0*
16	0,25 / 0*
18	0,20 / 0*
20	0,15 / 0*
22	0,10 / 0*

TABEL 4 (Aangepast naar tabel 5 uit (17)). Mogelijk om persoonlijk behandelschema op te stellen via https://hematologiegroningen.nl/protocollen/content/ib_tpchr.htm.
* toediening om de dag.

Er is geen toegevoegde waarde voor azathioprine (19), thalidomide (20), mycofenolaat mofetil (21) of plaquenil in de eerstelijns behandeling van chronische GVHD (SORT level B). Toevoeging van ciclosporine aan prednisolon laat geen significant verbeterde overleving of kortere behandelduur zien (22). Mogelijk is er

wel sprake van een steroïdsparend effect (SORT level B) waardoor de combinatie van ciclosporine met prednisolon wel wordt aangeraden.

Patiënten komen in aanmerking voor tweedelijns behandeling indien er sprake is van (23):

- Progressie ondanks behandeling met prednison 1mg/kg/dag gedurende minstens twee weken
- Stabiele ziekte ondanks behandeling met prednison >0,5mg/kg/dag gedurende minstens 1 maand
- Uitblijven van respons na 1 maand standaardbehandeling
- Niet kunnen afbouwen van prednison <1mg/kg/dag twee maanden na starten
- Significante toxiciteit van de behandeling

Het bepalen van de respons dient indien mogelijk plaats te vinden volgens de NIH Consensus development project criteria voor klinische studies bij chronische GVHD (24), zie ook appendix B. Hierbij is per orgaan aangegeven wat een complete respons, partiële respons of progressie behelst.

Internationaal is er geen consensus over de optimale tweedelijns behandelstrategie. Keuzes voor behandelingen worden nog steeds gemaakt o.b.v. ervaring, gebruiksgemak, risico op toxiciteit, lokale beschikbaarheid en kans op relapse van de primaire ziekte. Een overzicht van de beschikbare therapieën en mate van beschikbaar wetenschappelijk bewijs volgens de SORT criteria (25) staat gegeven in tabel 5, waarbij de werkgroep vooralsnog geen voorkeur uit kan spreken en derhalve de volgorde geen hiërarchie aangeeft. Dit onderstreept de noodzaak voor het verrichten van prospectieve klinische studies bij deze patiënten. Uiteraard verdient het dan ook de voorkeur om patiënten met refractaire chronische GVHD te behandelen in studieverband indien mogelijk. Op korte termijn zal er in Nederland waarschijnlijk worden gestart met een industrie gesponsorde studie naar JAK1/JAK2 inhibitie middels ruxolitinib voor deze patiënten categorie.

Patiënten met actieve chronische GVHD en/of behandeling hiervan dienen te worden beschouwd als immuungecompromitteerd waarbij profylaxe zoals in de setting van acute GVHD is gerechtvaardigd.

Behandeling	dosering	% respons	overleving	SORT level	Referenties
Extracorporele fotoferese (ECP)*:	12-24 weken	64-70% (CR+PR)	70-78% na 1 jaar	A	(26, 27)
Rituximab	375mg/m^2 iv 1x/week gedurende 4 weken	27-66% (CR+PR)	84% na 1 jaar	B	(28-30)
Imatinib	100-200mg/dag	30-79% (CR+PR, in ref 30 2%CR and 28% PR, in ref 31 37%CR and 42%PR)	84% na 1,5 jaar	B	(30-33)
Rituximab + nilotinib	RTX 375mg/m^2 iv 1x/week gedurende 4 weken met aansluitend nilotinib 2d200mg ged. 6 maanden	71% (8% CR, 63% PR)	96,5% na 1 jaar	B	(34)
MSC	1-4 infusies van 0.6 x10^6 cellen/kg/ lichaamsgewicht	74% (21%CR, 53%PR)	78% na 2 jaar	B	(35)
Mycofenolaat mofetil	2x/dag 1000mg (mediane dosis Onishi *et al.* 1500mg/dag)	7/11** en 6/23 (CR+PR)	onbekend	B	(36, 37)
Ruxolitinib	2x/dag 5-10mg	85% (7%CR, 78%PR)	97.4% na 6 maanden	B	(13)
Ibrutinib	1x/dag 420mg	67% (21% CR, 45% PR)	onbekend	B	(38)
Methotrexaat	7,5mg/m^2 /week	78% (50%CR, 28%PR)	92% na 1 jaar	B	(39)
Bortezomib	Dosis escalatie schema	5/10 ptn PR	onbekend	C	(40)

TABEL 5. Huidige behandelmodaliteiten voor chronische GVHD, rangschikking op basis van niveau van wetenschappelijk bewijs.
*: In Nederland alleen mogelijk in VU MC Amsterdam; **: slechts 1/11 patiënten had extensive chronische GVHD

CONCLUSIE:

Ondanks vooruitgang op verschillende gebieden in de transplantatiegeneeskunde blijft GVHD een frequent voorkomend probleem met significante mortaliteit en morbiditeit. Vooral chronische GVHD kan zich op verschillende wijzen uiten en meerdere orgaansystemen aantasten, wat vaak leidt tot een verminderde kwaliteit van leven. De huidige behandelstrategieën voor zowel acute als chronische GVHD zijn vaak niet gebaseerd op fase 3-studies. Mede daardoor lopen, behoudens het gebruik van corticosteroïden, de behandelstrategieën uiteen, vooral in het geval van steroïd-refractaire GVHD. Door een gebrek aan gedegen vergelijkende studies is het moeilijk een hiërarchie te adviseren in de behandelingsmogelijkheden. We beogen met deze richtlijn een voorzet te doen voor een meer uniforme behandeling van deze patiëntencategorie. Waar mogelijk heeft behandeling in studieverband de voorkeur. We pleiten voor het nauwkeurig en correct scoren van GVHD en de respons op behandeling. Multidisciplinaire behandeling van (met name chronische) GVHD met aandacht voor kwaliteit van leven is een must.

NIH score formulier chronische GVHD (vertaald van (16))

	SCORE 0	SCORE 1	SCORE 2	SCORE 3
PERFORMANCE SCORE:	– Asymptomatisch en normaal functionerend (ECOG 0; KPS of LPS 100%)	– Symptomatisch, volledig ambulant, echter beperkt in zware fysieke handelingen (ECOG 1, KPS of LPS 80-90%)	– Symptomatisch, ambulant, in staat tot zelfzorg, >50% van wakkere tijd uit bed (ECOG 2, KPS of LPS 60-70%)	– Symptomatisch, hulpbehoevend bij zelfzorg, >50% of wakkere tijd in bed (ECOG 3-4, KPS of LPS <60%)
HUID[1] **Gradering % lichaamsoppervlakte (BSA)** **GVHD verschijnselen die gegradeerd dienen te worden middels BSA:** **Vink aan:** – Maculopapulaire rash/erytheem – Lichen planus-achtige kenmerken – Sclerotische kenmerken – Papulosquameuze laesies of ichthyose – Keratosis pilaris-achtig beeld	– Niet aangedaan	– 1-18% BSA	– 19-50% BSA	– >50% BSA
Gradering huidverschijnselen:	– Geen sclerotische verschijnselen		– Oppervlakkige sclerotische verschijnselen (in staat tot oppakken huid)	**Alle aanvinken die van toepassing zijn** – Diepe sclerotische verschijnselen – Niet in staat oppakken huid – Verminderde mobiliteit – Ulceratie

	SCORE 0	SCORE 1	SCORE 2	SCORE 3
Andere huidverschijnselen NIET gegradeerd middels BSA: **Vink alle aan die van toepassing zijn:** – Hyperpigmentatie – Hypopigmentatie – Poikiloderma – Ernstige of gegeneraliseerde pruritus – Haargroei aangedaan – Nagels aangedaan – Afwijking aanwezig maar volledig verklaard door NIET GVHD oorzaak, specificeer: ……..				
MOND Lichen planus achtige verschijnselen aanwezig: – Ja – Nee	– Geen symptomen	– Milde symptomen, met zichtbare afwijkingen, echter geen significante beperking van orale intake	– Matige symptomen met zichtbare afwijkingen, **met** gedeeltelijke beperking van orale intake	– Ernstige symptomen, met zichtbare afwijkingen, **met** forse beperking van orale intake
– *Afw. aanwezig maar volledig verklaard door niet GVHD oorzaak, specificeer* ……..				
OGEN Keratoconjunctivitis sicca (KCS) bevestigd door oogarts: – Ja – Nee – Niet onderzocht	– Geen symptomen	– Milde droogheid ogen zonder invloed op ADL (□ 3 x per dag bevochtigende oogdruppels noodzakelijk)	– Matige droogheid van de ogen met invloed op ADL (>3x per dag bevochtigende oogdruppels noodzakelijk of traanbuisplugjes), **ZONDER** nieuwe visusstoornis ten gevolge van KCS	– Ernstige droogheid van de ogen met significante invloed op ADL (speciale bril of scleralenzen noodzakelijk om klachten te verminderen) **OF** niet in staat te werken door oogklachten **OF** visusstoornissen ten gevolge van KCS
– *Afw. aanwezig maar volledig verklaard door niet GVHD oorzaak, specificeer* ……..				

	SCORE 0	SCORE 1	SCORE 2	SCORE 3
LEVER	– Normaal totaal bilirubine en ALAT of AF < 3 x BNW	– Normaal totaal bilirubine met ALAT ≥ 3-5 x BNW of AF ≥ 3 x BNW	– Verhoogd totaal bilirubine maar ≤ 50 µmol/L of ALAT > 5 x BNW	– Verhoogd totaal bilirubine > 50 µmol/L
– *Afw. aanwezig maar volledig verklaard door niet GVHD oorzaak, specificeer* ……				
LONGEN[3] **Symptoom-score:**	– Geen symptomen	– Milde symptomen (kortademigheid na het beklimmen van 1 trap)	– Matige symptomen (kortademigheid na lopen over gelijkvloerse ondergrond)	– Ernstige symptomen (kortademigheid in rust; zuurstoftherapie noodzakelijk)
Long-score: Longfunctietesten: – niet uitgevoerd	– FEV1 ≥ 80%	– FEV1 60-79%	– FEV1 40-59%	– FEV1 □ 39%
– *Afw. aanwezig maar volledig verklaard door niet GVHD oorzaak, specificeer* ……				
GEWRICHTEN EN FASCIA P-ROM score: Schouder (1-7: Elleboog (1-7): Pols/vinger (1-7): Enkel (1-4):	– Geen symptomen	– Milde strakheid van armen of benen, normale of mild verminderde range of motion (ROM) **EN** geen invloed op ADL	– Strakheid van armen of benen **OF** gewrichtscontracturen, erytheem veroorzaakt door fasciitis, matig verminderde ROM **EN** milde tot matige invloed op ADL	– Contracturen **MET** significant verminderde ROM **EN** significante invloed op ADL (niet mogelijk om zelf veters te strikken, knoopjes los te maken etc.)
– *Afw. aanwezig maar volledig verklaard door niet GVHD oorzaak, specificeer* ……				

	SCORE 0	SCORE 1	SCORE 2	SCORE 3
Andere indicatoren, klinische manifestaties of complicaties gerelateerd aan cGVHD (vink aan en geef een gradering naar gelang de ernst (0-3) gebaseerd of functionele beperking wanneer mogelijk (geen = 0, mild = 1, matig = 2, ernstig = 3)				
Ascites (serositis)___ Myasthenia Gravis___ Polymyositis___ Thrombocyten <100,000/µl ___		Pericardvocht___ Nefrotisch syndroom___ Gewichtsverlies > 5%[2] zonder GI symptomen___		Pleuravocht___ Perifere neuropathie___ Eosinofilie > 500µl___ Anders___
TOTALE ERNST GVHD **(Naar mening van beoordelaar)**	– Geen GVHD	– Milde GVHD	– Matige GVHD	– Ernstige GVHD

