Role of clinical markers of inflammation in assessing chronic graft versus host disease activity and severity

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This dissertation has been made at the Experimental Immunology and Transplantation Branch, National Cancer Institute (NCI), National Institutes of Health (NIH) in Bethesda, Unites States in collaboration with Division of Hematology, Department of Internal Medicine, School of Medicine, University of Zagreb.

Mentor 1: Prof Boris Labar
Mentor 2: Prof Steven Z Pavletic

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Finally, I would like to thank my family for enormous support, love and patience.

Contents

List of abbreviations

AA	aplastic anemia
AEC	absolute eosinophil count
aGVHD	acute graft versus host disease
ALC	absolute lymphocyte count
Allo-HSCT	allogeneic hematopoietic stem cell transplantation
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
ANC	absolute neutrophil count
APC	antigen presenting cell
APR	acute phase reactants
ATG	anti-thymocyte globulin
BAFF	B-cell activating factor
BM	bone marrow
BOS	bronchiolitis obliterans syndrome
cGVHD	chronic graft versus host disease
CRP	C reactive protein
CD	cluster of differentiation
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CMV	cytomegalovirus
CT	computed tomography
DC	dendritic cell
DLI	donor lymphocyte infusion
DNA	deoxyribonucleic acid
EBV	Epstein–Barr virus
ECP	extracorporeal photopheresis
ESR	erythrocyte sedimentation rate
FEV1/FVC	forced expiratory volume in first second/ forced vital capacity
FSH	follicle stimulating hormone
GI	gastrointestinal
GVT	graft-versus-tumor effect
HLA	human leucocyte antigen
HPV	human papyloma virus
HSV	herpes simplex virus

IBMTR	International Bone Marrow Transplant Registry
IFN	interferon
IL-1	interleukin
IL2ra	IL2 receptor alpha
IRB	institutional review board
JAK	Janus kinase
KPS	Karnofsky performance status
LH	luteinizing hormone
MCP1	monocyte chemotactic protein 1
MCS	mental component summary
MDS	myelodysplastic syndrome
mHAg	minor histocompatibility antigens
MHC	major histocompatibility complex
MM	multiple myeloma
MSC	mesenchymal stem cells
mTOR	mechanistic target of rapamycin
NCI	National Cancer Institute
NIH	National Institutes of Health
PBSC	peripheral blood stem cell
PCR	polymerase chain reaction
PDGFR	platelet-derived growth factor
PFT	pulmonary function test
PNH	paroxysmal nocturnal hemoglobinuria
PSC	physical component summary
PST	prior systemic therapies
PT	prothrombin time
PTH	parathyroid hormone
PTT	partial thromboplastin time
PUVA	psoralen plus ultraviolet light therapy
RIC	reduced intensity conditioning
ROM	range of motion
RV	residual volume
SCID	severe Combined Immunodeficiency
SIL-2RL	soluble interleukin-2 receptor alpha

SSc	systemic sclerosis
T reg	T regulatory
TBI	total body irradiation
TCD	T cell depleted
TGFβ	transforming growth factor beta
TLR9	Toll-like receptor 9
TNF	tumor necrosis factor
TP	total protein
TRM	transplant related mortality
TSH	thyroid - stimulating hormone
UCB	umbilical cord blood
VOD	veno-occlusive disease
vWF	von Willebrand factor
WBC	white blood cells

1. Introduction

1.1. Allogeneic hematopoietic stem cell transplantation

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a curative treatment for various hematological malignant and non-malignant otherwise fatal diseases. In 1957. Nobel prize winner E.D. Thomas performed first human twin transplant for leukemia. [1] In 1959 Mathé performed first bone marrow transplants for radiation accident victims. [2] In the 1960s, additional information regarding the HLA system became available; the serologic HLA typing method was developed resulting with first successful HLA-matched sibling transplant for SCID in 1968. [3,4,5] First successful complete engraftment and survival of over 1 year was reported by Mathé et.al as well as description of acute and chronic GVHD in men. [6,7] In 1970's clinical bone marrow transplantation takes off, in early 70's Thomas performed first successful bone marrow transplantation for severe aplastic anemia and in 1977. one hundred patients with acute leukemia were treated by chemotherapy, total body irradiation, and allogeneic bone marrow transplantation from HLA matched sibling donor. Ninety-four patients were engrafted and only one patient rejected the graft. Thirteen patients are alive with a marrow graft, on no maintenance antileukemic therapy, and without recurrent leukemia 1-4.5 years after transplantation. [8] The principle of treating malignant hematological diseases by allogeneic stem cell transplant is to permit allogenic, immunologically competent cells to act against the host's leukemic cells. [6] Such an effect may be achieved by administration of high dose chemotherapy as part of the conditioning regimen followed by allogeneic stem cell transplant infusion. The donor immune system recognizes residual tumor cells as foreign and eradicates them via the graft-versus-leukemia (GVL) effect. Barnes and Loutit first described the graft versus tumor effect of transplanted spleen cells in experimental murine models and Mathé in humans. [9,7] The first direct demonstration of clinical GVT effect was the successful application of DLIs to treat relapsed CML. [10] The graft versus host disease was first described as "secondary syndrome" in humans and runting syndrome in mice. Since 1980's – 2000's the improvement were made in supportive care, GVHD prophylaxis, better management of early complications, new stem cell sources, new indications, DNA-based tissue typing, new conditioning regimens with less toxicity were introduced, resulting in improved outcomes, older patients appropriate for transplant, the rise cord blood transplantation, etc. Nevertheless, acute and chronic GVHD remain a major contributor to transplant-related deaths and very significant barrier to successful allo-HSCT. [11, 12]

The number of allogeneic transplantations continues to increase with more than 25 000 performed annually. [12] Now days patients are followed for 10 or more years after allo-HSCT. Recent study by Gooley et al. had shown substantial reduction in the hazard of death related to allogeneic transplantation and improved long-term survival after allo-HSCT due to reduction in organ damage, infection and severe acute graft versus host disease (GVHD). [13] However long-term survivors experience the burden of long-term complications such as chronic GVHD, metabolic, endocrinology abnormalities, decreased quality of life and secondary malignances. Mortality rates remain twice as high as that of the general population among 15-year survivors of HCT and relapse and chronic GVHD were the leading cause of premature death in survivors more than 2 years after allo-HSCT. [14]

1.2. Graft-versus-host-disease

Fifty years ago Billingham formulated three requirements for the development of GVHD: the graft must contain immunologically competent cells; the recipient must express tissue antigens that are not present in the transplant donor; and the recipient must be incapable of mounting an effective response to eliminate the transplanted cells. [15]

Important changes in clinical considerations

The time of onset became an arbitrary criterion, and it has become more meaningful to define the disease on the basis of clinical and histological findings. Accordingly, the commonly use day -100 posttransplantation cutoff to separate acute from chronic GVHD is no longer satisfactory and the 2005 NIH consensus defined that clinical manifestations rather than time from transplant should determine the presence of acute or chronic GVHD. (Figure 1.) NIH classification includes persistent, recurrent or late acute GVHD (after day-100) and an overlap syndrome (with both acute and chronic GVHD features). [16]

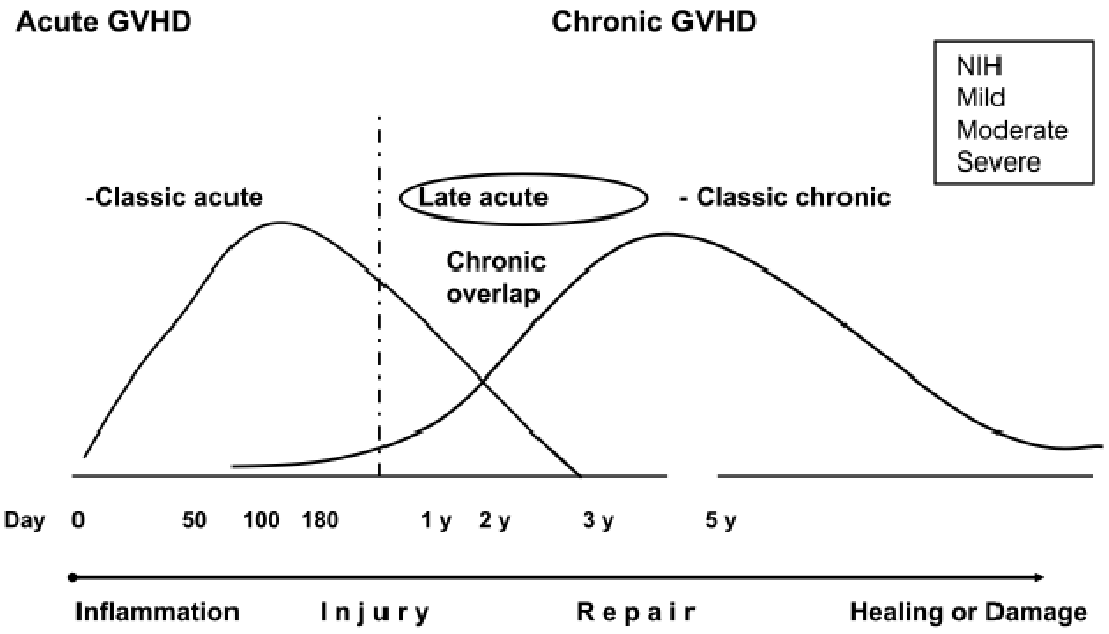


Figure 1. GVHD classification after NIH consensus *Courtesy of Prof SZ Pavletic*

1.2.1. Acute graft versus host disease

Acute GVHD remains a common complication after allo-HSCT and represent one of the most significant barriers to successful allo-HSCT and significant cause of treatment failure after transplantation, accounting for a substantial portion of early transplant morbidity and mortality. The most important factors that are responsible for alloreactivity include donor-host tolerance mechanisms and the use of immunosuppression. The three key major events in pathophysiology of acute GVHD include: 1) tissue damage from the conditioning regimen – leading to activation of antigen presenting cells and secretion of proinflammatory cytokines such as IL-1 and TNF-α 2) donor T-cell activation against recipient antigen in the context of MHC, and 3) an inflammatory response manifested by T-cell cytotoxic response against the host tissues (skin, gut, or liver). [17] (Figure 2.)

Polymorphisms for cytokines that are involved in "cytokine storm" are also risk factors for developing GVHD. [18]

The incidence of aGVHD is directly related to the degree of HLA mismatch. [19]

Class I HLA (A, B, and C) antigens are expressed on almost every nucleated cell in the organism and class II HLA (DR, DQ, and DP) are primarily expressed on hematopoietic cells (monocytes, dendritic cells, B-cells). In addition to class I and class II HLA antigens also "minor" histocompatibility antigens, such as HY and HA-3 represent a target for both GVHD and GVL. [20]

Clinically relevant, grade II-IV acute GVHD occurs in 35-45 % of patients who receive grafts from matched related donors, and in 60-80% in recipient's one antigen mismatched unrelated donor grafts. [21, 22]

The broad category of aGVHD includes classic acute GVHD (maculopapular erythematous rash, gastrointestinal symptoms and cholestatic hepatitis), occurring within 100 days after transplant or donor lymphocyte infusion, while persistent recurrent or late aGVHD (usually seen after withdrawal of immunosuppression) occurs beyond 100 days of transplantation or DLI. Both aGVHD subentities should occur without the presence of diagnostic or distinctive cGVHD manifestations. The newly defined entity, "late onset" of aGVHD has been shown to be highly associated with poor survival when cGVHD where reclassified according to new definition.[23,24]

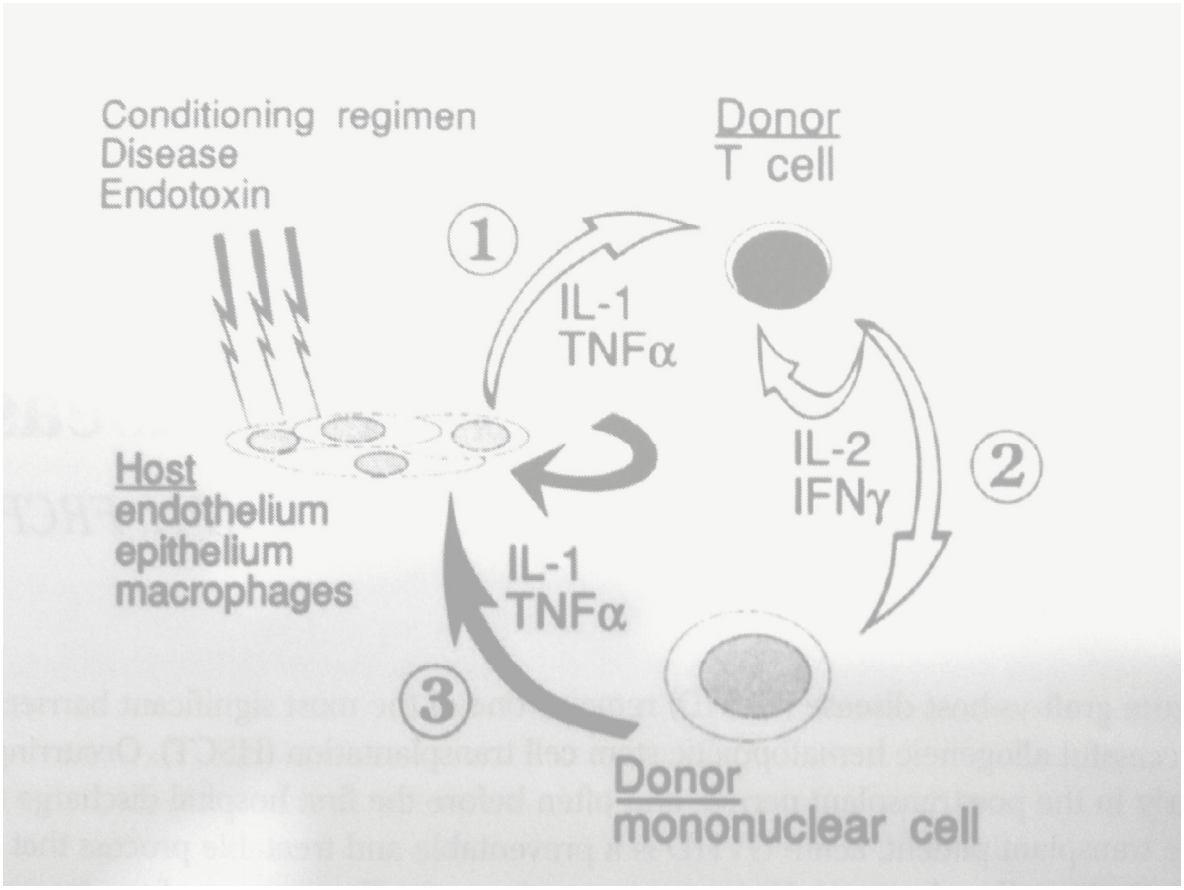


Figure 2. Pathophysiology of aGVHD *Internet source*

The conditions that increase risk for acute GVHD are listed in Table 1. [25]

Table 1. Risk factors for acute graft versus host disease

Donor recipient factors	Stem cell graft factors	Transplant related
HLA mismatch	PBSC > BM > UCB	Myeloablative > RIC
ABO incompatibility		
Unrelated donor		
Older donor		
Multiparity		
CMV seropositivity		

Combination of cyclosporine and pulse doses of methotrexate is the most often use pharmacologic prophylaxis of acute GVHD. Cyclosporine inhibits IL-2 mediated T-cell inhibition via inhibition of calcineurin. Methotrexate impairs purine synthesis in T-cells and prevents T-cell expansion. Other immunosuppressants such as tacrolimus, sirolimus (mTOR inhibitor) or mycophenolate-mofetil are also used. Clinical manifestations of acute GVHD include skin changes, diarrhea and liver impairment. Skin is the most commonly and usually the first affected organ and nearly half of the patients have skin involvement as only GVHD manifestation. The most common sign of aGVHD of the skin is maculopapular exanthema. Typically, a rash is appearing on palms and soles and it is highly suggestive for aGVHD and the presence of rash at these localizations helps differentiate between aGVHD and medicamentosus rash that generally spares these areas. The gastrointestinal tract is second most commonly affected organ. Symptoms of the upper GI involvement include anorexia, nausea, and dyspepsia, and the symptoms involving lower GI tract include profuse watery diarrhea, crampy abdominal pain, bleeding and in most severe form paralytic ileus. Cholestatic jaundice (hyperbilirubinemia) is the most common manifestation of the liver involvement. As per Glucksberg or IBMTR scales each organ is given an individual stage and these stages are combined to overall grade of GVHD. The overall grades are classified as I (mild), II (moderate), III (severe) and IV (very severe). [26] Only isolated skin aGVHD limited to a small surface area (stage I or II) can be treated with topical steroids. The standard primary therapy for grade II-IV acute GVHD are systemic corticosteroids (methylprednisolone 0.5 to 2 mg/kg, depending on center). Concurrently, patients are continued on calcineurin inhibitor based GVHD prophylaxis. Systemic corticosteroids are lympholytic and rapidly inhibit the inflammatory cytokine cascade. The second line therapies in steroid refractory disease (progression after 3 days on corticosteroid therapy and no improvement after 5-7 days) include: sirolimus, mycophenolate mofetil, methotrexate, antithymocyte globulin, infliximab (anti-TNF alpha) and ECP. [27] An early response to corticosteroids is a significant predictor of outcome. The most established prognostic factors for poor survival and mortality are grade III-IV severity and refractory disease. [28,29]

1.2.2. Chronic graft versus host disease (cGVHD)

Chronic GVHD is a multisystem disorder and the leading cause of non-relapse morbidity and mortality in survivors after allo-HSCT, but it is also associated with lower malignancy relapse rate, presumably because of graft-versus-leukemia effects. [30,31,32] Chronic GVHD is the single major factor determining long-term outcome and quality of life after HCT. [33] The incidence of disease occurrence is approximately 50% of transplant recipients. [34] Patients with cGVHD have poor quality of life, impaired functional status, inability to work, and need for ongoing chronic care, which also has important impact to health-related costs. [35]

They often require prolonged immunosuppressive treatment for an average of 2-3 years, which than puts them in danger of infection and unwanted consequences of corticosteroid treatment. Typical clinical manifestations are very protean and may reflect active tissue inflammation such as erythematous rash, oral erythema and lichenoid changes as well as more chronic processes such as sclerotic skin changes, joint contractures or fasciitis of the subcutaneous tissue. [33] It may often appear similar to systemic autoimmune diseases such as systemic sclerosis or Sjogren's syndrome. Despite recent progress in cGVHD severity staging [36] there are no reliable clinical measures of disease activity to differentiate active inflammation from residual tissue damage.

CGVHD Consensus Conference held in 2005 at the National Institutes of Health, USA, produced recommendations regarding cGVHD diagnosis, staging, histopathology, response criteria, biomarkers, ancillary and supportive care, and design of clinical trials.

These recommendations provided scoring system based on number of organs involved, severity and functional disability. In 2014, second cGVHD NIH Consensus Conference updated these recommendations. [37]

Very recent study from the Center for International blood and marrow transplant research showed increasing incidence of cGVHD in last 12-year period. In the multivariate analysis the period from 2004-2007 was associated with higher risk of cGVHD when compared with the earlier time periods (1995-1999 and 2000-2003). In the multivariate analysis the use of bone marrow with an unrelated donor and PBSC graft with all categories of donor group was associated with higher risk of cGVHD as compared with use of bone marrow with a matched sibling donor. Also, patients who developed cGVHD, non-relapse mortality has decreased over time, but at 5 years there were no differences among different time periods. [38]

1.2.2.1. Pathophysiology

The pathophysiology of cGVHD remains unclear. The disease is characterized by a combination of allogeneic and auto-immune dysregulation with significant immune deficiency. Impaired responses by both T (Treg, Th1 and Th2) and B cells lead to cytokine and antibody production and inflammation. [39,40,41] In mouse model, Th1 cytokines (IL-2 and IFN-γ) can reduce cGVHD and Th2 cytokines such as IL-4, IL4, IL-5, IL-10, and IL-13 can increase cGVHD, [42] but mouse models do not replicate human cGVHD, which can be associated with either Th1 or Th2 cytokine imbalance supported by the results of various studies: Nakamura et al. showed that IL-4-producing CD8+ T-cells were was significantly higher in patients with cGVHD than in patients without cGVHD and may be an immunological hallmark of cGVHD [43]; Ritchie et al showed that increased TNF-α and IFN-γ transcription predicted for the onset of extensive chronic GVHD [44], and Cavet et al showed that IFN-γ and IL-6 gene polymorphisms associate with cGVHD. [45] In cGVHD patients treated with ECP Th1 cells always increased during therapy, supporting the hypothesis that a more favorable immune balance contributes to clinical responses. [46]

Role of thymic regulation

The immune reconstitution after HSCT is happening via thymic-independent (mature donor T-cells from the graft) and thymic-dependant pathway (production of naïve T cells from donor hematopoietic stem cell). [47,48] Tymic damage is caused both by conditioning regiment and acute GVHD. [49] Dysregulation of thymic function and failure of negative selection is certainly one of the causes of cGVHD. (Figure 3.) CD4+ cells that express receptors with high affinity for "self-antigens" are normally deleted. CD4+ T cells generated de novo from donor stem cells appear to mediate the evolution of CGVHD from acute GVHD [50]. In fact, cGVHD occurs, even though it may not be preceded by acute GVHD. Zhang et al. found that host thymus is not required for the induction of cGVHD and that quiescent autoreactive T and B cells in transplants from non-autoimmune donors might be activated and expanded to cause cGVHD with autoimmune manifestations. [51]

T regulatory (CD4+CD25+) cells

Treg cells are characterized by their constitutive expression of the IL-2 receptor α chain (CD25). A FOXP3, a member of forkhead family of transcription factors was shown to be highly expressed in Treg cells. In mouse models adoptive transfer of *ex vivo* expanded CD4+CD25+T cell can prevent GVHD. [52,53] In humans, studies results are controversial. Some studies have shown that patients with cGVHD have elevated Tregs [54] and other reported decreased Tregs numbers. [55,56] The mechanism by which Tregs suppress cGVHD remains uncertain, but there is evidence that suppression is mediated by cytokines, such as transforming growth factor TGF-β and interleukin IL-10, or by contact with plasmacytoid dendritic cells through indoleamine 2,3-dioxygenase. [57] The adoptive transfer of Tregs in animal models of GVHD has demonstrated their efficacy, which suggests that Tregs can be exploited in the clinical setting. [53] Giorgini et al. showed that alloantigen-driven expansion is critical for the effectiveness of Tregs, and suggested that cellular therapy with alloantigen-induced Tregs in combination with glucocorticoids could prevent cGVHD after immune reconstitution. [58] Extracorporeal photopheresis increases levels of circulating functional Tregs in cGVHD patients [59] and recently, a novel photodepleting approach was found to both preserve and expand Treg numbers while selectively eliminating CD4+ effector T cells from patients with cGVHD. [60]