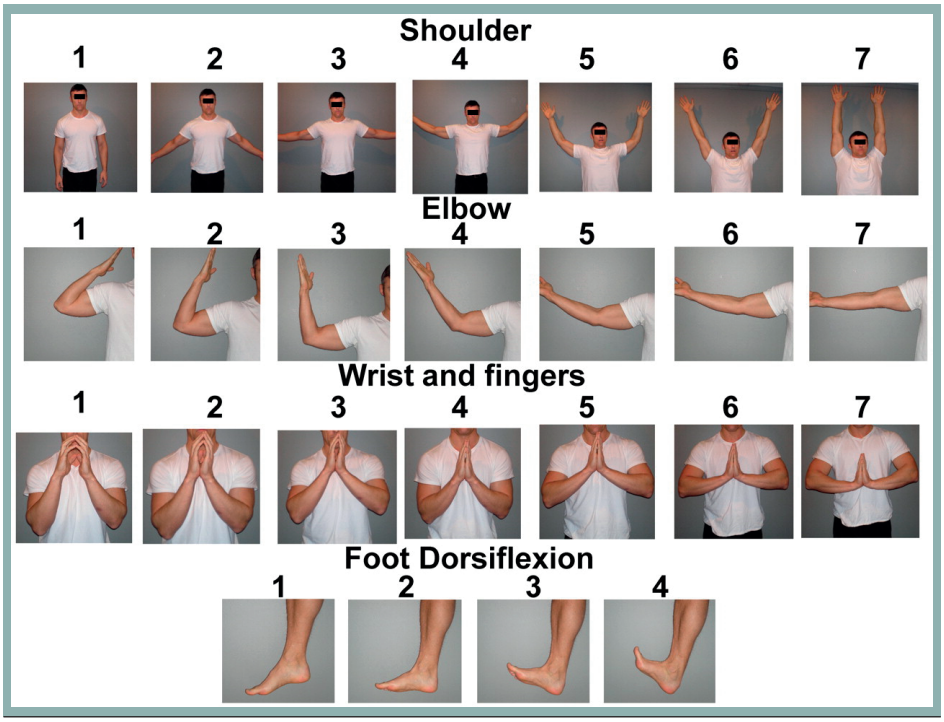
ECOG = Eastern Cooperative Oncology Group; KPS, Karnofsky Performance Status; LPS, Lansky Performance Status; BSA, body surface area; ADL, algemene dagelijkse levensverrichtingen; AF, alkalische fosfatase; ALAT, alanine aminotransferase; BNW, boven de normaalwaarde

[1] Huidgradering dient zowel het percentage huidoppervlakte (BSA) dat is aangedaan als het type huidafwijking te beschrijven. Indien hiertussen een discrepantie bestaat OF indien er oppervlakkige sclerotische laesies aanwezig zijn (score 2), maar er sprake is van verminderde mobiliteit of ulceratie (score 3), dan dient de hoogste score te worden gebruikt in de definitieve gradering.

[2] Gewichtsverlies in 3 maanden.

[3] Longgradering dient te worden uitgevoerd door middel van zowel de symptomen als de FEV1. FEV1 moet in de uiteindelijke score worden gebruikt indien er een discrepantie bestaat tussen symptomen en FEV1-waarden.

[4] Uitgevoerd door specialist of speciaal opgeleide beoordelaar.



Photographic Range of Motion (P-ROM)
(P-ROM figure adapted from figure 2 from (41)).

APPENDIX B

Overzichtstabel responsbepaling NIH Consensus Development project on criteria for clinical trials in chronische GVHD (24).

Responsbepaling in chronische GVHD klinische studies gebaseerd op klinische parameters

Orgaan	Complete respons	Partiele respons	Progressie
Huid	NIH huid score 0 na eerdere betrokkenheid	Afname NIH huid score met ≥1 punt	Toename NIH huid score met ≥1 punt, behalve van 0 naar 1
Ogen	NIH oog score 0 na eerdere betrokkenheid	Afname NIH oog score met ≥1 punt	Toename NIH oog score met ≥1 punt, behalve van 0 naar 1
Mond	NIH gemodificeerde OMRS 0 na eerdere betrokkenheid	Afname NIH gemodificeerde OMRS met ≥2 punten	Toename NIH gemodificeerde OMRS met ≥2 punten
Oesophagus	NIH oesophagus score 0 na eerdere betrokkenheid	Afname NIH oesophagus score met ≥1 punt	Toename NIH oeosphagus score met ≥1 punt, behalve van 0 naar 1
Hoge tractus digestivus	NIH hoge tr. digestivus score 0 na eerdere betrokkenheid	Afname NIH hoge tr. digestivus score met ≥1 punt	Toename NIH hoge tr. digestivus score met ≥1 punt, behalve van 0 naar 1
Lage tractus digestivus	NIH lage tr. digestivus score 0 na eerdere betrokkenheid	Afname NIH lage tr. digestivus score met ≥1 punt	Toename NIH lage tr. digestivus score met ≥1 punt, behalve van 0 naar 1
Lever	Normaal ALAT, AF en totaal bilirubine na eerdere verhoging van 1 of meer van deze parameters	Afname van ≥ 50%	Verhoging van 2x de ULN
Longen	- Normaal %FEV1 na eerdere betrokkenheid - Indien longfunctietesten niet beschikbaar een NIH long symptoom score van 0 bij eerdere betrokkenheid	- Toename van ≥10% voorspelde absolute waarde van %FEV1 - Indien longfunctietesten niet beschikbaar een afname van NIH long symptoomscore met ≥1 punt	- Afname van ≥10% voorspelde absolute waarde van %FEV1 - Indien longfunctietesten niet beschikbaar een toename van NIH long symptoomscore met ≥1 punt, behalve van 0 naar 1
Gewrichten en fascie	Zowel NIH gewricht en fascie score 0 als P-ROM score 25 na eerdere betrokkenheid bij tenminste 1 meting	Afname NIH gewricht en fascie score met ≥1 punt OF toename P-ROM score met 1 punt voor willekeurig gewricht	Toename NIH gewricht en fascie score met ≥1 punt OF afname P-ROM score met 1 punt voor willekeurig gewricht
Globaal	Door clinicus gescoorde totale ernst cGVHD score 0	Door clinicus gescoorde ernst totale cGVHD score afname ≥2 punten op 0-10 punten schaal	Door clinicus gescoorde ernst totale cGVHD score toename ≥2 punten op 0-10 punten schaal

OMRS: Oral Mucosa Rating Scale
ULN: Upper Limit of Normal
AF: Alkalisch Fosfatase
P-ROM: Photographic Range Of Motion

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6

Potential beneficial effects of cytomegalovirus infection after transplantation

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ABSTRACT

Cytomegalovirus (CMV) infection can cause significant complications after transplantation, but recent emerging data suggest that CMV may paradoxically also exert beneficial effects in two specific allogeneic transplant settings. These potential benefits have been underappreciated, and are therefore highlighted in this review.

Firstly, after allogeneic hematopoietic stem cell transplantation (HSCT) for acute myeloid leukemia (AML) using T cell and Natural Killer (NK) cell-replete grafts, CMV reactivation is associated with protection from leukemic relapse. This association was not observed for other hematologic malignancies This anti-leukemic effect might be mediated by CMV-driven expansion of donor-derived memory-like NKG2C^{+} NK cells and V$\delta 2^{neg}\gamma\delta$T cells. Donor-derived NK cells probably recognize recipient leukemic blasts by engagement of NKG2C with HLA-E and/or by the lack of donor (self) HLA molecules. V$\delta 2^{neg}\gamma\delta$T cells probably recognize as yet unidentified antigens on leukemic blasts via their TCR.

Secondly, immunological imprints of CMV infection, such as expanded numbers of V$\delta 2^{neg}\gamma\delta$T cells and terminally differentiated TCR$\alpha\beta^{+}$ T cells, as well as enhanced NKG2C gene expression in peripheral blood of operationally tolerant liver transplant patients, suggest that CMV infection or reactivation may be associated with liver graft acceptance. Mechanistically, poor alloreactivity of CMV-induced terminally differentiated TCR$\alpha\beta^{+}$T cells and CMV-induced interferon (IFN)-driven adaptive immune resistance mechanisms in liver grafts may be involved.

In conclusion, direct associations indicate that CMV reactivation may protect against AML-relapse after allogeneic HSCT, and indirect associations suggest that CMV infection may promote allograft acceptance after liver transplantation. The causative mechanisms need further investigations, but are probably related to the profound and sustained imprint of CMV infection on the immune system.

INTRODUCTION

While the positive impact of host-microbiota interaction on human health is being extensively studied in recent years, possible beneficial effects of life-long persistent viruses on human health remain a whole new world to explore. One of the most prevalent viruses among humans is cytomegalovirus (CMV). The sero-prevalence of CMV ranges from 30-100% depending on socioeconomic and ethnic background. CMV generally remains quiescent in healthy individuals, but can cause severe disorders in immunocompromised individuals, such as patients after hematopoietic stem cell transplantation (HSCT) or solid organ transplantation (SOT). Paradoxically, accumulating recent evidence suggests that CMV infection after transplantation may also have beneficial effects, particularly in protection against leukemic relapse following HSCT for Acute Myeloid Leukemia (AML) and in promoting graft acceptance after liver transplantation (LTx). Recent research has shed first light on potential immunological mechanisms behind these surprising beneficial effects. Here, we discuss recent evidence for these two potential benefits of CMV infection after transplantation and emerging insights into the immunological mechanisms that may be involved.

Anti-leukemic effect of CMV reactivation after allogeneic HSTC for AML

CMV reactivation is a frequent and major complication after HSCT, causing a variety of organ-specific diseases, including pneumonia, encephalitis, and gastrointestinal disease. Prior to the age of prophylactic and pre-emptive treatment of CMV reactivation, CMV-pneumonia was the most common infectious cause of death after HSCT. Despite advances in diagnostic techniques and treatment strategies, CMV sero-positivity remains to be associated with inferior outcome, especially after myelo-ablative HSCT(1-3). However, paradoxical observations that CMV reactivation may protect against leukemic relapse after allogeneic HSCT for AML go back to the mid 1980's (4), and have been confirmed in a series of recent studies which we summarize in **Table 1**.

In a homogeneous cohort of adult AML patients monitored by the pp65 antigenemia assay and treated with preemptive anti-CMV therapy, patients with early CMV replication after allo-HSCT had a significantly reduced risk to develop relapse within 10 years after transplantation (5). In a large cohort of allo-HSCT patients treated for different hematologic malignancies, Green *et al*. confirmed the anti-leukemia effect of early CMV replication detected by pp65 in AML patients, but did not observe such effect in acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML), lymphoma, and myelodysplastic syndrome

(MDS) patients (6). Interestingly, pre-transplant CMV seropositivity was associated with an increased risk of relapse, which was confirmed in another study (1). Therefore, not pre-transplant CMV serostatus, but actual CMV reactivation seems to contribute to the observed beneficial effect.