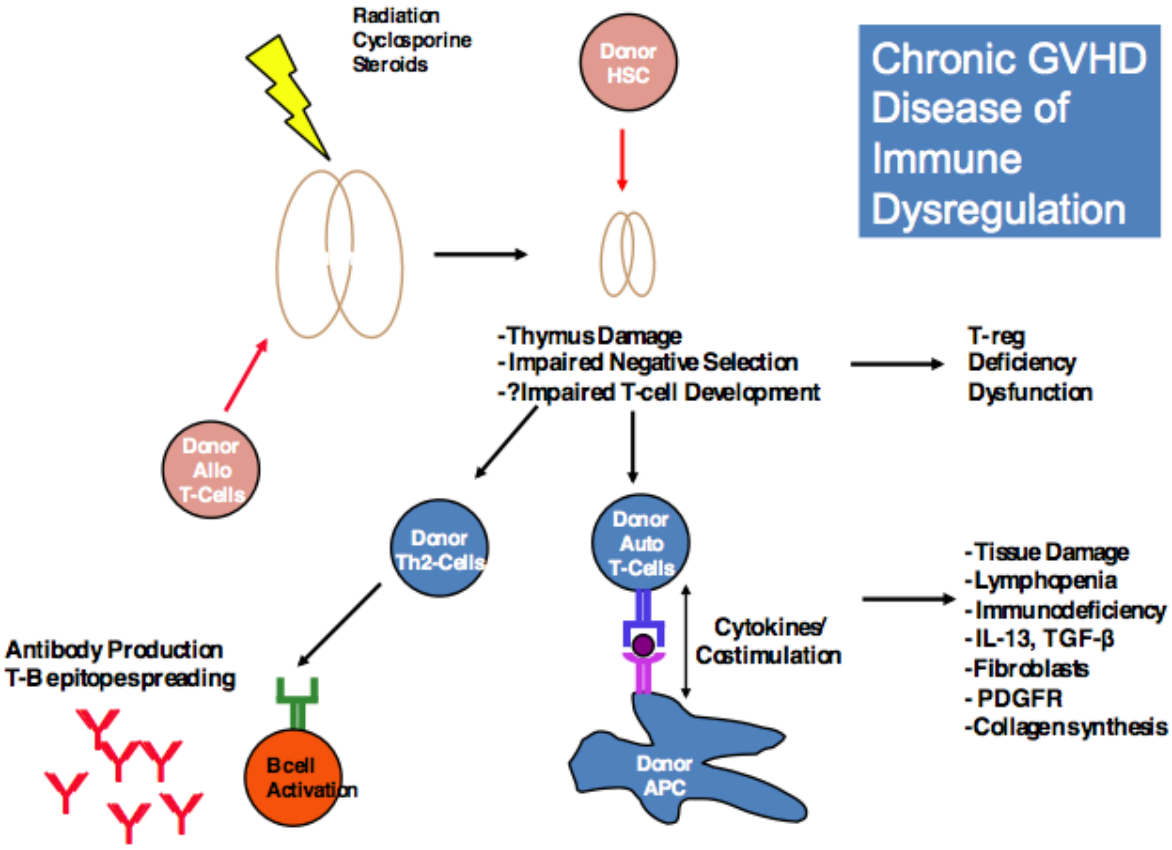


Figure 3. Immune dysregulation in cGVHD *Courtesy of Prof SZ Pavletic*

cGVHD and autoimmunity

On the other hand B-cell plays significant role in autoimmune component of cGVHD pathogenesis. Although cGVHD occurs in allogeneic transplant setting it shows some similarities with autoimmune diseases suggesting dysregulation in humoral immunity as well. It has been shown that patients with cGVHD have more circulating autoantibodies (anti-nuclear, anti-mitochondrial, anti-smooth muscle, anti-parietal) [61,62] and higher levels of B cell activating factor (BAFF) in their sera. [63,64,65] As BAFF levels are high after allo-HSCT, B cells are not through negative selection are likely positively selected during B cell recovery. In a study performed by Patriarca et al it was shown that patients who developed autoantibodies showed faster B-cell recovery, based on significant increase of B cell subset.

BAFF high levels and autoantibody production suggest a critical breakdown in peripheral B cell tolerance in patients with cGVHD and represents a model for aberrant persistence of allo- and auto-reactive B cells after transplantation and failure of normal B cell tolerance checkpoints. As a result, there is persistence of donor B cells reactive to recipient antigens and secretion of pathologic allo- and auto-antibodies. [66]

On the other hand these autoantibody positive patients showed abnormally low levels of serum immunoglobulins, which indicate, prolonged functional impairment. Also, donor B-cell responses to recipient HY antigens have been associated with the development of cGVHD in the setting of gender-mismatched alloHSCT. [67] This hypothesis is confirmed with anti-CD20 monoclonal antibody successful treatment in steroid-refractory cGVHD. [68,69] It has been shown that cGVHD patients with hypergammaglobulinemia have a significantly increased BAFF/B-cell ratio and serum autoantibodies (ANA, anti-dsDNA) compared to patients with hypogammaglobulinemia. In addition, hypergammaglobulinemia was significantly associated with sclerodermous form of skin cGVHD in multivariate regression analysis. [65] It has been shown that antibodies to platelet-derived growth factor (PDGF) in patients with sclerotic cGVHD have the capacity to induce both tyrosine phosphorylation of the PDGF receptor and type I collagen gene expression in fibroblasts, leading to fibrosis. [63]

Moreover, B cells are essential in many functions other than antibody secretion; direct antigen-presentation with priming of T lymphocytes and secretion of cytokines that modulate the intensity and type of immune response. [70] Increased levels of TLR9 expression have been documented in B-cells from cGVHD patients, suggesting an improved ability of these cells to act as APCs and to sustain a chronic inflammatory environment. [71]

Profibrotic-Inflammatory Cytokines

Scleroderma-like changes in cGVHD occur in up to 13%–16% of patients. [72] Many similarities have been described between SSc and cGVHD. T-cells (CD4+) are necessary for the disease, but several lines of evidence point to a pivotal role of cytokines, mainly TGF-β, in the development of fibrotic changes. [73]

These observations initiated the treatment with a thyrosine-kinase inhibitors (in use for treatment of chronic myeloid leukemia) of PDGFR, c-KIT, BCR-ABL and share potent antifibrotic and anti-inflammatory properties, being powerful dual inhibitors of both PDGF-R and TGF-β pathways like imatinib with good responses in steroid refractory/dependent cGVHD. [74]

Spoerl et al showed that inhibition of JAK1/2 signaling resulted in reduced proliferation of effector T-cells and suppression of proinflammatory cytokine production in response to alloantigen in mice. They treated six treated patients with steroid-refractory GVHD with ruxolitinib. All patients responded with respect to clinical GVHD symptoms and serum levels of proinflammatory cytokines (suppression). Ruxolitinib impaired differentiation of CD4 (+) T cells into IFN-γ- and IL17A-producing cells, and promoted tolerogenic Treg cells. [75]

Role of eosinophils

Increased peripheral blood eosinophils are known to be associated with cGVHD, i.e. a particular form of fasciitis, eosinophilic fasciitis, a scleroderma-like process in which the fascia is inflamed with eosinophilic infiltration. Results of a pilot study showed sparing effect of Montelukast (cysteinyl leukotriene receptor-1 antagonist) that targets eosinophils in treatment of cGVHD. [76]

Inflammatory response

After activation, DC and B cells start to secrete inflammatory cytokines. As a marker of activated T cells, soluble interleukin-2 receptor alpha (sIL-2RL) has been reported to correlate with severity of aGVHD and cGVHD. [77]

1.2.2.2. Risk factors

Previous acute GVHD

Chronic GVHD may be a later manifestation of alloreactive acute GVHD, a result of tissue damage caused by acute GVHD or treatment aimed to acute GVHD or share the same risk factors because both acute and chronic GVHD stem for alloreactivity.

In a study performed by Flowers et al for all risk factors associated with cGVHD (use of female donors for male recipients, grafting with mobilized blood cells), point estimates and confidence intervals were not significantly changed after adjustment for prior acute GVHD that suggests the mechanisms involved in acute and chronic GVHD are not entirely congruent and that cGVHD is not simply the end stage of acute GVHD. [78] (Figure 4.)

Peripheral blood stem cells as transplant source

A meta analysis performed by Cutler et al showed that relative risk for development of cGVHD is much higher after peripheral blood than bone marrow transplantation. [79] The underlying immunologic factors affecting the appearance of cGVHD in peripheral blood and bone marrow recipients are not completely understood. High CD34+ counts may be important factor, since cGVHD did not correlate with CD3+ counts. Higher doses of CD34+ cells (> 8.0 x106/kg) were associated with significantly increased risk of clinical extensive cGVHD. [80]

HLA disparity between recipient and donor

Chronic GVHD occurs in approximately one-third of patients receiving HLA-identical sibling transplants, half of patients undergoing HLA non-identical related HSCT, and two-thirds of those undergoing matched unrelated HCT. [31,34] Minor HLA antigen mismatches are also recognized in the development of cGVHD when a male recipient receives cells from female donor, especially when donor had prior pregnancy or transfusions. Miklos et al showed that antibody responses to H-Y minor histocompatibility antigens correlate with cGVHD. [67]

Age of the recipient and donor

Adult transplant recipients develop cGVHD more often (46%) than pediatric patients (13%). Allo-HSCT after RIC in high-risk patients (older age) also resulted in high incidence of cGVHD. Older donor age (more than 30 years old) is associated with increased risk of cGVHD development. [33,78,34]

Infection

Some reports link cytomegalovirus (CMV) infection with chronic GVHD. CD13 is aberrantly expressed in CMV-infected individuals, and antibodies to CD13 have been associated with chronic GVHD. [81,82]

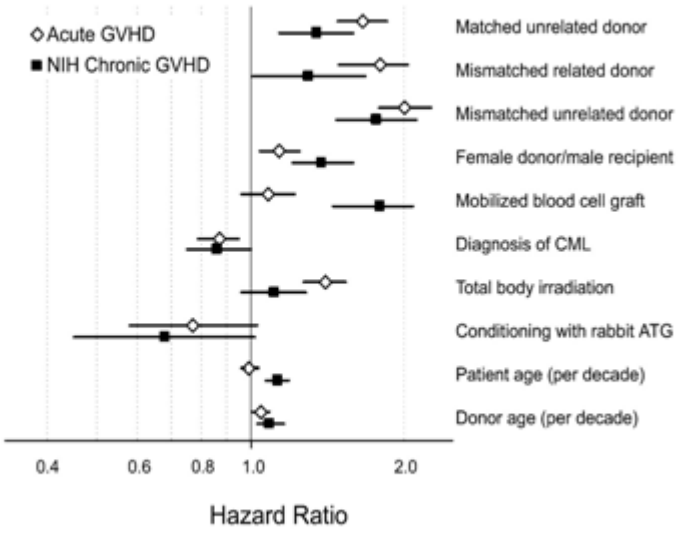


Figure4. *Multivariate risk factor profiles for grades 2-4 acute GVHD and NIH cGVHD Hazard ratio and 95% CI for each risk factor* [78]

Thrombocytopenia

A low platelet count in cGVHD patients is among the most consistent and strongest negative survival predictors across cGVHD studies in both allogeneic bone marrow transplantation (allo-BMT) and allogeneic peripheral stem cell transplantation (allo-PBSCT). [83–85,86–91] Patients with cGVHD and persistent thrombocytopenia demonstrate poorer responses to therapy, experience higher mortality rates from infection or, less often, from hemorrhage. [86,92] Low platelet counts were also reported as a marker for a group of patients with severe cGVHD who have increased incidence of transplant-related complications and a higher mortality rate. [84–86,90,92,93,94,95] The thrombocytopenia in cGVHD is not usually associated with disease relapse or graft rejection, but significantly correlates with increased non-relapse mortality, [96] indicating the existence of additional poorly understood pathophysiological mechanisms that could generate the association of thrombocytopenia and negative outcome of cGVHD.

Although thrombocytopenia in cGVHD patients is strong predictor of poor survival in many cGVHD studies, such correlation is still neither clearly explained nor well understood. Several possible mechanisms of thrombocytopenia in the cGVHD setting were proposed: transplant-related thrombocytopenia, malignancy relapse, microangiopatic thrombocytopenia, drug-induced thrombocytopenia, immune-mediated thrombocytopenia, hypersplenism, infection, cytokine-induced thrombocytopenia (increased TGF-β, low thrombopoietin level, other cytokines). [83]

Type of onset

Chronic GVHD that evolves directly from aGVHD; progressive-onset has worse prognosis than quiescent or de novo onset.