The association between early CMV replication after allo-HSCT and reduction of AML relapse risk was further confirmed by four independent recent studies from different countries (7-10). However, this association was not observed in patients who did not receive a myeloablative conditioning regimen (7) or were treated with an *in vivo* T-cell depleting therapy, such as ATG or alemtuzumab (10, 11). In three of these studies CMV reactivation was determined by PCR. In contrast, one recent registry study, included 5310 AML patients, showed no benefit of CMV reactivation for AML relapse risk after allo-HSCT (12). However, in this study 28% of AML patients did not receive myeloablative therapy and 27% of AML patients were treated by *in vivo* T/Natural Killer (NK)-cell depleting therapy. In addition, the methods for evaluation of CMV reactivation were unknown, which may have resulted in different definitions of CMV reactivation. A recent meta-analysis of 6 studies, including the recent registry study (12), with 8511 AML patients that received mainly T-cell–replete grafts and were not treated with T-cell depleting therapy, confirmed that CMV reactivation after allo-HSCT results in a substantial reduction of the risk of relapse (HR=0.6, 95% CI=0.43-0.84, p=0.003) (13).

Thus, the evidence of a protective association between CMV replication and leukemic relapse in AML patients appears compelling, but only under specific transplantation conditions (7, 10, 11). However, it should be emphasized that only three studies reported an improved overall survival in AML patients with CMV replication after HSCT (5, 8, 10), while the majority of studies found that the anti-leukemic effect did not translate into improved survival. Indeed, other studies found either no difference in survival between patients with and without CMV replication (6, 7), or reported that CMV replication was associated with worse survival due to increased non-relapse mortality (9, 12).

The only evidence available in the cord blood transplantation setting is a recent registry study (14), which showed a trend to reduced AML relapse in patients with CMV reactivation. However, like in the registry study of Teira *et al.* (12), the methods used to detect CMV reactivation are unknown, and part of the patients received T-cell depleting therapy.

Whether CMV reactivation can protect against relapse after allo-HSCT for other hematological malignancies is controversial. While Ito *et al.* found a decreased risk of relapse in CML patients after CMV reactivation within 100 days after allo-

HSCT (15), two other studies did not confirm this finding (6, 9). Most studies did not observe any beneficial effect of CMV reactivation after allo-HSCT for acute lymphoblastic leukemia (ALL), myelodysplastic syndrome (MDS), or lymphomas (6, 9, 14, 16), but a recent study by Koldehoff *et al.* reports a reduced relapse incidence in non-Hodgkin lymphoma (NHL) patients (17). Because of contradictory results reported for other hematological malignancies, results on associations between CMV reactivation and relapse after allo-HSCT derived from mixed populations of patients with different hematological malignancies are difficult to interpret (3, 18).

In addition, apart from one study where more severe (grade II-IV) acute GVHD was observed in patients with CMV reactivation (10), CMV reactivation was not associated with acute or chronic GVHD and remained an independent risk factor for AML relapse in multivariate analyses in which GVHD was included (Supplementary Table 1), however this was not always reported. Available data therefore suggest that protection of AML relapse cannot be merely explained by an increased CMV-induced allogeneic immune response.

Possible mechanisms of anti-leukemic effects of CMV after allo-HSCT for AML

CMV reactivation does not protect against AML relapse when T- and/or NK cells are depleted *in vivo* or *in vitro*, suggesting that CMV reactivation requires a reconstitution of donor-derived T cells and/or NK cells to reduce leukemic relapse (7, 10, 11). Although recently a direct pro-apoptotic effect of CMV on acute leukemia cell lines has been shown (19), CMV is generally thought to be non-cytolytic, but instead to protect infected cells from apoptosis in order to delay cell death and maintain viral replication. Therefore, the anti-leukemic effects of CMV infection after allo-HSCT for AML are probably mainly caused by cross-reactivity of CMV-stimulated innate and adaptive immune responses with cancer cells. CMV infection leaves a deep and life-long imprint on the human immune system. Two types of immune cells that are expanded during CMV infection have been postulated to be involved in CMV-induced protection against AML. These are: natural killer (NK)-cells and $V\delta2^{neg}\gamma\delta$ T cells (**Figure 1**).

A CMV reactivation after allogeneic HSCT induces a long-lasting expansion of, mainly donor-derived, memory-like NK cells, or CMV-adapted NK cells, with enhanced functional properties compared to conventional NK cells. This CMV-induced memory-like NK-cell population is characterized by low expression of CD56, expression of CD57, lack of the inhibitory NKG2a receptor and expression of the activating heterodimeric receptor CD94-NKG2C (20, 21). The memory-associated features of these CMV-induced NK cells include secondary expansion and enhanced capacity to produce IFN-γ upon CMV reactivation (20, 22, 23). Once

induced, their expansion is not limited to CMV reactivation, as stimulation via the low affinity Fc receptor IIIa (CD16) by IgG, as well as pro-inflammatory cytokines, can contribute to the expansion, persistence, and functional properties of CMV-induced memory-like NK cells (21, 24, 25). The enhanced functional properties of CMV-induced donor-derived NKG2C^{+} memory NK cells compared to conventional NK cells are caused by epigenetic remodeling resulting in increased proliferative responses as well as cytokine production (21, 24, 26). Interestingly, expansion of these cells after HSCT is not only associated with protection from CMV reactivation (27), but also trended to be associated with a reduced rate of AML relapse (28).

The mechanism by which this NK-cell subset recognizes leukemic blasts may be related to the switch in expression from the inhibitory NKG2A to the activating NKG2C, both receptors for HLA-E, which is expressed on leukemic blasts. Alternatively, in partially HLA-mismatched HSCT, the anti-leukemic effect may be related to the general mechanism of NK-cell self-tolerance, which is mediated by inhibitory receptors, such as killer cell immunoglobulin-like receptors (KIR), that recognize self-MHC class I molecules. According to the "missing-self hypothesis", recipient AML-cells can be targets for cytotoxicity of donor-derived NKG2C^{+} NK cells, induced upon CMV infection, as they lack donor HLA molecules (23).

γδ T cells are involved in the first line of host immune defense to microbial pathogens, and expand in the circulation during CMV infection. They also show some adaptive features, such as accelerated expansion upon CMV reactivation (29, 30). The main γδ T cell subset in peripheral blood expresses T cell receptors encoded by the Vδ2 and Vδ9 gene segments, and is referred to as Vδ2^{pos}γδ T cells. γδ T cells that express Vδ1, Vδ3 or Vδ5, but not Vδ2, TCR are collectively designated as Vδ2^{neg}γδ T cells. The latter reside mainly in intestinal and skin epithelia, spleen, and liver (30). Interestingly, a strong and durable expansion of circulating Vδ2^{neg}γδ T cells occurs upon CMV reactivation after allo-HSCT (31, 32). This phenomenon is unique for CMV infection and does not occur after infections with other viruses after HSCT (31). Involvement of CMV-induced Vδ2^{neg}γδ T cells in protection from leukemic relapse has been suggested by studies showing that expansion of circulating Vδ2^{neg}γδ T cells after HSCT is associated with improved leukemia-free survival (33) and that CMV-reactive Vδ2^{neg}γδ T cells isolated from CMV-infected HSCT recipients cross-react to primary AML cells (32).

Vδ2^{neg} γδ T cells recognize leukemic blasts via their TCR, while CD8αα probably serves as a co-receptor in antigen recognition (29). They probably recognize

novel antigens in similar manner as Vδ2^{pos}γδ T cells. Quite different from αβ-TCR, Vδ2^{pos}γδ TCR recognize conformational changes in proteins in an antibody-like way (34-36). In addition, natural cytotoxicity receptors, especially NKp30, may play a role in tumor cell recognition by Vδ2^{neg} γδ T cells (37).

Association between CMV infection and graft acceptance in SOT

In experimental animal SOT models, both acute CMV infection and CMV reactivation have been shown to prevent or disrupt graft acceptance, which is thought to result from cross-reactivity of virus-specific T cells to allo-antigens (38). In addition, in the clinical setting CMV infection after SOT is generally associated with an increased risk of acute and chronic allograft rejection and like for HSCT, CMV infection is a major cause of morbidity and mortality. These data have been summarized in excellent reviews (39-41). Therefore sophisticated strategies have been implemented to detect CMV early and treat pre-emptively.

However, a main issue in the interpretation of the observed associations between CMV infection and graft rejection is that rejections often occur before CMV infection, and therefore it is difficult to prove cause and effect (39, 42). Two recent studies suggest that the link between CMV infection and acute as well as chronic rejection after kidney transplantation are far less significant than previously thought (42, 43). A CMV infection significantly impacts the immune system leaving a clear fingerprint of memory inflation in the T cell compartment, resembling features of immune ageing or senescence (44, 45). This memory inflation is accelerated during CMV reactivation under immunosuppressive medication after SOT (46). Nevertheless, CMV-specific T cells that cross-react to donor cells are only transiently present in the circulation of CMV-infected kidney transplant recipients, and their presence is not associated with inferior graft function (47). Several recent studies even suggest that under certain conditions there may even be an opposite association between CMV infection and graft rejection. Firstly, in elderly kidney transplant recipients CMV-seropositivity was associated with CD4$^+$ T cell immune-senescence and freedom of acute rejection (48). Secondly, donor-specific T-cell hypo-responsiveness, i.e. reduced frequencies of donor-specific but not of third party-specific T cells, and reduced immunological graft damage were observed in patients with strong CMV-specific T cell responses after kidney and heart transplantation (49, 50). Thirdly, increased numbers of terminally differentiated CD8$^+$ T cells, as well as CD4$^+$ T cells lacking CD28, both T cell subsets associated with CMV latency, in the circulation prior to kidney transplantation have been associated with a lower risk for acute rejection (51, 52). Finally, primary CMV infection following LTx is associated with accumulation of terminally differentiated CD8$^+$ T cells in the circulation as well as with donor-specific CD8$^+$ T

cell hypo-responsiveness and a reduced incidence of acute rejection episodes late after transplantation (53).

As compared with other solid organ grafts, liver grafts display unique immunological features, and LTx is the only setting in which a significant proportion of patients can eventually discontinue immunosuppressive medication without undergoing rejection, a phenomenon known as spontaneous operational tolerance (54). Recent prospective immunosuppression withdrawal studies have shown that operational tolerance can be achieved in about 40% of stable adult LTx patients and 60% of stable pediatric LTX patients (55-57). Interestingly, studies from two different centers found expanded numbers of peripheral $V\delta1^+\gamma\delta$ T cells and an increased peripheral $V\delta1/V\delta2$ $\gamma\delta$ T cell ratio in tolerant compared to non-tolerant LTx patients (58-60). In addition, tolerant pediatric LTx recipients exhibit an increased intra-graft $V\delta1/V\delta2$ ratio (61). A high peripheral $V\delta1/V\delta2$ $\gamma\delta$ T cell ratio has even successfully been used as a biomarker to select liver transplant (LT) patients for immunosuppression withdrawal (56). Since durable expansion of circulating $V\delta1^+\gamma\delta$ T cells and an increased peripheral $V\delta1/V\delta2$ ratio after CMV infection has been observed in all types of SOT including LTx (53, 62-65), these observations suggest an association between CMV infection and tolerance after LTx. Such relationship is further supported by a recent study which showed that primary CMV infection after LTx is associated with both expansion of circulating $V\delta1^+\gamma\delta$ T cells and donor-specific $CD8^+$ T cell hypo-responsiveness (53). CMV-responsive $V\delta2^-$ $\gamma\delta$ T cells have been implicated in antibody mediated rejection after kidney transplant recipients (66), suggesting a different role for these cells after liver transplantation, which may be related to the lower impact of antibodies in liver graft rejection compared to kidney graft rejection.

Increased numbers of circulating terminally differentiated $CD8^+$ T cells expressing co-inhibitory receptors is another feature shared by CMV infection (53, 67) and operational tolerance (56) after LTx, again suggesting a possible association. Finally, comparison of gene expression patterns in circulating leukocytes between tolerant and non-tolerant LTx recipients revealed over-expression of NK-cell-related genes in tolerant patients. Interestingly, KLRC4, one of genes encoding NKG2C, which is induced on circulating NK cells by CMV infection and reactivation both after HSCT (20-22, 27) and SOT, including LTx (68-70), was found to be over-expressed in tolerant LTx patients in two different cohorts (59, 71).

Thus, although a direct association between CMV infection and graft acceptance after LTx has not been demonstrated, the presence of sustained immunological imprints of CMV infection in operationally tolerant, but not in non-tolerant, LT patients is strongly suggestive for such association.

Possible mechanisms of graft acceptance in SOT after CMV infection

How CMV restrains alloreactivity after LTx remains elusive, and whether CMV-induced peripheral immune cell signatures play a causative role in promoting liver transplant tolerance is as yet unknown. Although $V\delta1^{+}\gamma\delta$ T cells under certain conditions may have immune-regulatory properties (72, 73), it is as yet unknown whether they can contribute to liver graft acceptance. However, the massive peripheral expansion of terminally differentiated $CD8^{+}$ T cells expressing co-inhibitory receptors upon CMV infection after LTx may contribute to reduced T cell allo-reactivity to organ grafts, since these cells show impaired functional responses to allo-antigens (53, 67) (**Figure 1**). In addition, as terminally differentiated $CD8^{+}$ T cells poorly infiltrate in organ grafts, they might not contribute to graft rejection even when functionally competent to respond to allo-antigens (53, 74).

CMV infection of the liver graft may also promote resistance of liver grafts to allogeneic attack by triggering type I IFN production (75) and recruitment of T-helper 1 cells that produce IFN-γ (76), which is strikingly absolutely needed for liver transplant tolerance in animal models (77). IFN-γ produced by graft-infiltrating T cells critically contributes to immunological tolerance of liver grafts in experimental animals by induction of intra-graft PD-L1 expression that leads to T cell apoptosis (78). Indeed, interaction of PD-L1 expressed in the liver graft with the co-inhibitory receptor PD-1 on graft-infiltrating T cells also counter-regulates rejection activity against liver grafts in humans (79) (**Figure 1**). Although this mechanism of CMV-driven graft-anti-host resistance is speculative, a role for intra-graft IFN signaling in development of operational tolerance after LTX has been suggested by an immunosuppression withdrawal trial in HCV-infected LT patients in which operationally tolerant patients were observed to overexpress interferon-stimulated genes as well as PD-L1 and PD-1 in their liver graft (56). Such so-called 'adaptive immune resistance' mechanisms, in which expression of immunosuppressive molecules, such as PD-L1, is induced in response to IFN type I and/or IFN-γ produced by infiltrating immune cells, are also utilized by tumors to escape immune attack (80). In addition, recent data show that IFN-type I signaling during chronic viral infections may promote $CD8^{+}$ T cell exhaustion and impair memory T cell responses against unrelated antigens (81, 82), connecting IFN-signaling to terminal differentiation and exhaustion of $CD8^{+}$ T cells.

Finally, one or more of the well-established immune evasion strategies of CMV to establish latency may be involved, such as production of viral IL-10, which may exert systemic immunosuppressive effects (83).

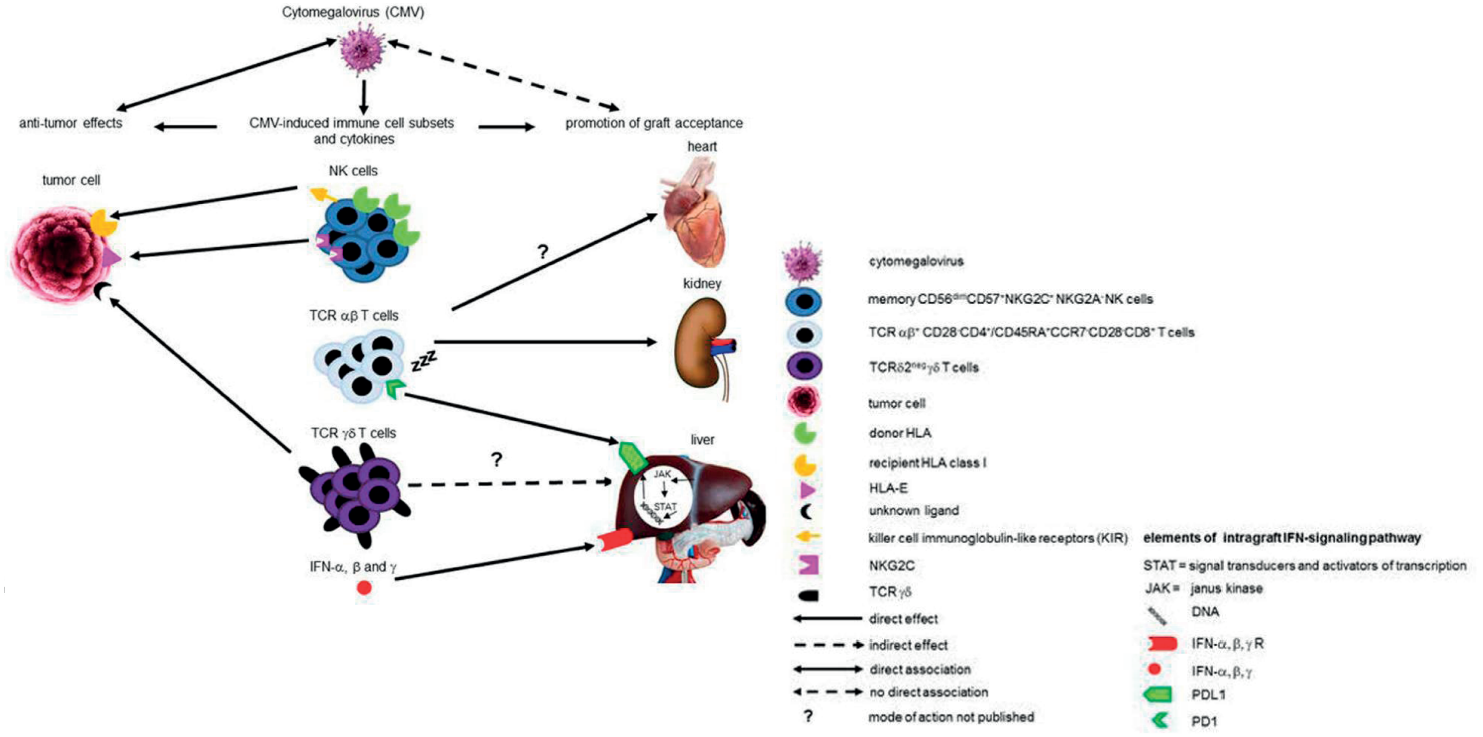
Cytomegalovirus (CMV)
anti-tumor effects
CMV-induced immune cell subsets and cytokines
promotion of graft acceptance
tumor cell
NK cells
heart
kidney
TCR αβ T cells
TCR γδ T cells
liver
IFN-α, β and γ
JAK
STAT
?
cytomegalovirus
memory CD56dim CD57+ NKG2C+ NKG2A- NK cells
TCR αβ+ CD28- CD4+/CD45RA+ CCR7- CD28- CD8+ T cells
TCRδ2neg1 γδ T cells
tumor cell
donor HLA
recipient HLA class I
HLA-E
unknown ligand
killer cell immunoglobulin-like receptors (KIR)
NKG2C
TCR γδ
direct effect
indirect effect
direct association
no direct association
mode of action not published
elements of intragraft IFN-signaling pathway
STAT = signal transducers and activators of transcription
JAK = janus kinase
DNA
IFN-α, β, γ R
IFN-α, β, γ
PDL1
PD1

FIGURE 1: Schematic overview of (potential) beneficial effects of CMV infection after transplantation
This schematic overview illustrates the (potential) beneficial effects exhibited by different CMV-induced immune cell subsets and intra-graft IFN-signaling pathways after transplantation. Compelling evidence exists for anti-leukemic effects of CMV-induced donor-derived memory-like NK cells after HSCT in AML patients, and two mechanisms have been described. One involves enhanced expression of the activating NK-receptor NKG2C, at the expense of the inhibitory NKG2A, interacting with HLA-E expressed by AML-blasts. The other mechanism involves the "missing self" principle, as recipient tumor cells do not express donor HLA class I and are therefore a target for killing by donor-derived NK cells as a result of lack of inhibition via donor-HLA class I-recognizing inhibitory KIR. CMV-induced TCR$\delta 2^-$ $\gamma\delta$ T cells have also been associated with anti-leukemic effects after HSCT, probably via recognition of an as yet unknown ligand by their TCR. Evidence implicating CMV-specific TCR $\alpha\beta$ T cells in preventing AML relapse after HSCT is lacking.
In addition, CMV-induced immune cell subsets have been associated with graft acceptance and liver-transplant tolerance. Evidence merely consists of associations and no detailed mechanistic insights are available yet. Induction of terminally differentiated TCR$\alpha\beta$ T cells with low alloreactivity by CMV infection in various types of solid organ transplantations may be involved in development of graft acceptance. CMV-induced circulating TCR$\delta 2^-$ $\gamma\delta$ T cells are associated with liver transplant tolerance but probably not functionally involved. Over-expression of NKG2C in peripheral blood is associated with both CMV infection and graft acceptance after liver transplantation, but whether a causal exists between NKG2C$^+$ NK cells and graft acceptance is unknown. Apart from CMV-induced immune cell subsets, intra-graft IFN- α,β and γ production, which can be induced by CMV, has been associated with liver transplant tolerance by induction of PD-L1 expression in the graft, thereby counteracting the host immune response.

Study (Ref.)	Effect	Patients	Adults / pediatric patients	Myeloablative pre-conditioning	T/NK-depleted graft	Antibody-based in vivo T-cell depletion	Donors
Elmaagacli *et al.* (5)	Positive in AML	AML n=266	adults	All	no	no	sibs 118 (44%); MUD 148 (56%)
Manjappa *et al.* (7)	Positive in AML	AML n=264	adults	206 (78%)	no	46 (17%) ATG	MRD 108 (41%); MUD 156 (59%)
Jang *et al.* (8)	Positive in AML	AML n=74	median age 35; range 15-59 years	68 (92%)	not mentioned	11 (15%) ATG or alemtuzumab	MRD 31 (42%); MUD 43 (58%)
Green *et al.* (6)	Positive in AML	AML n=761, ALL n=322, CML n=646, lymphoma n=254, MDS n=371	2306 adults / 260 children	659 (87%) of AML patients	39 (5%) of AML patients	not mentioned	sibs 397 (52%); MUD 351 (46%); haplo 12 (2%)
Takenaka *et al.* (9)	Positive in AML	AML n=1836, ALL n=911, CML n=223, MDS n=569	median age 46; range: 16-74 years	1381 (75%) of AML patients	no	no	MRD 989 (54%); MUD 847 (46%)
Bao *et al.* (10)	Positive in AML sub-group	AML n=227	adults+children, median age 35; range 2-63 years	All	no	117 (52%) ATG	sibs 110 (49%), MUD 57 (25%), haplo 60 (26%)
Ramanathan *et al.* (14)	Positive trend in AML	AML n=925, ALL n=759	adults + children, median age 28; range 1-79 years	680 (74%) of AML patients	not mentioned	part of patients, number not mentioned	CB
Teira P *et al.* (12)	Negative in AML	AML n=5310, ALL n=1883, CML n=1079, MDS n=1197	median age 48; range 1-83 years	3809/5310 (72%)	149/5310 (3%)	1439/5310 (27%)	sibs 4071 (43%); MUD 3481 (37%)
Ito *et al.* (15)	Positive in CML	CML n=110	adults+children, median age 36; range 13-69 years	97 (88%)	97 (88%)	no	sibs 110 (100%)