1.2.2.3. Survival

Recent study by Arora et. al showed that in the multiple regression model, increasing recipient age, the presence of and higher grade of prior aGVHD, early onset of cGVHD (< 5 months), higher serum bilirubin at cGVHD onset, lower Karnofsky performance status at cGVHD onset, presence of thrombocytopenia at cGVHD onset (platelet count of < 100x109/L), transplantation from a mismatched URD or other related donor versus an HLA-identical sibling donor, disease status at transplantation (intermediate or advanced versus early), GVHD prophylaxis, and gender mismatch (female donor to male recipient versus male donor to male recipient) were significantly associated with a higher risk of mortality. [97]

Factors associated with a decreased risk of NIH chronic GVHD were the use of rabbit ATG in the pretransplant conditioning regimen and a diagnosis of CML. [98,34]

1.2.2.4. Diagnosis

Diagnosis of cGVHD is made based on established NIH Consensus criteria [16] and requires the following: distinction from acute GVHD, the presence of at least one diagnostic feature such as skin or oral mucosa lichen planus-like changes, poikiloderma, deep sclerotic features of chronic GVHD (Table 2.) or the presence of at least one distinctive clinical manifestation confirmed by biopsy or laboratory tests, (evaluation by ophthalmologist, gynecologist) or radiology (Table 3.) and exclusion of other possible diagnoses.

Table 2. Diagnostic cGVHD features

Diagnostic features of cGVHD	
Skin	Poikiloderma
	Lichen planus-like
	Morphea-like
	Lichen sclerosus-like Deep sclerotic features
Mouth	Lichen-type
	Hyperkeratotic plaques
	Restriction of mouth opening from sclerosis
Genitalia	Lichen planus-like
	Vaginal scarring
Gastrointestinal tract	Esophageal web
	Strictures or stenosis in the
	upper to mid third of the esophagus
Lung	Bronchiolitis obliterans
	diagnosed with lung biopsy

Fascia, Joints	Fasciits
	Joint stiffness or contractures secondary
	to sclerosis

Table 3. Distinctive cGVHD features

Distinctive features of cGVHD	
Skin	Depigmentation
Nails	Dystrophy
	Longitudinal ridging, splitting or brittle features
	Pterygium unguis
	Nail loss (symetric; affects most nails)
Scalp and body hair	New onset of scarring or nonscarring scalp alopecia (after recovery from chemotherapy)
	Scaling, papulosquamous lesions
Mouth	Xerostomia
	Mucocele
	Mucosal atrophy
	Pseudomembrane
	Ulcers
Genitalia	Erosions
	Fissures
	Ulcers

Eyes	Cicatrical conjunctivitis
	New-onset dry, grity, or
	Keratoconjuntivitis sicca
	Confluent areas of punctate keratopathy
Lung	Bronchiolitis obliterans
	diagnosed with PFTs and radiology
Muscles, joints, fascia,	Myositis or polymyositis

1.2.2.5. Classification

Classic: presence of at least one diagnostic or distinctive manifestation of cGVHD without features characteristic of acute GVHD.

Overlap: presents at any time post-HCT with features of both chronic GVHD and acute GVHD.

1.2.2.6. Onset

De novo: no prior aGVHD

Quiescent: prior aGVHD with resolution

Progressive: onset of chronic GVHD without resolution of prior existing acute GVHD with inferior overall survival. [99]

1.2.2.7. Staging

Global cGVHD scoring

Mild cGVHD involves only 1 or 2 organs or sites (except the lung), with no clinically significant functional impairment (maximum of score 1 in all affected organs or sites).

Moderate cGVHD involves (a) at least 1 organ or site with clinically significant but no major disability (maximum score of 2 in any affected organ or site) or (b) 3 or more organs or sites with no clinically significant functional impairment (maximum score of 1 in all affected organs or sites). A lung score of 1 is also moderate cGVHD.

Severe cGVHD indicates major disability caused by cGVHD (score of 3 in any organ or site). A lung score of 2 or greater is also severe cGVHD. [16] (NIH score sheet- Figure 5.)

	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, capable of self-care, >50% of waking hours out 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 *Clinical features:* ☐Maculopapular rash ☐Lichen planus-like features ☐Papulosquamous lesions or ichthyosis ☐Hyperpigmentation ☐Hypopigmentation ☐Keratosis pilaris ☐Erythema ☐Erythroderma ☐Poikiloderma ☐Sclerotic features ☐Pruritus ☐Hair involvement ☐Nail involvement % BSA involved ☐	☐No Symptoms	☐<18% BSA with disease signs but NO sclerotic features	☐19-50% BSA OR involvement with superficial sclerotic features "not hidebound" (able to pinch)	☐>50% BSA OR deep sclerotic features "hidebound" (unable to pinch) OR impaired mobility, ulceration or severe pruritus
MOUTH	☐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
EYES	☐No symptoms	☐Mild dry eye symptoms not	☐Moderate dry eye symptoms	☐Severe dry eye symptoms

	SCORE 0	SCORE 1	SCORE 2	SCORE 3
GI TRACT	☐ No symptoms	☐ Symptoms such as dysphagia, anorexia, nausea, vomiting, abdominal pain or diarrhea without significant weight loss (<5%)	☐ Symptoms associated with mild to moderate weight loss (5-15%)	☐ Symptoms associated with significant weight loss >15%, requires nutritional supplement for most caloric needs OR esophageal dilation
LIVER	☐ Normal LFT	☐ Elevated Bilirubin, AP*, AST or ALT <2 x ULN	☐ Bilirubin >3 mg/dl or Bilirubin, enzymes 2-5 x ULN	☐ Bilirubin or enzymes > 5 x ULN
LUNGS FEV1 ☐ DLCO ☐	☐ No symptoms ☐ FEV1 > 80% OR LFS=2	☐ Mild symptoms (shortness of breath after climbing one flight of steps) ☐ FEV1 60-79% OR LFS 3-5	☐ Moderate symptoms (shortness of breath after walking on flat ground) ☐ FEV1 40-59% OR LFS 6-9	☐ Severe symptoms (shortness of breath at rest; requiring O_2) ☐ FEV1 ≤39% OR LFS 10-12
JOINTS AND FASCIA	☐ 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 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.)
GENITAL TRACT	☐ No symptoms	☐ Symptomatic with mild signs on exam AND no effect on coitus and minimal discomfort with gynecologic exam	☐ Symptomatic with moderate signs on exam AND with mild dyspareunia or discomfort with	☐ Symptomatic WITH advanced signs (stricture, labial agglutination or severe ulceration) AND severe pain

* AP may be elevated in growing children, and not reflective of liver dysfunction

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

Esophageal stricture or web___	Pericardial Effusion___	Pleural Effusion(s)___
Ascites (serositis)___	Nephrotic syndrome___	Peripheral Neuropathy___
Myasthenia Gravis___	Cardiomyopathy___	Eosinophilia > 500µl___
Polymyositis___	Cardiac conduction defects___	Coronary artery involvement___

Platelets <100,000/µl ___ Progressive onset

OTHERS: _____

Figure 5. cGVHD score sheet

1.2.2.8. Clinical manifestations

Skin: Diagnostic signs for skin cGVHD include lichen planus-like eruption (plaques with a silvery or shiny appearance), poikiloderma, morphea-like superficial sclerotic features (localized patchy areas) or lichen sclerosus-like lesions (discrete gray to white moveable papules plaques), deep sclerotic features ("thickened or tight skin", caused by deep and diffuse sclerosis over a wide area) (Figure 6.).

Mouth: Lichen planus-like changes (white lines and lacy-appearing lesions of the palate, buccal mucosa or lips), hyperkeratotic plaques, or decreased mouth opening because of the sclerotic features of the skin cGVHD.

Genital tract in women: Vaginal scarring or stenosis and lichen planus-like changes.

Lung: clinical manifestations include dyspnea on exertion, cough, or wheezing. The only diagnostic sign is biopsy proven bronchiolitis obliterans. BO is clinically diagnosed if 1) FEV1/FVC ratio <0.7 and FEV1 <75% of predicted. 2) Evidence of air trapping or small airway thickening or bronchiectasis on high-resolution chest computed tomography (with inspiratory and expiratory cuts), residual volume (RV) >120%, or pathologic confirmation of constrictive bronchiolitis. 3) Absence of infection in the respiratory tract, documented with investigations directed by clinical symptoms, such as radiologic studies (radiographs or computed tomographic scans) or microbiologic cultures.

Muscles, joints and fascia: Joint stiffness, fasciitis or contractures due to sclerosis.

Gastrointestinal tract: Esophageal web, stricture or concentric rings documented by endoscopy.

Eyes: Diagnostic signs (diagnosed by ophtalmologist) include cicatrial conjunctivitis, keratoconjunctivitis sicca and punctate keratopathy.

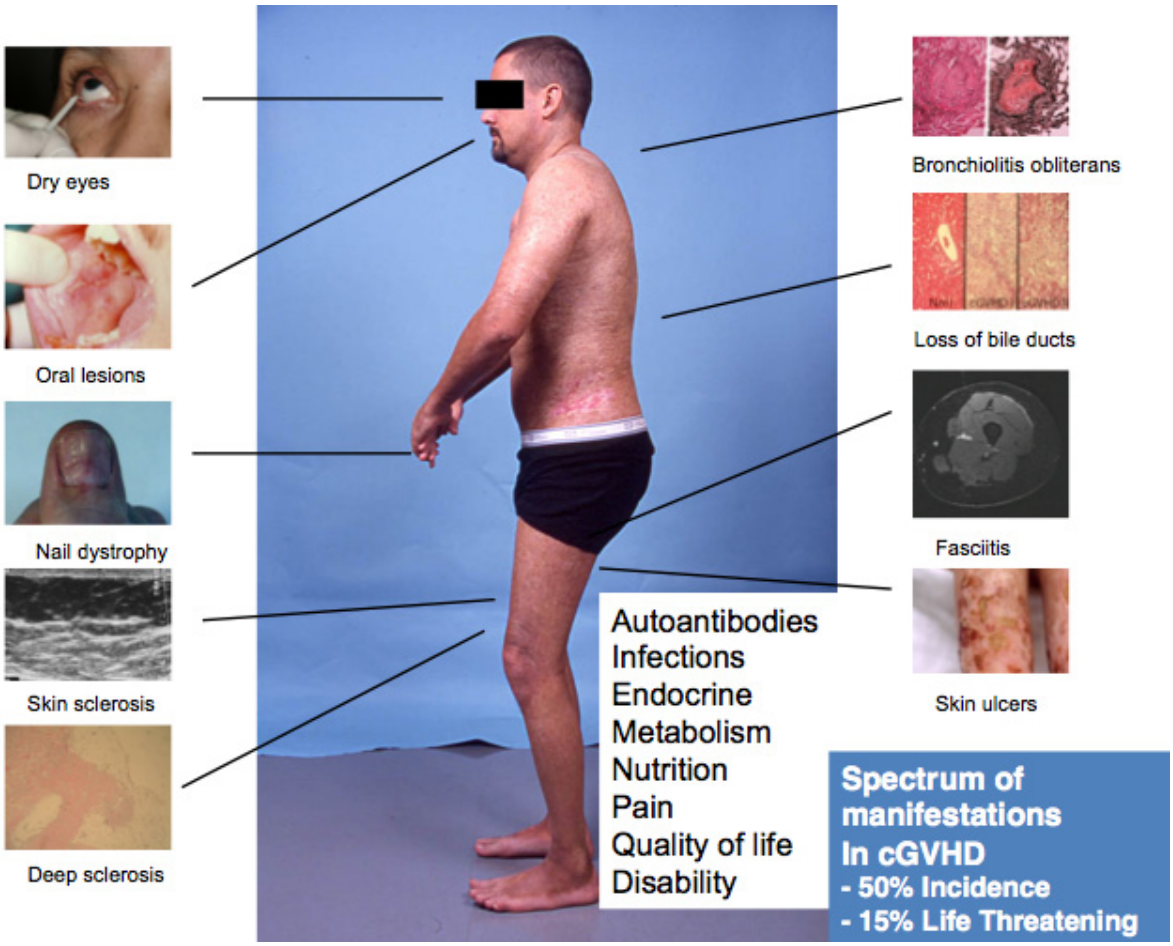


Figure 6. Spectrum of manifestations in cGVHD Courtesy of Prof SZ Pavletic

1.2.2.9. Prevention

Although many recipient risk factors associated with increased cGVHD are not modifiable, and include older age, underlying diagnosis, lack of an HLA-matched donor, while other modifiable factors are associated with lower incidence of cGVHD such as choosing a better donor (male, younger), use of bone marrow rather than peripheral blood, [100] and limitation of CD34+ and T-cell dose [80] infused may reduce the risk of cGVHD. If the recipient is male, then avoidance of a female donor, especially someone multiparous, may decrease the risk of chronic GVHD. Donor ABO compatibility and CMV seronegativity have also been associated with lower risks of cGVHD. While umbilical cord blood is currently a graft source of last resort in adults, it appears to be associated with lower rates of chronic GVHD. [101]

Prophylaxis by combined immunosuppression

Various combined regimens (cyclosporine+methotrexate, cyclosporine+mofetil-micophenolate, prednisone +tacrolimus, etc.) have been used for cGVHD prevention but none of them is highly effective.

Prophylaxis by T-cell depletion

Ex vivo: Methods of T-cell depletion include the use a single monoclonal antibody targeting several cell subpopulations (e.g. alemtuzumab), or selective removal of T, B, and NK-cells, as well as positive selection of CD34 or CD133 progenitors. [102,33] Because poor immune reconstitution after TCD grafts and relapse (GVL effect), the prevention of GVHD does not mean better outcome, on the contrast overall and leukemia free survival has been inferior comparing to patients receiving unmanipulated transplants. [103]

In vivo: In vivo T-cell depletion (as part of the preparative regiments) with antibodies (rabbit or horse ATG, alemtuzumab) administration prevents GVHD by targeting and down regulating incoming donor T-cells and reduces the host immune response in favor of engraftment. In vivo T-cell depletion is successful in aGVHD prevention but results in cGVHD prevention are less evident, [104] except in a study by Finke et al. where it was shown that addition of ATG to GVHD prophylaxis with cyclosporine and methotrexate resulted in decreased incidence of acute and chronic GVHD without an increase in relapse, non-relapse mortality or infection rate. [105]

1.2.2.10. Treatment

Treatment of the chronic GVHD requires multidisciplinary approach. Patients require joint care of specialists' team including dermatology, dental, ophthalmology, gynecology, physical medicine etc.

a) systemic therapy: immunosuppressive or immunomodulatory agents

b) topical and symptomatic therapy

c) supportive care

a) Systemic therapy

First-line:

The mainstay of cGVHD treatment is systemic therapy. First-line treatment of chronic GVHD consists of steroids alone or in combination with calcineurin inhibitors and is based on randomized trials. [106] The recommended dose of steroids (prednisone or methylprednisolone) is 1 mg/kg/day. The generally recommended approach involves continued administration of the calcineurin inhibitor used for GVHD prophylaxis together with prednisone initially at 1 mg/kg/day. The combination of steroids with calcineurin inhibitors (cyclosporine or tacrolimus) is particularly indicated in treatment of moderate or severe cGVHD or for those with less severe disease but with high-risk features (thrombocytopenia <100, progressive onset or bilirubin >2 mg/dl at onset). [34] Combination use of cyclosporine and prednisone confirmed, however, beneficial steroid sparing effects of cyclosporine as demonstrated by lower incidence of avascular bone necrosis in patients in the combination arm. [107]

For mild cGVHD case the use of topical immunosuppressant (topical calcineurin inhibitors, topical steroids, phototherapy) for oral mucosa, eye and skin.

Response should be assessed not before eight weeks of treatment have been finished, or until up to 3-6 months of treatment have been finished in the case of deep skin sclerosis. Strategies for the tapering the dose of prednisone vary, but as a general preference, one should use the minimum dose that is sufficient to control cGVHD manifestations. First line treatment achieves remission in approximately 20% of adult and 50% of pediatric patients. [108] Currently, no uniformly accepted definition of steroid refractory cGVHD is available. Generally, accepted criteria for steroid refractory cGVHD are (1) progression despite immunosuppressive treatment using 1 mg/kg/day of prednisone for 2 weeks, (2) stable disease

if 4 to 8 weeks on ≥0.5 mg/kg/day of prednisone, and (3) inability to taper below 0.5 mg/kg/day of prednisone. [106]

Second-line:

In case of first-line steroid-based therapy failure due to progression or refractory disease, second line treatment is indicated. The list of drugs for salvage therapy is long (Table 4.), there is no standard treatment, and trial-and-error remains the major way to identify an effective treatment of the individual. [34] Response rates vary from 25-75% (photopheresis) and these responses are most commonly incomplete or not durable. [23,109] Polypharmacy is common in cGVHD patients, but no more than three immunosuppressive agents shod be given, as combination of more drugs does not lead to improvement but leads to increased risk of toxicity and infections. [108]

The median duration of treatment is approximately 2 years in patients who had HCT with marrow cells and 3.5 years in those who had HCT with peripheral blood stem cells. [110]

Table 4. List of 30 agents used in secondary therapy [111]

- Acitretin/etretinate
- Alefacept
- Alemtuzumab
- Antithymocyte globulin
- Azathioprine
- Bortezomib
- Clofazimine
- Daclizumab
- Extracorporeal photopheresis (ECP)
- Etanercept
- Halofuginone
- Imatinib
- Infliximab
- Interleukin-2
- Lidocaine
- Mesenchymal stem cells (MSC)
- Methotrexate
- Montelukast
- Mycophenolate mofetil
- Pentostatin
- Pravastatin
- Psoralen/UVA
- Rituximab
- Sirolimus
- Steroids (pulse)
- Thoraco-abdominal radiation
- T-regulatory cell infusions
- Thalidomide
- Ursodeoxycholic acid
- UVB

b) Topical and symptomatic therapy

Topical therapy includes corticosteroids, calcineurin inhibitors, PUVA for skin or mouth, and topical estrogens, corticosteroids, calcineurin inhibitors for gynecological manifestations.

c) Supportive and ancillary care

Prolonged immunosuppressive therapy including steroids is often necessary to control disease manifestations and severity. Treatment, combined with delayed and impaired immune reconstitution associated with cGVHD, increases the risk of infections and other complications. Clinical manifestations of cGVHD can persist for prolonged periods of time, causing significant morbidity. Some of these changes, such as contractors, may be irreversible. Infection is the most common cause of mortality in patients with cGVHD, and prophylaxis of infections requires special focus. The immune defects in cGVHD are broad, including macrophage function, antibody production, and T-cell function. All patients with cGVHD are considered at risk for infection with encapsulated bacteria, particularly Streptococcus pneumoniae. Prophylactic antibiotics (penicillin V K) should be given to all patients with cGVHD during immunosuppressive treatment. Most experts recommend Haemophilus influenzae B conjugate or influenza vaccinations, since the risk of adverse outcomes is low. No live viruses should be given. IVIg should be considered for patients who have recurrent infections and IgG levels less than 400 mg/dl. Invasive mould infections are also one of the major concerns in patients under immunosuppressive treatment, and antifungal therapy is also necessary, especially when the corticosteroid dosage is more than 0.5-1.0 mg/kg/day. All patients should receive Pneumocystis carinii prophylaxis (trimethoprim-sulfamethoxazole, which also ensures prophylaxis against Toxoplasma and Nocardia). Some centers use long-term antiviral prophylaxis to prevent recurrent herpes simplex and varicella zoster virus infection. Cytomegalovirus (CMV) disease monitoring after day 100 is recommended in patients with active cGVHD, history of CMV reactivation and lymphopenia.

Monitoring, surveillance for malignances and management of medication toxicities (e.g. hypertension, renal dysfunction etc) is necessary. Organ specific interventions include: for skin photoprotection, topical emollients, antipruritic agents, topical corticosteroids; for eyes photoprotection, artificial tears, contacts lens, ointments, topical steroids, topical cyclosporine, punctual occlusion, autologous eye drops, surveillance for cataract and infection; for mouth oral hygiene, topical steroids or topical calcineurin inhibitors, topical analgesics, surveillance for malignancy and infection; for lungs bronchodilatators, pulmonary rehabilitation, oxygen, surveillance for infection; for gynecological tract in women topical

estrogens, topical calcineurin inhibitors, topical steroids, vaginal dilatators, surveillance for infection (HPV, HSV) and malignances; for musculoskeletal system physical therapy, bone densitometry, therapy for osteopenia or osteoporosis, surveillance for decreased range of motion; for neurologic system treatment of neuropathic syndromes, calcineurin levels monitoring, seizure prophylaxis, blood pressure control, EMNG monitoring.

1.2.2.11. Biologic markers of chronic GVHD

Lots of studies are published and trying to identify cGVHD biomarker that could provide clinically useful information (correlation with development, diagnosis and prognosis of disease). An ideal biomarker should be highly sensitive and specific, reflecting the current status of disease; should be related to the disease activity and/or severity in accordance with the clinical evolution; should anticipate clinical changes before they occur; and should add independent information about the risk or prognosis that is reproducible and feasible. The NIH definition of a biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic response to a therapeutic intervention. [112]

The disease is a result of a Th1 and Th2 impaired function. There are data suggesting that development of cGVHD may be a Th2-mediated process, because of the increased production of IL-4, IL-5, IL-10 cytokines. [113] A group of investigators identified an IL-10 promoter gene polymorphism known to be associated with a lower production of IL-10 correlated with cGVHD development and another that higher levels of IL-10 at the fourth month post-transplant is associated with development of cGVHD due to Th2 predominance. [114,40] High levels of Th1 cytokines (IL-1, TNF-α, INF-γ) have been found in the sera of cGVHD patients [39], in contrast to low levels of INF-γ described by another group.

Various studies suggested that risk for cGVHD development may be associated with donor-recipient genetic polymorphism, deficiency in regulatory immune cell populations (NK, Treg, DC2), and variation in inflammatory and immunoregulatory mediators post-alloHSCT (increased TNF-α, IL-10 and BAFF, and decreased TGF-β and IL-15). CGVHD is associated with alteration in immune cell populations (increased CD3+ T cells, Th17, CD4+ and CD8+ effector memory cells, monocytes, CD86 expression, BAFF/B cell ratio, and deficiency of Treg, NK cells, and naïve CD8+ T cells). [115]

Most studies support increased pro-inflammatory cytokines in chronic GVHD cases, including TNF-, IL-6, IL-1β, IL-8, sIL-2R, and IL-1Ra. Validated proteomic work from suggests that BAFF, sCD13, elafin, IL-2Rα, MIG (CXCL9), and anti-dsDNA may distinguish cGVHD cases from non-chronic GVHD controls with high accuracy. [115]

1.3. Inflammation and acute phase reactants

1.3.1. Inflammation

Inflammation is a complex organism response to biological, chemical or physical insult. In the acute phase, leukocytes, primarily granulocytes, migrate along a chemotactic gradient to the site of injury in a carefully orchestrated effort that is mediated by cytokines and acute phase reactants to remove the stimulus or cells damaged by injury and to initiate healing. Persistent inflammation as a result of prolonged exposure to stimulus or an inappropriate reaction to self molecules can lead to the chronic phase in which the active immune cell populations shift to include a mononuclear phenotype, and tissue damage and fibrosis can occur. Chronic inflammation is reported to contribute to numerous diseases including allergy, arthritis, asthma, atherosclerosis, autoimmune diseases, diabetes, and cancer, and to conditions of aging. The inflammatory process involves multiple physiological systems with the immune system playing a central role. In the acute phase, platelets and granulocytic cells such as basophils/mast cells, neutrophils and eosinophils are activated; producing and releasing a number of soluble mediators that stimulate and regulate the inflammatory response.