TABLE 1: Summary of recent studies on the association of post-transplant CMV replication and relapse of haematological malignancies after HSCT *(continued)*

Stem cell source	CMV-detection	Endpoint	Relapse rate			Effect of CMV reactivation on AML-relapse	Comments
			with CMV reactivation	without CMV reactivation	P-value		
BM 45(17%); PBSC 221 (83%)	pp65 antigenemia	cumulative incidence of AML-relapse at 10 years after HSCT = 33% (95% CI, 27%-40%)	10 year CIR AML 9%	10 year CIR AML 42%	<0.0001	*CMV infection: HR=0.2, 95%CI=0.1-0.4, P<0.0001	
BM 23 (9%); PBSC 240 (91%)	PCR	cumulative incidence of AML-relapse at 6 years after HSCT = 43%	6 year CIR AML 38,9%	6 year CIR AML 59%	0.03	*CMV infection: HR=0.53, 95%CI=0.33–0.83, P=0.015	Effect restricted to patients receiving myelo-ablative conditioning
BM 5 (7%); PBSC 69 (93%)	PCR	cumulative incidence of AML-relapse at 5 years after HSCT = 31%	patient numbers not mentioned			*CMV infection: HR=0.21, 95%CI=0.08–0.54, P=0.001	
BM 301(40%); PBSC 460 (60%)	pp65 antigenemia	cumulative incidence of AML-relapse at 1 year after HSCT = 25,2%	1 year CIR AML 26.5%	1 year CIR AML 32.7%	0.19	*CMV infection: HR=0.56, 95%CI=0.3–0.9, P=0.02 ***	Effect restricted to AML patients and no effect on overall mortality
BM 1267 (69%); PBSC 569 (31%)	pp65 antigenemia	cumulative incidence of AML-relapse at 5 years after HSCT = 26,5%	5 year CIR AML 22,4%	5 year CIR AML 29,6%	<0.01	*CMV infection: HR=0.77, 95%CI=0.59–0.99, P=0.04	Effect restricted to AML patients
BM 50 (22%), PBSC 125 (55%), BM+PBSC 52 (23%)	PCR	cumulative incidence of AML-relapse at 3 years after HSCT = 26%	3 year CIR AML 22% Subgroup: non ATG patients 3 year CIR AML 8,9%	3 year CIR AML 29,7% Subgroup: non ATG patients 3 year CIR AML 26,7%	0.237 0.016	*CMV infection: HR=0,28, 95%CI=0.1-0.79, P=0.016)	Effect restricted to subgroup of patients NOT receiving ATG in conditioning (n=110)
CB	unknown	cumulative incidence of AML-relapse at 3 years after HSCT = 35%	patient numbers not mentioned			*CMV infection: HR=0.895% CI= 0.62–1.04, P = 0.097	Trend restricted to AML patients
BM 2475 (26%); PBSC 6994 (74%)	unknown	cumulative incidence of AML-relapse at 3 years after HSCT =38%	patient numbers not mentioned			*CMV infection: HR=0.97 95%CI=0.86–1.1, P=0.6 (all AML pts)	Also no effect in ALL, CML, MDS
BM 27 (25%), PBSC 83 (75%)	pp65 antigenemia and PCR	cumulative incidence of CML relapse = 49% relapse after median follow-up of 6,2 years	patient numbers]not mentioned			*CMV infection: HR=0.533, 95%CI=0.29-0.99, P=0.045	

Study (Ref.)	Effect	Patients	Adults / pediatric patients	Myeloablative pre-conditioning	T/NK-depleted graft	Antibody-based in vivo T-cell depletion	Donors
Koldehoff *et al.* (17)	Positive in NHL	B-cell lymphoma n=94, T- and NK-cell neoplasms n=42	adults	107 (79%)	not mentioned	57 (42%) ATG	sibs 36 (26%), MUD 80 (59%), MUD-MM 20 (15%)
Mariotti *et al.* (16)	Negative in NHL	B-cell lymphoma n=265	adults	75 (26%)	no	124 (45%) ATG or alemtuzumab	sibs 147 (55%), MUD 50 (19%), MUD-MM 68 (26%)

TABLE 1: Summary of recent studies on the association of post-transplant CMV replication and relapse of haematological malignancies after HSCT
ALL: Acute Lymphoblastic Leukemia; AML: Acute Myeloid Leukemia; BM: bone marrow; CB: Cord Blood; CIR: Cumulative Incidence of Relapse; CML: Chronic Myeloid Leukemia; D/R: Donor/Recipient; haplo: Haploidentical donor; MDS: Myelo Dysplastic Syndrome; MM: Mismatched; MRD: Matched Related Donor allogeneic HSTC; MUD: Matched Unrelated Donor allogeneic HSCT; NHL: Non Hodgkin Lymphoma; PBSC: peripheral Blood Stem Cells; sib: sibling donor allogeneic HSCT.
* multivariate analysis; **Center for International Blood & Marrow Transplant (CIBMTR) database; *** Effect calculated on cumulative incidence of AML-relapse at 100 days after HSCT

Concluding remarks

CMV infection, although generally disadvantageous in immunosuppressed subjects, paradoxically has beneficial effects in HSCT and probably also in LTx recipients. More research is definitely needed to substantiate and better understand these enigmatic observations. Whereas the anti-leukemic effect of CMV infection after HSCT in AML-patients is firmly established, prospective studies are required to investigate whether there is a direct association between CMV infection after LTx and development of allogeneic liver graft acceptance. Such studies should be accompanied by extensive peripheral and intra-graft immune profiling to begin to understand the putative immunological mechanisms linking CMV infection with tolerance after LTX. Similarly, studies aiming to further unravel the immunological mechanisms linking CMV infection and prevention of AML-recurrence after HSCT are required. Once those benefits and the underlying mechanisms have been firmly established, development of therapeutic approaches to mimic the beneficial effects of CMV infection after HSCT for AML and after LTx becomes an interesting, although challenging aim. Development

	Stem cell source	CMV-detection	Endpoint	Relapse rate			Effect of CMV reactivation on AML-relapse	Comments
				with CMV reactivation	without CMV reactivation	P-value		
	BM 11 (8%); PBSC 125 (92%)	pp65 antigenemia and PCR (after June 2011)	cumalative incidence of NHL relapse at 5 years after HSCT = 31%	5 year CIR NHL 22%	5 year CIR NHL 38%	<0.013	*CMV infecton: HR=0.29, 95%CI=0.11-0.76, P<0.014	
	BM 26 (10%), PBSC 239 (90%)	pp65 antigenemia	cumulative incidence of B-cell lymphoma relapse at 5 years after HSCT = 42%	5 year CIR NHL 34%	5 year CIR NHL 50%	0.42	*CMV infection: HR=1.0, 95% CI=0.6-1.6; P=0.9	

of a CMV vaccine that induces similar immunological imprints as CMV infection, might be a way to achieve this goal.

DISCLOSURES

Dr. Litjens, Drs. van der Wagen, and Dr. Kwekkeboom have nothing to disclose. Prof. Kuball reports grants from Novartis, and Miltenyi Biotech. He is scientific co-founder and CSO of gadeta (www.gadeta.nl) and inventor on multiple patents on γδ TCR receptors and isolation strategies for engineered immune cells.

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SUPPLEMENTAL MATERIALS

Study (Ref.)	Patients	Adults / pediatric patients	Myeloablative pre-conditioning	T/NK-depleted graft	Antibody-based in vivo T-cell depletion	Donors
Elmaagacli *et al.* (5)	AML n=266	adults	All	no	no	sibs 118 (44%); MUD 148 (56%)
Manjappa *et al.* (7)	AML n=264	adults	206 (78%)	no	46 (17%) ATG	MRD 108 (41%); MUD 156 (59%)
Jang *et al.* (8)	AML n=74	median age 35; range 15-59 years	68 (92%)	not mentioned	11 (15%) ATG or alemtuzumab	MRD 31 (42%); MUD 43 (58%)
Green *et al.* (6)	AML n=761, ALL n=322, CML n=646, lymphoma n=254, MDS n=371	2306 adults / 260 children	659 (87%) of AML patients	39 (5%) of AML patients	not mentioned	sibs 397 (52%); MUD 351 (46%); haplo 12 (2%)
Takenaka *et al.* (9)	AML n=1836, ALL n=911, CML n=223, MDS n=569	median age 46; range: 16-74 years	1381 (75%) of AML patients	no	no	MRD 989 (54%); MUD 847 (46%)
Bao *et al.* (10)	AML n=227	adults+children, median age 35; range 2-63 years	All	no	117 (52%) ATG	sibs 110 (49%), MUD 57 (25%), haplo 60 (26%)
Ramanathan *et al.* (14)	AML n=925, ALL n=759	adults + children, median age 28; range 1-79 years	680 (74%) of AML patients	not mentioned	part of patients, number not mentioned	CB
Teira P *et al.* (12)	AML n=5310, ALL n=1883, CML n=1079, MDS n=1197	median age 48; range 1-83 years	3809 (72%)	283/9469 (3%)	2503/9469 (27%)	sibs 4071 (43%); MUD 3481 (37%)

SUPPLEMENTARY TABLE 1 (*continued*)

Stem cell source	CMV-detection	Grade II-IV acute GVHD (with /without CMV reactivation)	Comments	cGVHD
BM 45(17%); PBSC 221 (83%)	pp65 antigenemia	187 pts (70%), not dissected with respect to CMV reactivation	Cumulative incidence of grade II-IV GVHD in pp65 antigenemia 35% (compared to 15% in pts with grade 0-I GVHD)	no percentages mentioned, in MV analysis disease stage, cGVHD development and CMV replication were independent predictors of reduced relapse risk
BM 23 (9%); PBSC 240 (91%)	PCR	100 pts (37%), 44% in CMV reactivation cohort vs. 56% in non-CMV cohort (n.s.)		32% in CMV reactivation cohort vs. 36% in non-CMV cohort (n.s.)
BM 5 (7%); PBSC 69 (93%)	PCR	26 pts (35%), 17 of 52 (33%) in early CMV replication cohort vs. 9 of 22 (41%) in no early CMV replication cohort (n.s.)		33 of 43 pts (77%) with early CMV replication versus 9 of 17 pts (53%) without early CMV replication) (n.s.)
BM 301(40%); PBSC 460 (60%)	pp65 antigenemia	582 of 761 (76%), not dissected with respect to CMV reactivation	Controlling for acute GVHD (grades 0-2 vs 3-4) did not change the association between CMV reactivation and relapse in any of the disease groups	353 of 761 (46%) AML patients had cGVHD, not specified between CMV positive/negative patients
BM 1267 (69%); PBSC 569 (31%)	pp65 antigenemia	630 of 1836 AML pts (34%), not dissected with respect to CMV reactivation		808 of 1836 (44%) AML patients had cGVHD, not specified between CMV positive/negative patients
BM 50 (22%), PBSC 125 (55%), BM+PBSC 52 (23%)	PCR	93 pts (41%), 60% in CMV reactivation cohort vs. 40% in non-CMV reactivation cohort (p=0.039)		65 of 118 (53%) in CMV reactivation cohort vs 57 of 109 (47%) in non-CMV reactivation cohort (n.s.)
CB	unknown	388 pts (42%), not dissected with respect to CMV reactivation	AML D − /R+: 43% (95% CI, 39–47%); AML D − /R − : 40% (95% CI, 34–45%), in MV analysis positive CMV serology had no impact on incidence of aGVHD or cGVHD in AML	not mentioned
BM 2475 (26%); PBSC 6994 (74%)	unknown	37% of all patients, not dissected with respect to CMV reactivation	Incidence of Grade II-IV aGVHD by day 100 was similar across the 4 D/R serology cohorts regardless of disease	Incidence of cGVHD not different across the 4 D/R serology cohorts.