Acute-phase responses (APR) are systemic reactions that reflect organ site inflammation in acute and chronic diseases. [116] It is characterized by increased plasma concentration of acute phase proteins driven by various cytokines release. Major acute phase proteins include: transport proteins (ceruloplasmin, haptoglobin), complement system (C3, C4, C5), coagulation system (fibrinogen, vWF, plasminogen and antithrombin III) and other (CRP, serum amyloid A, ferritin, IL-1RA and α2-macroglobulin) [117,116]. Many cytokines and chemokines contribute to inflammation; some facilitate leukocyte chemotaxis to the site of injury, while others modulate immune cell function. The cytokines that are best known for stimulating and perpetuating inflammatory responses are IL-6, IL-1, IL-2, TNF-α, IFN-γ, and transforming growth factor (TGF)-β. IL-6 was originally identified as a B-cell differentiation factor, and increased levels of this cytokine have been associated with polyclonal B cell activation and chronic inflammation. In the initial phases of acute inflammation, IL-6 mediates the acute phase response. IL-6 levels remain high in chronic inflammatory processes leading to enhanced survival and growth of lymphocytes and macrophages that perpetuate inflammation. IL-1 has a number of direct and indirect activities that promote inflammation including the stimulation of the production of other cytokines and the release of prostaglandins. These promote the generation of cytotoxic effector cells and synergize with

colony stimulating factors to increase the production of inflammatory cells in the bone marrow. IL-2 augments NK cell activity, stimulates the production of inflammatory cytokines such as IL-1 and IFN-γ and enhances macrophage cytotoxicity. It also contributes to chronic inflammation by stimulating the proliferation of antigen specific T- and B-lymphocytes. TNF-α enhances inflammation and is important in the process of removing dead and dying cells through apoptosis. TNF- α has been shown to upregulate the expression of Class I and II major histocompatibility complex (MHC) molecules on certain cell types resulting in cell activation and cytokine release. IFN-γ is a potent activator of macrophages. It stimulates the production of IL-1 and TNF-α and enhances the expression of Class II MHC molecules on immune cells and vascular endothelium. The latter is of particular importance in allowing inflammatory cells to move through the vasculature into tissues or a site of injury. TGF-β is important in the regulation of tissue repair and regeneration following injury. It is produced by a number of immune and nonimmune cell types and is important in the regulation of the inflammatory response by inhibiting the production of proinflammatory cytokines such as IL-2, IFN-γ.

Cytokines responsible for synthesis of APR in hepatocytes are: TNF-α, IL-1β, INF-γ, TGF-β and particularly IL-6 produced by macrophages and monocytes. [118] Serum levels of IL-6 and CRP often correlate. These cytokines suppress the synthesis of albumin. Hypoalbuminemia is a frequent during inflammation. [119] Despite it is called acute phase reactant its level can and often is increased and used for disease activity monitoring in chronic inflammatory states and autoimmune diseases such as rheumatoid arthritis. [117,120] They are also called "positive acute phase proteins" because their concentration in serum increases during inflammatory state. There are also "negative acute phase proteins" like albumin, transferin and transthyretin whose synthesis during inflammation is decreased (Figure 7.).

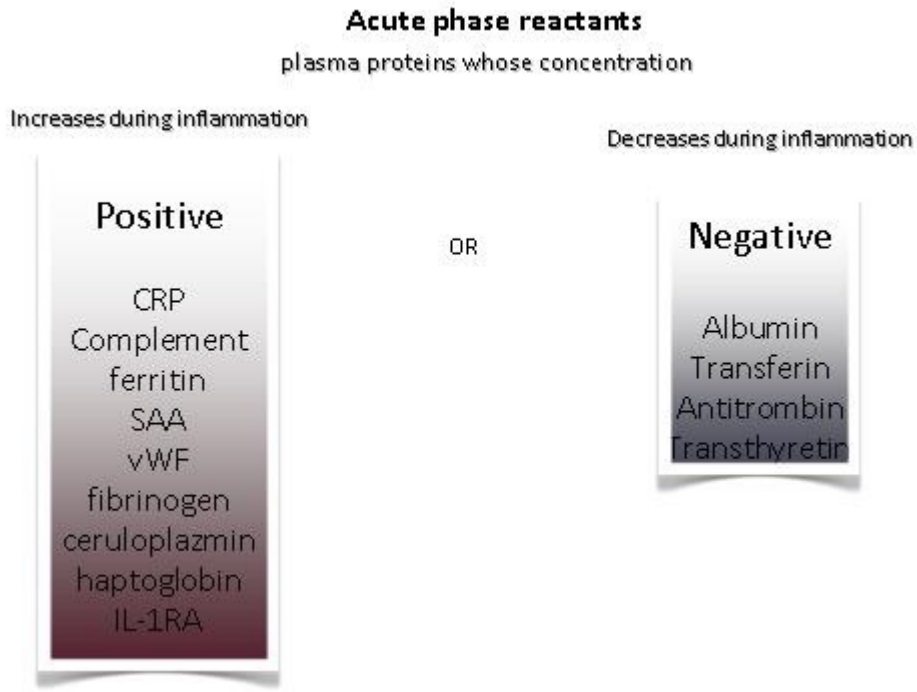


Figure 7. Acute phase reactants whose concentration increases or decreases during inflammation

1.3.2. C-reactive protein (CRP)

CRP is a beta globulin and it is the best known acute phase serum protein which is widely used as marker of infection and intensity of inflammatory process. It has proinflammatory as well as anti-inflammatory effects. Proinflammatory effects include ability to activate classical complement cascade, which is different from activation by antibody, binding to FcγRI and FcγRII on the surface of leucocytes, which activates them. Anti-inflammatory effects include binding to apoptotic and necrotic cells thus facilitating opsonisation and phagocytosis by macrophages. [121,122] A very important property of CRP is the ability to bind C1q to activate the classical complement cascade and enhancing the capacity for defense against stimuli. CRP is absent or present in very low concentrations in normal serum. Many studies demonstrate that normal CRP levels in the American population are less than 2 or 3 mg/L. [123,124] Minor CRP elevation (3-10 mg/L) has been regarded as a marker of "low grade inflammation". Values greater than 10 mg/L are generally accepted to be regarded as reflecting clinically significant inflammation. [124,125,126] On the contrary markedly elevated levels of CRP are strongly associated with infection. [127]

Low grade inflammation differs from acute inflammation. It is usually associated with some chronic condition in which classic clinical signs of inflammation are missing. Also lots of data from the epidemiological studies had shown positive correlation between minor CRP elevation and underlying atherosclerosis, metabolic syndrome and risk of cardiovascular events as well as conditions including obesity, type 2 diabetes, asthma and neurodegenerative diseases — are all characterized by chronic low-grade inflammation. [128,129,130]

Patients with cGVHD have enhanced expression of the inflammatory cytokines TNF-α, IL-6, TGF-β, IL-1β and IFN-γ and decreased levels of anti-inflammatory cytokines such as IL-10, as is seen in APR. [39,62,116,131,132,133,134] A number of acute phase reactants have well established roles in monitoring clinical outcomes for systemic inflammatory and autoimmune diseases. [116, 135] CRP (C-reactive protein) and erythrocyte sedimentation rate (ESR) correlate with activity of rheumatoid arthritis.[14] CRP has also been shown elevated in 46% of SSc patients. [136] This is in contrast to systemic lupus erythematosus in which CRP values are typically normal or only modestly elevated and decreased levels of complement components C3 and C4 are associated with active disease. [137,126] Therefore, it is essential to validate these tests in individual disease settings.

Increased levels of CRP are strongly associated with major transplant-related complications like veno-occlusive disease (VOD) and acute GVHD. [138,139] Also conditioning with TBI was associated with significantly elevated levels of CRP. [138,139]

1.3.3. Complement system

Complement system is organized system of serum proteins and important part of antigen-nonspecific part of the immune response. The complement system is a complex network of proteins that participate in the acute inflammatory response through their enzymatic activity, effects on mediator release, chemotaxis and vascular permeability, and the ability to enhance phagocytosis through opsonization of microbes. C3 and C4 are acute phase proteins and their levels increase in acute phase response. Complement levels are usually normal or decreased in autoimmune diseases. C3 and C4 are proteins whose plasma concentration increases in terms of inflammation. [116]

1.3.4. Ferritin

Ferritin concentration in plasma is an indicator for organism iron stores. Though an elevated ferritin level is used as surogate for iron stores, it may be elevated in other circumstances, including inflammation and may also occur in association with abnormal liver function tests and longer disease duration. Ferritin is an acute phase reactant which concentration rises in acute phase reaction under influence of cytokines such as IL-1 and TNF. [140] Also, ferritin has been incorporated into prognostic scoring systems for patients undergoing myeloablative allogeneic transplantation for acute leukemia and MDS. [141] Iron overload is known to have an immunomodulatory effect, influences innate and acquired immune responses. Reduced CD8+ T-cell counts have been observed in patients with iron overload caused by thalassaemia or haemochromatosis. [142] Several studies have reported the association between iron overload and transplant related complications such as sinusoidal obstruction syndrome, infection and idiopathic pneumonia syndrome. [143] In a few case reports, hemosiderin deposits were described in cGVHD related myopathy. [144] Beside that hyperferritinemia is associated with lower incidence of cGVHD, high relapse rate and decreased survival. [145,146]

1.3.5. Albumin

Albumin is quantitatively the most important protein. Albumin synthesis is regulated by cytokines, hormones, nutritional status and serum oncotic pressure. [147] Hypoalbuminemia is a reflection of hepatic synthesis dysfunction and lots of other conditions such as a malnutrition, nephrotic syndrome and inflammation. Albumins are negative phase reactants. Their levels are falling during the inflammation. [119]

2. Hypotheses

Inadequate or increased production of proinflammatory cytokines is associated with chronic GVHD. Their production are usually increased in active and severe cGVHD and decreased in inactive and moderate cGVHD. The proinflammatory cytokines can lead to increased or decreased (depending on function) synthesis of acute phase reactants. So the acute phase reactants of inflammation are expected to be increased in active or severe cGVHD and lower in inactive or moderate cGVHD. If it is so, these acute phase reactants - laboratory markers of inflammation can serve as indicators of activity and severity of cGVHD. They also could be a valuable indicator of treatment response and follow-up after treatment of cGVHD.

3. Aims and purpose of the research

Aims

1. To determine the level of laboratory markers of inflammation (routinely measured in clinical practice) in cGVHD in relation to disease activity and severity

2. To identify clinical and biological markers of cGVHD activity

3. To identify laboratory indicators of inflammation predictive for prognosis and survival

Purpose and expected scientific contribution of the research

As there are no standard measures to define activity of cGVHD, this research would pioneer the identification, and possible clinical implementation of laboratory markers of inflammation relevant to the disease activity assessment, with the goal of early treatment and prevention of irreversible organ damage, as well as disease monitoring and treatment response.

4. Patients, methods and plan of investigation

4.1. Patients

The research was done at the Experimental Transplantation and Immunology Branch, National Cancer Institute, National Institutes of Health in Bethesda, MD, USA in collaboration with Division of Hematology, Department of Internal Medicine of School of Medicine, University of Zagreb, within a protocol "Leukemias and hematopoietic stem cell transplantation".

The research included 189 adult patients median 48 years old [18-70] who were enrolled in the National Cancer Institute protocol "Natural History Study of Clinical and Biological Factors Determining Outcomes in Chronic Graft-Versus-Host Disease".

4.2. Methods

4.2.1. Plan of investigation

Research plan

1. To determine distributions profiles for markers of inflammation of interest in this cGVHD population.
2. To determine in univariate and multivariate analysis whether there is any statistically significant correlation between these biomarkers and cGVHD study endpoints and investigate their role in the context of other clinical parameters. Multivariate analysis will be done adjusted for the time post transplant.