Study (Ref.)	Patients	Adults / pediatric patients	Myeloablative pre-conditioning	T/NK-depleted graft	Antibody-based in vivo T-cell depletion	Donors
Ito *et al.* (15)	CML n=110	adults+children, median age 36; range 13-69 years	97 (88%)	97 (88%)	no	sibs 110 (100%)
Koldehoff *et al.* (17)	B-cell lymphoma n=94, T- and NK-cell neoplasms n=42	adults	107 (79%)	not mentioned	57 (42%) ATG	sibs 36 (26%), MUD 80 (59%), MUD-MM 20 (15%)
Mariotti *et al.* (16)	B-cell lymphoma n=265	adults	75 (26%)	no	124 (45%) ATG or alemtuzumab	sibs 147 (55%), MUD 50 (19%), MUD-MM 68 (26%)

SUPPLEMENTARY TABLE 1: Summary of recent studies on the association of post-transplant CMV replication and GVHD

a/cGVHD: acute/chronic Graft versus Host Disease; ALL: Acute Lymphoblastic Leukemia; AML: Acute Myeloid Leukemia; BM: bone marrow; CB: Cord Blood; CIR: Cumulative Incidence of Relapse; CML: Chronic Myeloid Leukemia; D/R: Donor/Recipient; haplo: Haploidentical donor; MDS: Myelo Dysplastic Syndrome; MM: Mismatched; MRD: Matched Related Donor allogeneic HSTC; MUD: Matched Unrelated Donor allogeneic HSCT; NHL: Non Hodgkin Lymphoma; PBSC: peripheral Blood Stem Cells; sib: sibling donor allogeneic HSCT.

† no percentages available for aGVHD gr II-IV

Stem cell source	CMV-detection	Grade II-IV acute GVHD (with /without CMV reactivation)	Comments	cGVHD
BM 27 (25%), PBSC 83 (75%)	pp65 antigenemia and PCR	27 pts (34%), not dissected with respect to CMV reactivation	aGVHD grade II-IV not included in MV analysis as p>0,05	65 of 110 (59%) in all patients, not specified beteween CMV positive/negative patients
BM 11 (8%); PBSC 125 (92%)	pp65 antigenemia and PCR (after June 2011)	55 pts (44%),not dissected with respect to CMV reactivation		no percentages mentioned, in MV analysis non-sensitivity to chemotherapy, grade II-IV aGVHD and cGVHD were decisive predictors of Non Relapse Mortality.
BM 26 (10%), PBSC 239 (90%)	pp65 antigenemia	49% GVHD gr I-IV, 10% GVHD gr III-IV †, 11,3% in pp65+ pts aGVHD gr III-IV vs. 9,1% in pp65- pts (n.s.) †	131 pts (49%) had aGHVD gr I-IV, no difference between pts with CMV (52%) or without CMV (48%) reactivation	35% developed cGVHD, 50% of patients with CMV reactivation and 50% without CMV reactivation (n.s.)

7

General discussion

Lotte van der Wagen

SUMMARY

Despite the application of allogeneic SCT since 1957 (1) we have not been able to eradicate GVHD as a significant contributor of treatment related mortality (TRM) and morbidity. In the last years, much progress has been made in unravelling the complex pathophysiology of GVHD. These insights have led to new therapies and will hopefully further contribute to complete eradication of GVHD. Unfortunately we are not there yet and as hematologists we still have to counsel our patients about the complex risks involving allogeneic SCT.

In this thesis we conducted two clinical trials and analyzed data, clinical parameters as well as soluble and cellular markers, to provide insight into the pathophysiology of GVHD. We also propose new therapeutic options which partly have already been adopted in current clinical practice.

Acute Graft versus Host Disease and treatment with MSC

As is discussed in the introduction there are numerous treatment options for steroid-refractory GVHD patients (2). However these therapies are often based upon small phase I/II trials or even case series. Furthermore they often provide very limited data on biological parameters or possible biomarkers to guide therapy choices.

In 2008, Le Blanc *et al.* (3) published a landmark study regarding the use of Mesenchymal Stromal Cells (MSC) for steroid-refractory acute GVHD. Since then, there have been multiple groups that have repeated this setup in adult as well as pediatric cohorts (3-11). In **chapter 2.1** we describe the results of our clinical trial in patients with steroid-refractory acute GVHD (12). We show a clinical resolution of all GVHD related symptoms lasting for at least 1 month (CR_{GVHD}) in 50% of patients. Median time from first infusion of MSC to reach CR_{GVHD} was 53,5 days (range 3–116). CR_{GVHD} patients have a significantly improved one-year overall survival (OS) as compared to $NonCR_{GVHD}$ patients (79 versus 8%, $p<0.001$).

As we aimed to establish predictive factors for response and OS we conducted extensive immune monitoring on peripheral blood samples at regular intervals. We show a six soluble biomarker-panel consisting of Interleukin 2 Receptor α (IL2Rα), Tumor Necrosis Factor receptor 1 (TNF-R1), hepatocyte growth factor (HGF), IL-8, elafin and regenerating islet-derived protein 3α (Reg3α) as proposed by Levine *et al.* to be predictive for mortality when measured before MSC administration (HR 2.924; CI 1.485–5.758) (13). However we could not confirm Suppression of Tumorigenicity 2 (ST2) to be predictive for mortality before MSC administration. As working mechanisms of MSC are not fully elucidated it has been proposed that they act via regulatory cellular subsets (14-18). We measured a comprehensive panel of cellular subsets and observed an increase in myeloid dendritic cells (mDCs) in the peripheral blood in CR_{GVHD} patients starting two weeks after MSC infusion (Figure 3, chapter 2.1). We did not observe significant differences in regulatory T-cells between CR_{GVHD} and $NonCR_{GVHD}$ patients as reported by Jitschin *et al.* (19).

Since T-cells have been described to be functionally impaired after incubation with MSC we tested overall proliferation rate of T-cells as well as responses against viral and tumor-associated antigens. However we did not observe impaired functionality or responses in T-cells after application of MSC in a mixed lymphocyte reaction (Figure 4, chapter 2.1).

All variables were tested in a univariate Cox regression analysis and all variables with a probability level of <20% were entered in the multivariate model. At baseline (day 0), only patient age and the Levine soluble biomarker formula

were associated with one-year mortality (Table 3, chapter 2.1). At 2 weeks after the first MSC infusion, the number of immature mDC1 and the level of ST2 were the most important factors associated with one-year mortality.

This phase II clinical trial showed similar response rates as reported by others and no infusional toxicity (2). Overall survival is strongly associated with reaching CR_{GVHD}. However we could not reproduce earlier reported cellular and soluble biomarkers such as ST2. This could be due to the limited patient numbers and although 48 patients is one of the largest cohorts reported, due to many variables such as different primary diseases, conditioning regimens, age of patients and different organ systems affected by GVHD, this patient category is notoriously difficult to validate biomarkers.

Furthermore, the setup of a phase II trial always limits the power to interpret the efficacy of the therapy. To address this fundamental issue of showing efficacy of MSC, we started a randomized phase III trial in collaboration with HOVON. This trial, named HOVON112, will randomize patients with grade 2-4 acute GVHD to receive standard first line therapy with steroids or to receive steroids plus MSC. The role of MSC as a second line agent is currently being investigated in the HOVON113 trial, where steroid-refractory acute GVHD patients are randomized between mycophenolate mofetil (MMF) plus placebo or MMF plus MSC. The outcome of these critical trials will determine the definitive place of MSC in the treatment of GVHD, both trials are currently recruiting (www.hovon.nl/studies). The only other randomized phase III trial with MSC was performed with the off-the-shelf product Remestemcel-L (Prochymal). In this study 244 patients were included and were randomized in a 2:1 ratio to receive standard second-line GVHD treatment plus placebo or second-line GVHD treatment plus 8 infusions of Remestemcel-L. Unfortunately these results were only published in an abstract and never in a peer-reviewed journal (20). The conclusion in this abstract is that 'results suggest addition of Prochymal produced significant improvement without additive toxicity in patients with steroid-refractory GVHD involving visceral organs' (20). However, the study failed to meet its primary endpoint and therefore raised doubts regarding the effectiveness of MSC. We have further discussed and looked into the dilemmas regarding MSC and the variables possibly affecting effectiveness in **chapter 3**.

The search for robust biomarkers in MSC treated patients continues

Since performing clinical trials with Advanced Therapy Medicinal Products (ATMP) has proved to be challenging, we currently are still searching for early biomarkers to help guide therapy choices in this patient category. Some of the

same problems that arise when comparing clinical results of studies with MSC treated GVHD patients also arise when looking at biomarkers in this population. Patients with steroid-refractory GVHD represent a rather heterogeneous group with different primary diseases, conditioning regimens, graft composition etc. Therefore finding differences in small cellular subsets amidst 'background noise' proposes a challenge. We try to overcome these challenges in **chapter 2.2**. Here we analyzed our multicolor flow cytometry data from the clinical trial described in chapter 2.1 using Elimination of Cells Lying in Patterns Similar to Endogeneity (ECLIPSE), a new non-subjective method to discover small cell populations (21). Subsequently we performed Discriminant Analysis of MultiAspect CYtometry (DAMACY) (22) on the cell populations found by ECLIPSE, to objectively classify patients as responders or non-responders.

In the ECLIPSE model of patients that had survived one year after initiation of MSC therapy, a cell population was found expressing Blood Dendritic Cell Antigen 1 (BDCA-1), which was hardly present in the deceased patients at 2 weeks after infusion of MSC (Chapter 2.1 figure 2). Using DAMACY patients could be classified according to this marker with 73% accuracy (a specificity of 0.70 and a sensitivity of 0.75) to the correct group. BDCA-1 positive cells are representative of our earlier reported cellular biomarker myeloid Dendritic Cells type 1 (mDC1).

We then continued to test this marker in the same unbiased fashion by means of ECLIPSE in blood samples from the patients from the hospital exemption cohort. However we could not validate mDC1 to be different between responders and non-responders and no good DAMACY classification could therefore be constructed. Possible explanations for this inability to validate this cellular biomarker could be of a technical nature as the samples of patients in the hospital exemption cohort were measured on a different flow cytometer with slightly different antibodies. However this underlines the sensitivity of FACS measurements and the importance of the validation of clinical biomarkers in independent cohorts as these are not solid outcomes and therefore not yet currently applicable in clinical practice. Possibly a proteomics approach might be more robust as analyses are more automated and standardized as was recently shown for ST2 and Reg3α in the MAGIC consortium (23). However immune monitoring of cellular subsets in GVHD patients will remain a valuable tool to provide biological insights and new techniques in data processing such as ECLIPSE and DAMACY algorithms could prove vital for providing an unbiased analysis method.

MSC properties influence clinical outcome

As the randomized phase III trial with Remestemcel-L raised questions about the effectiveness of MSC for treating steroid-refractory acute GVHD a debate started on possible differences regarding this industrial MSC product versus academic MSC products (20, 24). Obvious differences between the two are the number of passages in the laboratory before clinical use which is usually 3 in academic trials, but is much higher in the off-the-shelf industrial product Remestemcel-L. Multiple passaging may alter the working mechanisms by means of epigenetic reprogramming and senescence (25-27). Other groups have therefore developed manufacturing protocols to generate MSC of more consistent potency (28). However, these findings led us to extend the clinical trial cohort described in chapter 2.1 (n=48) with the data from our hospital exemption cohort (n=54) in **chapter 3** in order to perform more in-depth analyses of MSC product characteristics and clinical outcome.