Investigate in a preliminary fashion if there are any statistical differences between laboratory markers of inflammation and control values in patients without chronic GVHD**.**

All patients included into the study underwent a four-day, one-time visit evaluation by a multi-disciplinary team that included experts in dermatology, ophthalmology, dentistry, rehabilitation medicine, gynecology, pain and palliative, and hematopoietic cell transplantation. Patient evaluation also included comprehensive history and physical examination, functional measurements and quality of life (QOL) assessments. In addition, patients also undergo extensive sub-specialist evaluation with in-depth subspecialty grading of the key organs, such as the Schubert Scale for oral involvement, Schirmer's tear test and eye exam, and NIH Skin Response Scale. Clinical assessments and laboratory data were recorded at the time of the visit using the pre-defined data collection forms that included NIH score sheet, Clinician activity assessment (form A, Figure 9. A), patient report form (form B, Figure 9. B) and subspecialists evaluations forms. For all patients laboratory assessment have been performed: complete blood count, platelets, CRP, ESR, C3, C4, total complement, IgA, IgG, IgM, total proteins, albumin, beta-2 microglobulin, ferritin and parathyreoid hormone.

Research plan is given in Figure 8.

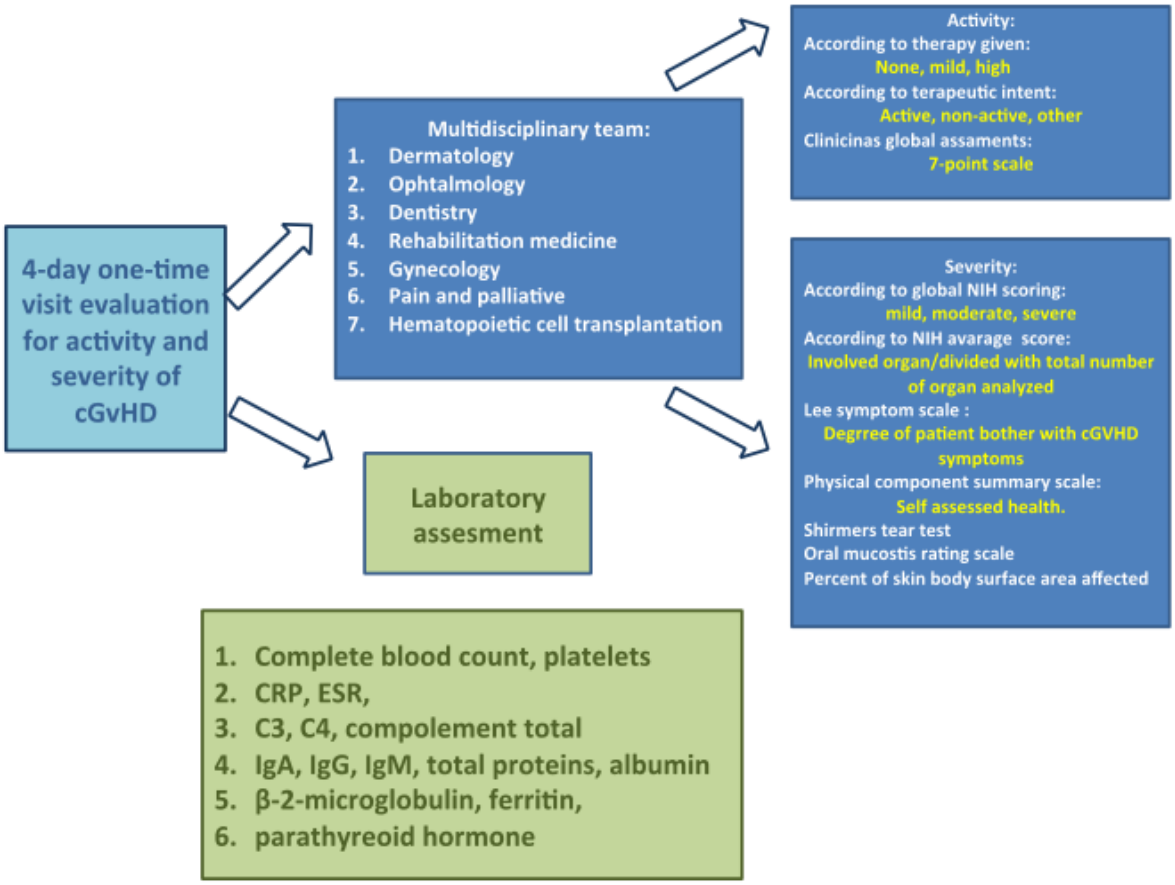
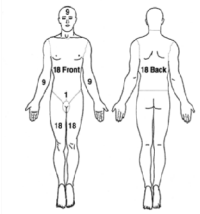


Figure 8. Research plan

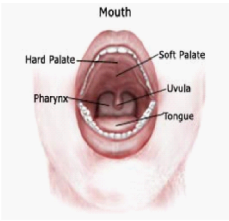
A. Form A

Today's Date: _____ Patient Name: _____ Current Weight: _____

CHRONIC GVHD ACTIVITY ASSESSMENT- CLINICIAN

Component	Findings	Scoring (see skin score worksheet)
Skin 	Erythematous rash of any sort	% BSA (max 100%)
	Superficial sclerosis	% BSA (max 100%)
	Deep sclerosis (hidebound/non-pinchable or subcutaneous sclerosis/Fasciitis)	% BSA (max 100%)
	Ulcer(s): select the largest ulcerative lesion, and measure its largest dimension in cm and mark location of ulcer	Location: _____ Largest dimension: _____cm
Eyes Bilateral Schirmer's Tear Test (without anesthesia) in persons 9 years or older	Right Eye: mm of wetting	Left Eye: mm of wetting

Mouth



Mucosal change	No evidence of cGvHD		Mild		Moderate		Severe	
Erythema	None	0	Mild erythema or moderate erythema (<25%)	1	Moderate (≥25%) or Severe erythema (<25%)	2	Severe erythema (≥25%)	3
Lichenoid	None	0	Hyperkeratotic changes(<25%)	1	Hyperkeratotic changes(25-50%)	2	Hyperkeratotic changes (>50%)	3
Ulcers	None	0	None	0	Ulcers involving (≤20%)	3	Severe ulcerations (>20%)	6
Mucoceles*	None	0	1-5 mucoceles	1	6-10 scattered mucoceles	2	Over 10 mucoceles	3
	Column Total		Column Total		Column Total		Column Total	
	Oral surfaces score: vermillion lips, labial and buccal mucosa, tongue (dorsal,lateral&ventral), and soft palate.		*Mucoceles scored for lower labial and soft palate only				**Total score for all mucosal changes**	

Blood Counts	Platelet Count K/uL	ULN K/uL	Total WBC K/uL	ULN K/uL	% Eosinophils %					
Liver Function Tests	Total serum bilirubin mg/dL	ULN mg/dL	ALT U/L	ULN U/L	Alkaline Phosphatase U/L	ULN U/L				

Gastrointestinal-Upper GI • Early satiety OR • Anorexia OR • Nausea & Vomiting	*0= no symptoms* *1=mild, occasional symptoms, with little reduction in oral intake during the past week* *2=moderate, intermittent symptoms, with some reduction in oral intake during the past week* *3=more severe or persistent symptoms throughout the day, with marked reduction in oral intake, on almost every day of the past week*
Gastrointestinal-Esophageal • Dysphagia OR • Odynophagia	*0= no esophageal symptoms* *1=Occasional dysphagia or odynophagia with solid food or pills during the past week* *2=Intermittent dysphagia or odynophagia with solid foods or pills, but not for liquids or soft foods, during the past week* *3=Dysphagia or odynophagia for almost all oral intake, on almost every day of the past week*
Gastrointestinal-Lower GI • Diarrhea	*0= no loose or liquid stools during the past week* *1= occasional loose or liquid stools, on some days during the past week* *2=intermittent loose or liquid stools throughout the day, on almost every day of the past week, without requiring intervention to prevent or correct volume depletion* *3=voluminous diarrhea on almost every day of the past week, requiring intervention to prevent or correct volume depletion*
Lungs • Bronchiolitis Obliterans	Pulmonary Function Tests with Diffusing Capacity (attach report for person> 5 yrs old) FEV-1 % Predicted Single Breath DLCO (adjusted for hemoglobin) % Predicted

Health Care Provider Global Ratings:
In your opinion, do you think that this patient's chronic GvHD is mild, moderate or severe?

1= mild
2=moderate
3=severe

Where would you rate the severity of this patient's chronic GvHD symptoms on the following scale, where 0 is cGVHD symptoms that are not at all severe and 10 is the most severe cGVHD symptoms possible:

0 1 2 3 4 5 6 7 8 9 10

cGvHD symptoms not at all severe — Most severe cGvHD symptoms possible

Over the past month would you say that this patient's cGvHD is

+3= Very much better
+2= Moderately better
+1= A little better
0= About the same
-1=A little worse
-2=Moderately worse
-3=Very much worse

Functional Performance (in persons >4 years old) • Walk Time • Grip Strength	**Total Distance Walked in 2 Minutes:** *Number of laps: _____ (x 50 feet) + final partial lap: _____ feet = _____ feet walked in 2 minutes*	**Grip Strength (Dominant Hand)**		**Range of Motion:** ○ Not performed ○ Physical Therapy Report Attached
		Trial #1	**Trial #2**	**Trial #3**
		psi	psi	psi

Score	Lansky Performance Status Scale Definitions (circle from 0-100) (persons < 16 years old)	Karnofsky Performance Status Scale Definitions (circle from 0-100) (persons 16 years or older)
100	Fully active, normal	Normal no complaints; no evidence of disease
90	Minor restrictions in physically strenuous activity	Able to carry on normal activity; minor signs or symptoms of disease
80	Active, but tires more quickly	Normal activity with effort; some signs or symptoms of disease
70	Both greater restriction of and less time spent in play activity	Cares for self; unable to carry on normal activity or to do active work
60	Up and around, but minimal active play; keeps busy with quieter activities	Requires occasional assistance but is able to care for most personal needs
50	Gets dressed but lies around much of the day, no active play but able to participate in all quiet play and activities	Requires considerable assistance and frequent medical care
40	Mostly in bed; participates in quiet activities	Disabled; requires special care and assistance
30	In bed; needs assistance even for quiet play	Severely disabled; hospital admission is indicated although death not imminent
20	Often sleeping; play entirely limited to very passive activities	Very sick; hospital admission necessary; active supportive treatment necessary
10	No play; does not get out of bed	Moribund; fatal processes progressing rapidly
0	Unresponsive	Dead

B. Form B

Symptoms	Not present										As bad as you can imagine
Symptoms have been in the <u>last seven days</u>. Please fill in the circle below from 0 (symptom has not been present) to 10 (the symptom was as bad as you can imagine it could be) for each item.	0	1	2	3	4	5	6	7	8	9	10
Your **skin itching** at its WORST?	□	□	□	□	□	□	□	□	□	□	□
Your **mouth dryness** at its WORST?	□	□	□	□	□	□	□	□	□	□	□
Your **mouth pain** at its WORST?	□	□	□	□	□	□	□	□	□	□	□
Your **mouth sensitivity** at its WORST?	□	□	□	□	□	□	□	□	□	□	□

Eyes	What is your main complaint with regard to your eyes?	
	Please rate how severe is this eye symptom, between 0 (not at all severe) and 10 (most severe):	0 1 2 3 4 5 6 7 8 9 10
Vulvovaginal Symptom (females only)	Do you have any burning, pain or discomfort in the area of your vagina, vulva or labia? **OR** Do you have any discomfort or pain with sexual intercourse?	o Yes o No o Not applicable

<u>Patient Global Ratings:</u>

1. Overall, do <u>you</u> think that your chronic graft versus host disease is mild, moderate or severe?

1= mild

2=moderate

3=severe

2. Please circle the number indicating how severe your chronic graft versus host disease symptoms are, where 0 is cGVHD symptoms that are not at all severe and 10 is the most severe chronic GVHD symptoms possible.