First we compared baseline characteristics of patients from the original study cohort and the hospital exemption program (chapter 3, table 1). As the mean MSC dose per infusion was decreased in the hospital exemption program to $1.0x10^6$ cells/kg this was significantly different between the two cohorts, however all other clinical variables were the same including CR_{GVHD} response rate and one-year OS (chapter 3, table 1 and figure 1A). Overall 49% of patients reached CR_{GVHD}. One-year OS for the entire cohort was 41,6% with a significantly improved one-year OS for responding patients (83,7%) vs. non-responding patients (1,9%, log rank test p<0,001, chapter 3 figure 1). Causes of death were relapse of primary malignancy (9,7%), GVHD (43,5%), infection (32,3%) and other (14,5%).

We performed a multivariate Cox regression analysis to assess the importance of the different clinical variables as well as MSC donor variables on clinical outcome. The relevant factors contributing to one-year OS are patient age, GVHD severity or liver GVHD and MSC donor age. From these factors, MSC donor age is the only variable we can currently influence by integrating this into our MSC product selection procedure. Therefore these findings have implications for ongoing clinical trials as well as market approval of these ATMPs.

We then continued to investigate the explanation for this difference in survival due to MSC donor age. From the 12 bone marrow (BM) donors used to produce the MSC for the entire cohort of 102 patients, we could retrieve passage 1 or 2 material from 8 BM donors. These 8 donors had been used to treat the majority of 92,4% of patients. These cells were brought into culture and then tested for cell viability, T-cell suppressive capacity and RNA sequencing. We observed no differences in cell viability between donors, however MSC derived from young BM donors showed significantly increased T-cell suppression compared to MSC de-

rived from older BM donors (chapter 3, figure 2B). Still, the difference in clinical outcome cannot be solely explained by improved T-cell suppression in younger donors as the degree of T-cell suppression does not seem to impact one-year OS (chapter 3, figure 1C).

To further assess potential molecular mechanisms, we performed transcriptome analyses, which revealed as expected a clear clustering of MSC based on age of the BM donor. This is striking as patients were treated with generally regarded 'young' MSC donors with a maximum age of 33 years at time of BM donation. Apparently even in this relatively small age range already differences exist that contribute to the different clinical response. Differential expression analysis revealed 73 downregulated and 31 upregulated genes in the MSC derived from young (<10 years of age) versus older MSC donors. Pathways involved in apoptosis, MHCII expression and extracellular matrix formation seem to play a role. A recent paper by Hennrich *et al*. also highlights, by means of proteome analyses, age-effected pathways in MSC related to apoptosis (29). Possibly MSC derived from young donors can elicit a more potent immunosuppressive effect related to differences in apoptosis mechanisms. In particular our finding that apoptosis related genes are affected in young donors supports the recent observation that increase in apoptosis of MSC associates with efficacy (30).

Combining therapies to treat sclerotic chronic GVHD

In **chapter 4.1** we describe the phase II clinical trial we performed in patients with sclerotic chronic GVHD (31). B-cell depletion and tyrosine kinase inhibition have each separately shown effectiveness in decreasing chronic GVHD severity (32-40). We combined these strategies to improve outcomes. All patients had received several lines of treatment (range 1-5) before enrollment and were found to be steroid-refractory. In 24 patients eligible for analysis we observed an overall response in 71% of patients (two patients CR, 15 patients PR). Responding patients show a significant decrease (chapter 4.1 figure 2, p=0,0012) in chronic GVHD affected body surface area (BSA) at end of follow-up compared to baseline. Not only skin manifestations of chronic GVHD improved in 71% of patients, but we also observed responses in other affected organs such as the oral and gastro-intestinal tract in 74% and 62% respectively.

Long-term immunosuppressive therapy accounts for many side effects and therefore is an important contributing factor to the morbidity of chronic GVHD patients. We show corticosteroid dose could be tapered in responding patients but not in non-responding patients (chapter 4.1, figure 4). The observed responses were durable and lasted until the end of study follow-up when patients had already discontinued the study medication for 6 months. In terms of cost-

effectiveness this was also an interesting observation as most new agents under investigation for chronic GVHD require continuous administration. Toxicity was manageable, but dose reductions for nilotinib were needed regularly. Most reported side effects included fatigue, nausea, pain in extremities and prolonged QT interval on standard ECG monitoring. Of 29 treated patients, 38% experienced a serious adverse event (SAE) during the study (chapter 4.1, figure 5). The one-year OS was 96,5% with one patient who died due to progressive osteomyelitis with multiresistant bacteria.

Intriguingly, we found a low number of CD5+ B-cells as positive predictor for therapeutic responses in this study, as we also identified in our previous study with monotherapy rituximab (41). Responding chronic GVHD patients did not have significantly different T-cell numbers or regulatory T-cells either at baseline or during follow-up.

We included only 29 patients in our study of whom 24 were eligible for analyses. These small numbers did not allow for subgroup analyses. As severe chronic GVHD is an orphan disease it is difficult to perform large clinical trials. Ideally this combination of B-cell depletion and tyrosine kinase inhibition should be tested in a randomized phase III trial against other emerging treatments such as Jak 1/2 inhibition (ruxolitinib) and Bruton's tyrosine kinase inhibition (ibrutinib). As pharmaceutical companies don't have much financial incentive to set up such a trial we as clinicians should be smart in future trial design and incorporate immune monitoring and possibly crossover designs based on biomarkers.

Optimizing treatment using therapeutic drug monitoring

Also the limited tolerance of nilotinib poses a barrier for widespread clinical use of the combination of rituximab and nilotinib in chronic GVHD. Many kinase inhibitors show both exposure-response and exposure-toxicity relationships (42). Consequently therapeutic drug monitoring (TDM) is widely accepted and applied in the treatment of chronic myeloid leukemia (CML). In **chapter 4.2** we therefore performed a retrospective analysis of samples obtained from our prospective clinical trial cohort to study the exposure-response and exposure-toxicity relationships (43). Nilotinib trough concentrations were available for 18 patients of 24 that completed the study (11 responders and 7 non-responders). Nilotinib trough concentrations in responders were significantly higher than in non-responders (mean nilotinib trough level 1071 vs. 782 µg/L, p=0.03, chapter 4.2 figure 1). Interestingly, no significant difference in trough concentration was observed between patients treated with 300 and 200 mg b.i.d. (1067 vs. 974 µg/L, p = 0.66). We could not relate this to drug interactions, e.g., by use of azoles. Remarkably, no significant difference in nilotinib trough concentration was

found between patients with and without reported drug toxicity. We could not clearly link trough levels of nilotinib to observed toxicity, possibly due to the small sample size.

However, we observe a possible concentration-effect relationship for nilotinib on response of chronic GVHD indicating that TDM might improve response rates in this patient category. As TDM for tyrosine kinase inhibitors is widely accepted and integrated in CML care it should also be available for chronic GVHD patients. We propose to incorporate TDM in chronic GVHD care especially in patients showing no response to tyrosine kinase inhibitors since they might benefit from higher concentrations.

Consensus in the Dutch polder

In 2016 we set out to write a new guideline on behalf of the HOVON SCT working party concerning treatment options for acute and chronic GVHD. This resulted in a published guideline in 2018 which makes up **chapter 5** (44). The field of GVHD treatment has changed rapidly in recent years as has the entire field of allogeneic SCT. Graft manipulation by means of αβ-T-cell depletion or alemtuzumab, post-transplant cyclophosphamide, and the more standard use of prophylactic donor lymphocyte infusion (DLI) have changed the occurrence as well as timing of GVHD. Also recent insights in the pathophysiology of both acute and chronic GVHD as described in chapter 1 have led to the availability of more immunosuppressive agents for treating GVHD. In chapter 5 we provide an overview of treatment options that reflects the current practice in the Netherlands and provides the available scientific rationale. In tables 4 and 6 of chapter 5, the available treatment options are summarized and presented in a hierarchical order based on available scientific evidence. However, as shown by the Strength Of Recommendation Taxonomy (SORT) staging, evidence is often scarce further highlighting the need for well-designed prospective clinical trials. Currently, therapeutic decisions are often made based on experience of the treating physician and availability and reimbursement of the therapy.

CMV and allogeneic SCT, a difficult relationship

In **chapter 6** we give an overview of current literature regarding the possible beneficial effects of cytomegalovirus (CMV) reactivation after allogeneic SCT and solid organ transplantation (SOT) (45). In this thesis we focus on CMV reactivation in the setting of allogeneic SCT.

CMV reactivation is considered as a frequent complication after allogeneic SCT and left untreated can cause a variety of organ-specific diseases including pneumonia and encephalitis. Prior to the age of prophylactic and pre-emptive

treatment of CMV reactivation, CMV pneumonia was the most common infectious cause of death after allogeneic SCT. Also CMV reactivation has usually been considered as a possible initiating event in GVHD.

Despite advances in diagnostic techniques and treatment strategies, CMV seropositivity remains to be associated with inferior outcome, especially after myeloablative HSCT (46-48). However, paradoxical observations show that CMV reactivation may protect against leukemic relapse after allogeneic HSCT for AML go back to the mid-1980s (49), and have been confirmed in a series of recent studies (chapter 6, table 1) (50-55). We show that for a subgroup of allogeneic SCT recipients early CMV replication after allogeneic SCT seems to be beneficial. This holds true for patients with Acute Myeloid Leukemia (AML) treated with a myeloablative conditioning regimen and not receiving in vivo T-cell depletion such as ATG or alemtuzumab (50, 56-59). These intriguing observations suggest that CMV reactivation requires a reconstituted donor-derived T-cell and/or NK-cell reservoir to reduce the risk of leukemic relapse. Since CMV itself will probably not induce leukemic cell death, the effect is presumably mainly caused by cross-reactivity of the stimulated innate and adaptive immune response. Immune cells of interest in this response are natural killer (NK) cell and Vδ2negγδ T-cells, cells which attack CMV infected cells but are not reported to be involved in mediating GVHD (60). NK-cells may be induced upon CMV infection and then target recipient AML cells according to their lack of donor HLA molecules (61). On the other hand, circulating γδ T-cells are also expanded and have shown cross-reactivity to primary AML cells (62). When the exact underlying mechanisms are elucidated, therapeutic approaches can be developed to mimic the beneficial effects of CMV reactivation after allogeneic SCT. Possible routes for exploring this could be through a CMV vaccine.

Future directions

Whilst the indications for allogeneic SCT are still increasing and the patient population is expanding through the use of reduced toxicity conditioning regimens, happily the incidence of severe GVHD is decreasing. Due to many adjustments in conditioning regimens, T-cell depleting strategies, timing of DLI and improved GVHD prophylaxis the prevalence of this debilitating iatrogenic disease is slowly decreasing. The rise of more targeted immune therapies such as CAR T-cells will also replace allogeneic SCT for some current indications.

This does not mean we can diminish our efforts as there is still much progress to be made. Current treatment with allogeneic SCT is for a large part still a ‘black box’ of immune therapy. Then also some of the therapies we use to treat GVHD are for a large part a ‘black box’ such as MSC which working mechanisms are only

partly unraveled. When we expose our patients to cellular therapies we should try to learn as much as possible from this in order to keep improving our care. Future studies involving MSC should also consider the biological factors of MSC as well as intrinsic donor factors that can all contribute to their immunomodulatory capacity. Including extensive immune monitoring in clinical trials is mandatory and should probably also be introduced in standard clinical care to acquire big data sets that can show patterns not otherwise visible in smaller cohorts. The findings regarding MSC donor age as described in chapter 3 again emphasize the need to register data in clinical registries in order to pool data in larger cohorts to both show correlations as well as to collect possible rare and long-term adverse events. The current cellular therapy registry of the European society for Blood and Marrow Transplantation (EBMT) is such a tool for reporting and pooling data across Europe (63). Larger data sets will also allow for validating possible biomarkers, cellular as well as soluble, as robust biomarkers are still lacking whilst they could prove very useful in daily clinical practice. Decision-making could be done faster and biomarkers could help to personalize treatment, especially in the current era where we can choose between different classes of agents.

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NEDERLANDSE SAMENVATTING

Dit onderzoek gaat over de complicaties van stamceltransplantaties met stamcellen afkomstig van een donor. Voor de behandeling van een aantal vormen van leukemie en lymfeklierkanker en voor de behandeling van ernstige (aangeboren) afweerstoornissen, blijft een stamceltransplantatie van een donor de enige kans op genezing van de ziekte.

Een stamceltransplantatie van een donor wordt een allogene stamceltransplantatie genoemd. Door een stamceltransplantatie met donorcellen wordt er, behalve nieuwe bloedvormende cellen, ook een nieuw afweersysteem bij de patiënt getransplanteerd. Het nieuwe afweersysteem kan afwijkende cellen opsporen, bijvoorbeeld de leukemiecellen, en deze vernietigen. Dit effect wordt het 'graft versus leukemie' effect genoemd. T-cellen, een specifieke groep binnen de witte bloedcellen, spelen hierin een belangrijke rol.

Helaas heeft een allogene stamceltransplantatie ook een belangrijke keerzijde, namelijk het ontstaan van 'graft versus host' ziekte (GVHD). Dit houdt in dat het nieuwe afweersysteem zich óók tegen de gezonde cellen van de patiënt (de 'host') richt. GVHD kan zo ernstig verlopen dat patiënten hieraan overlijden.

De complexiteit van het menselijk afweersysteem is enorm, waardoor de uitdaging in 2019, ruim 60 jaar nadat de eerste allogene stamceltransplantatie is uitgevoerd, nog steeds bestaat uit het opwekken van het gewenste 'graft versus leukemie' effect zonder hierbij de schadelijke GVHD te krijgen.

In dit proefschrift komen studies aan bod naar verschillende behandelingen van respectievelijk acute GVHD en chronische GVHD met als doel de huidige behandelstrategieën te verbeteren. Ook hebben we een studie verricht naar een mogelijk gunstig effect van een virusinfectie na allogene stamceltransplantatie.

Acute GVHD

Acute GVHD treedt vaak op binnen 3 maanden na een allogene stamceltransplantatie doordat de donor T-cellen de gezonde cellen van de ontvanger herkennen als lichaamsvreemd. Hierdoor ontstaat er een immuunreactie in met name de huid, de darmen en de lever. De klinische symptomen variëren hierbij van lichte huiduitslag tot zeer ernstige diarree en leverfalen. Recent onderzoek van het Centrum voor Internationaal Bloed en Beenmerg Transplantatie (CIBMTR) laat zien dat 15% van de patiënten die een allogene stamceltransplantatie ondergaat, overlijdt aan GVHD.

Hoe vaak acute GVHD optreedt, is sterk afhankelijk van een aantal factoren:

- Hoe goed de donor past bij de ontvanger op bepaalde belangrijke eiwitten (HLA match)

- Stamcellen geoogst uit perifeer bloed geven meer GVHD dan stamcellen uit beenmerg of navelstrengbloed (de stamcelbron)
- Gelijke sekse van donor en ontvanger geeft minder GVHD
- De gebruikte afweer onderdrukkende medicijnen rondom de stamceltransplantatie (GVHD profylaxe)
- De gebruikte (chemo)therapie middelen of bestraling voorafgaand aan de stamceltransplantatie om het beenmerg van de ontvanger voor te bereiden (conditionering)

De belangrijkste behandeling van GVHD bestaat uit het onderdrukken van het (overactieve) afweersysteem met medicijnen zoals prednison. Ongeveer de helft van de patiënten reageert hier goed op, de andere helft niet en deze patiënten hebben nog zwaardere afweer onderdrukkende middelen nodig, zogenaamde tweedelijns behandeling. Welk tweedelijns middel het beste is voor welke patiënt is niet goed uitgezocht. Een van de behandelingen die wordt gegeven bestaat uit het toedienen van Mesenchymale Stromale Cellen (MSC). Dit zijn een speciaal type cellen uit het beenmerg van gezonde donoren die de afweerreactie kunnen onderdrukken.

In **hoofdstuk 2.1** worden de resultaten beschreven van een klinische studie met 48 patiënten met acute GVHD die werden behandeld met 3 infusies met MSC:
- 50% van de patiënten reageert hier goed op.
- Van de patiënten bij wie de therapie aanslaat, leeft na 1 jaar nog 79%, bij de patiënten die niet goed reageren leeft nog maar 8% na 1 jaar.
- Na toediening van MSC is een stijging van de immature myeloide dendritische cellen geassocieerd met een betere overleving.
- In de toekomst kan deze cellulaire biomarker mogelijk worden gebruikt om sneller van behandeling te wisselen indien deze niet lijkt aan te slaan.

In **hoofdstuk 2.2** is de flow cytometrie data van verschillende soorten immuuncellen geanalyseerd d.m.v. computer algoritmes om nog eerder en beter te kunnen voorspellen welke patiënten goed gaan reageren op de behandeling met MSC.
- Op dit moment zijn deze predictiemodellen echter nog niet goed genoeg om in de praktijk te kunnen gebruiken.

In **hoofdstuk 3** staan de kenmerken van de MSC beenmergdonoren op de uitkomsten van de patiënten centraal. Vanwege de succesvolle behandeling in de helft van de patiënten beschreven in hoofdstuk 2, hebben we uiteindelijk in totaal

102 patiënten met MSC behandeld. Door de toename in groepsgrootte konden we meer variabelen onderzoeken die mogelijk van invloed zijn op de uitkomst.

- Patiënten behandeld met MSC gekweekt uit beenmerg van beenmergdonoren onder de 10 jaar oud, hebben een grotere kans op overleving dan patiënten die zijn behandeld met MSC afkomstig van oudere beenmergdonoren.
- Nadere proeven in het laboratorium laten zien dat MSC van jonge donoren meer T-cel remming geven.
- Ook laten analyses naar de eiwitexpressie van de MSC van de jonge versus de oude donoren zien dat er andere genen aan- en uitgeschakeld staan.
- Bij het selecteren van geschikte beenmergdonoren kunnen deze verschillen in de toekomst worden meegenomen om de behandeling te verbeteren.

Chronische GVHD

Chronische GVHD is een geheel andere ziekte dan acute GVHD en lijkt veel meer op een auto-immuunaandoening. Na een stamceltransplantatie van een donor krijgt ongeveer 30-70% van de patiënten in enige mate last van chronische GVHD. Alle orgaansystemen kunnen worden aangetast en verbindweefseling ('fibrose') speelt hierin een belangrijke rol. De behandeling van chronische GVHD bestaat ook uit afweer onderdrukkende medicijnen zoals prednison. De behandeling is vaak langdurig waardoor er veel ernstige bijwerkingen ontstaan door de therapie.

Wij hebben een combinatie van twee medicijnen getest die afzonderlijk van elkaar wel enig effect hebben op chronische GVHD, maar nog niet eerder in de combinatie zijn gebruikt. Het eerste medicijn is rituximab, dit is een monoklonaal antilichaam tegen het eiwit CD20 wat aangrijpt op de B-cellen, een bepaald subtype van de witte bloedcellen. Het tweede medicijn is nilotinib, dit is een zogenaamde tyrosine kinase remmer welke bepaalde enzymen remt, o.a. ook de PDGF-receptor welke betrokken is bij de vorming van fibrose.

In **hoofdstuk 4.1** staan de resultaten van een klinische studie beschreven waarbij 29 patiënten met een ernstige vorm van chronische GVHD zijn behandeld met een combinatie van rituximab en nilotinib.

- Bij 71% van de patiëntengroep is een klinische verbetering opgetreden (complete respons + partiële respons).
- Bij de patiënten met een goede respons kon de prednison dosis ook worden afgebouwd.
- Patiënten die van tevoren een laag aantal CD5+ B-cellen hadden, hadden meer kans op een respons.

In **hoofdstuk 4.2** zijn bloedsamples van de patiënten uit deze klinische studie vervolgens gebruikt om de spiegel van nilotinib te meten.

- Het blijkt dat er een mogelijke relatie bestaat tussen de hoogte van de spiegel en de kans op respons.
- Bij patiënten die niet goed reageren op de behandeling kan de spiegel van nilotinib voortaan worden gemeten om te kijken of ze wel de juiste dosis krijgen.

Hoofdstuk 5 betreft een overzichtsartikel waarin alle huidige behandelingen voor acute en chronische GVHD op een rij zijn gezet. Ook wordt de recent gepubliceerde Nederlandse richtlijn beschreven. Deze richtlijn wordt gesteund door de HOVON (Stichting Hemato-Oncologie voor volwassenen Nederland) stamceltransplantatie werkgroep.

Mogelijk gunstig effect CMV infectie

De periode na een allogene stamceltransplantatie wordt gekenmerkt door een slechte afweer en hierbij een hoog risico op infecties. Het cytomegalovirus (CMV) komt frequent voor en kan indien het niet wordt behandeld leiden tot ernstige long- en herseninfecties. Om deze reden wordt CMV standaard gemonitord na het ondergaan van een allogene stamceltransplantatie. Indien er een (re)activatie van het virus optreedt, kan vroeg worden gestart met antivirale therapie.

Hoofdstuk 6 geeft een beschrijving van de recente literatuur betreffende CMV (re)activatie en allogene stamceltransplantatie.

- Een subgroep van patiënten lijkt baat te hebben van een CMV (re)activatie.
- Het betreft hier patiënten met een acute myeloide leukemie (AML) met een myeloablatieve conditionering en zonder T-cel depletie die minder relapse van hun leukemie krijgen.
- Mogelijk bestaat er cross-reactiviteit tussen de herkenning van CMV en resterende leukemiecellen.

Hoofdstuk 7 geeft een overkoepelende discussie van de resultaten van dit proefschrift.

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Collega's

Het lab

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CURRICULUM VITAE

Lotte Emilia van der Wagen was born on January 19 1983 in Ede, daughter of a nurse and a civil engineer. She grew up in Ede together with her two younger brothers. After secondary bilingual education at the Marnix College she entered medical school at Utrecht University in 2001.

During her internships it became clear that Internal medicine and especially Hemato-oncology had her special interest. In 2008 she started her residency in Internal medicine at the UMC Utrecht (supervisors prof. dr. D.H. Biesma, prof. dr. M.M.E. Schneider and prof. dr. H.A.H. Kaasjager). The peripheral part of her internal medicine training was performed in the St. Antonius Hospital in Nieuwegein (supervisor Dr. A.B.M. Geers).

Her research project commenced with a successful grant application at the Dutch Cancer Society. From May 2012 until August 2016 she worked in the research group of prof. dr. J.H.E. Kuball on translational research projects involving Graft versus Host Disease.

Since February 1st 2019 she has finished her Internal medicine and Hematology training and started as a hematologist at the UMC Utrecht with a special focus on stem cell transplantation.

Lotte is married to Rik van der Ende and together they have two beautiful daughters, Emi (2013) and Hanne (2015).