0 1 2 3 4 5 6 7 8 9 10

cGVHD symptoms not at all severe

Most severe cGVHD symptoms possible

3. <u>Compared to a month ago</u>, overall would you say that your cGVHD symptoms are:

+3= Very much better

+2= Moderately better

+1=A little better

0= About the same

-1=A little worse

-2=Moderately worse

-3=Very much worse

Figure 9. A. Form A; B. Form B

4.2.2. Exclusion of infection

To exclude the infection in patients with elevated CRP (>0.8 mg/dL) the following laboratory and clinical assessment have been performed:

- Infectious disease consult
- Microbiology assessment: specify urine and blood cultures, swabs, CT scan of the sinuses, PCR CMV and EBV DNA

Patients who received allogeneic hematopoietic stem cell transplant at the NIH without cGVHD served as the age and sex matched controls (N=17) for this study. All subjects signed NCI IRB approved informed consent.

4.2.3. Chronic GVHD definition criteria

4.2.3.1. Chronic GVHD activity:

Chronic GVHD activity was defined by:

a) Intensity of systemic immunosuppression at the time of evaluation: None, Mild = single agent prednisone<0.5 mg/kg/day; Moderate = prednisone ≥ 0.5 mg/kg/day and/or any single agent/modality; High = 2 or more agents/modalities ± prednisone ≥ 0.5 mg/kg/day. [148] Disease was considered more active if the need for systemic immunosuppression was higher.

b) Therapeutic intent at the time of visit/evaluation. The post-transplant course, history of cGVHD presentation, features, treatment, and therapeutic response were carefully documented in each subject participating in this study. Based on review of materials (prior medical records, including clinician progress notes, laboratory data, diagnostic tests/scans (e.g. PFTs, chest CT) and the in-depth comprehensive evaluation conducted over 4 days, after a detailed discussion we reached an interdisciplinary consensus on each case on the decision to increase, decrease or maintain the immunosuppressive regimen.

Disease was defined as "active" if the practitioner decided to increase systemic therapy due to worsening disease, to substitute systemic therapy due to lack of response or withdraw systemic therapy due to lack of response. Disease was defined as "non-active" if the practitioner decided to decrease systemic therapy because the cGVHD was improving, not to change current systemic therapy because cGVHD was stable or to alter systemic therapy only because of toxicity. If patients either had not been receiving any immunosuppressive therapy at the time of evaluation or did not meet any of the previously mentioned criteria, they were categorized as "other" (excluded from the analysis)

c) Clinician's global assessment of change over the past month (7-point scale): worsened (-3= very much worse, -2= moderately worse, -1= a little worse), unchanged (0= about the same), and improved (+1= a little better, +2= moderately better, +3= very much better). Based on our review of patient's medical history and comprehensive clinical exam and evaluation, clinician had reached a decision on cGVHD trajectory. This particular question of whether cGVHD is better or worse over the preceding month is derived from NIH cGVHD response criteria evaluations (form A) Figure 9.A, which was originally based on the literature experience in other disease settings. This scale is based on the clinician's subjective impression of cGVHD change over the past month and on patient's symptoms and overall clinical history over the previous month. The limitation of this proposed instrument is the lack of a baseline

comparison and the consideration that assessment is heavily influenced by patient reported symptoms. [149]

4.2.3.2. cGVHD severity:

cGVHD severity was defined by:

a) Global NIH scoring (reflects the degree of organ impact and functional impairment due to cGVHD):

Patients had mild cGVHD if only 1 or 2 organs (except lungs) were involved, with a maximum score 1 in all affected organs. Moderate cGVHD involved at least 1 organ with clinically significant, but not major disability (maximum score 2); or 3 or more organs with no clinically significant functional impairment (maximum score 1 in all affect organs). A lung score 1 was classified as moderate. Severe cGVHD indicated major impairment caused by cGVHD (score 3 in any organ). Lung scores of 2 or 3 were classified as severe. Organs scored included the skin, eyes, mouth, GI tract, liver, lungs, and joint/fascia. Of note, when scoring lung on the NIH score sheet, the lung function score (LFS) is used when pulmonary function tests (PFTs) are available and only in the absence of PFTs, are the symptoms used to grade lung. The LFS is computed by the extent of FEV1 and DLCO compromise (FEV1: >80%=1, 70–79%=2, 60–69%=3, 50–59%=4, 40–49%=5, <40%=6; DLCO: >80%=1, 70–79%=2, 60–69%=3, 50–59%=4, 40–49%=5, <40%=6; summary score (FEV1+DLCO): ⩽2=LFS 1, 3–5=LFS 2, 6–9=LFS 3, 10–12=LFS 4). When discrepancy existed between pulmonary symptom or PFT scores the higher value was used for final scoring. All but 3 patients had PFTs available for scoring in our study population (Figure 5). The genital area was scored in females only [16];

b) NIH average score which is a result of total NIH score for each of the organ systems divided by the total number of organ systems analyzed (8 for female and 7 for male);

c) Lee symptom scale: degree of patient bother with cGVHD symptoms. It is a 30-item symptom scale with 7 subscales which correlate highly with patients' self-assessed mild, moderate, and severe cGVHD manifestations [150];

d) Using the physical component summary (PCS) scale, drawn from the SF-36 v.2, a well validated measure of self-assessed health. The SF-36 Health Survey is a multi-purpose health survey which contains 36 questions. 36 items evaluate 8 factors: vitality, physical functioning,

bodily pain, general health perceptions, physical role functioning, emotional role functioning, social role functioning, and mental health. In addition to the individual subscale scores, 2 component summary scores, physical (PCS) and mental (MCS) are computed through aggregation of the subscales. The SF-36 is a generic measure of health status as opposed to one that targets a specific age, disease, or treatment group. It has proven useful in comparing general and specific populations, estimating the relative burden of different diseases, differentiating the health benefits produced by a wide range of different treatments, and screening individual patients [151,152];

e) Schirmer's tear test performed in each eye with anesthesia scored from 0-30 mm;

f) Oral Mucositis Rating Scale (OMRS) a rating scale (0-273) used to grade and measure oral changes including erythema, atrophy and ulceration [153];

g) Percentage of skin body surface area (BSA) affected by: erythema, moveable sclerotic skin manifestations, and non-moveable skin changes and fasciitis. [149]

4.2.4. Laboratory assessments

4.2.4.1. Markers of inflammation

- Chemistry: CRP, ferritin, complement total, C3, C4, albumin, ESR, IgG, IgM, IgA, beta-2 microglobulin, total protein
- Hematology: WBC, ANC (absolute neutrophil count), lymphocytes (absolute lymphocyte count), eosinophils (absolute eosinophil count), platelet count

4.2.4.2. Other laboratory assessment

- CBC with differential, hemoglobin, bilirubin, AST, ALT, GTT, AP, GGT, creatinine, urea, potassium, sodium, calcium, magnesium, phosphorus, lipid panel and triglycerides, PT, PTT, quantitative serum immunoglobulins, autoantibodies panel, T3, T4, TSH, T4 free, testosterone (free and total), LH, FSH, estradiol, PTH, vitamin D 25, vitamin D 1,25, peripheral blood chimerism, hepatitis B and C serology, PCR CMV DNA, urine analysis, cyclosporine or tacrolimus level, peripheral blood immunophenotypization, amylase and lipase (if GI symptoms are present)

Blood samples were submitted to the Department of Laboratory Medicine, Clinical Center, NIH for routine laboratory analysis. Serum albumin and total protein (TP) were analyzed with Synchron LX20 Chemistry Analyzer (Beckman Coulter Inc., Brea, CA) and Dimension Vista System (Siemens Healthcare Diagnostics Inc., Newark, DE). Agreement between the two analyzers (slope/intercept) was verified using debiased (Deming) regression analysis (Albumin: 0.99/0.09; TP: 1.03/0.02). Serum CRP was measured by turbidimetry and C3, C4, IgG (immunoglobulin G), IgM (immunoglobulin M) and IgA (immunoglobulin A), and were measured by nephelometry using Beckman Coulter IMMAGE Immunochemistry System and Siemens Dimension Vista System. The agreement between the two different methodologies was: CRP 0.96/0.39; C3 1.1/1.6; C4 0.96/0.5; IgA 0.95/8; IgM 1.02/ -3; IgG 0.98/30. Concentrations of serum beta-2-microglobulin, ferritin and parathyroid hormone were determined using a chemilumiluminescent immunometric assays on the Siemens Immulite 2500. ESR was analyzed on Excyte 40 Automated ESR Analyzer (Vital Diagnostics). CBC data was obtained using Automated Hematology Analyzers.

4.2.5. Statistical analyses

Univariate analyses between a set of laboratory and clinical predictors and a set of cGVHD activity and severity definitions were initially performed to screen for associations between laboratory markers of inflammation and outcomes of interest. Statistical methods used in these univariate analyses included the following: Wilcoxon rank sum test, Jonckheere-Terpstra trend test, [154] Kruskal-Wallis test, and Spearman rank correlation. Spearman correlations are interpreted as follows: $|r| > 0.70$=strong correlation; $0.5 < |r| < 0.7$=moderately strong correlation; $0.3 < |r| < 0.5$= weak to moderately strong correlation; $|r| < 0.3$=weak correlation. In view of the number of tests performed in univariate analyses, only p-values <0.01 are considered to be statistically significant while if $0.01 < p < 0.05$, the associations reflect strong trends. Laboratory parameters were compared with controls using a Wilcoxon rank sum test. Laboratory markers which were found to be potentially associated ($p<0.05$) with the outcomes of interest were then evaluated using univariate logistic regression analyses. Following univariate logistic regression analysis, multivariable logistic regression analysis was done to determine if any of the 24 laboratory parameters were associated with a set of outcomes after adjusting for a set of clinical and demographic parameters. Outcomes that were dichotomized were evaluated with respect to the significance of potential prognostic factors using univariate and then multiple logistic regression analysis. Outcomes that were classified into three ordered categories were evaluated for the effects of potential prognostic factors using logistic regression for ordered outcomes.

Survival analyses were done beginning at the date of entry onto the natural history protocol until death or last follow-up, since the intervals from HSCT to cGVHD diagnosis or from cGVHD to on-study were not associated with survival and the laboratory data were known only at the time of enrollment. Kaplan-Meier analyses and log-rank tests were used to determine the association between potential predictors and survival after entering on the trial. P-values determined after an initial analysis identified groups to form with differing prognosis were adjusted by multiplying the p-value by the number of implicit tests performed to arrive at the final grouping. Following these univariate analyses, Cox proportional hazards models were constructed to determine the joint association between the factors of potential interest and survival. All p-values are two-tailed, and except as noted above, have not been adjusted for multiple comparisons.

5. Results

5.1. Patient Characteristics

Clinical, demographic and cGVHD-related characteristics of patients are summarized in Table 5. and Table 6.

Median patient's age on study was 48 years [18-70 years] and 48% of patients were female and 52% were male. Median time from transplant to onset of cGVHD was 7 months [1.6-83]. Median time from transplant to enrollment was 37 months [4-258]. Median time from cGVHD diagnosis to enrollment was 23 months [0-222]. Median follow-up of surviving patients was 29.8 months [1-70]. The majority of patients (66%) had severe disease in terms of global NIH global score with a median of 4 organs involved [1-8]. Eighty patients (42%) had the progressive onset of the disease. (Figure 10.) One hundred forty (74%) patients received moderate or high intensity of immunosuppression and failed a median of 4 [range 0-9] prior systemic therapies. Seventy-one (38%) of the patients were scored as active. Fifty-seven patients (30%) were scored as worsened, 34 (19%) as improved and 64 (34%) as unchanged by clinician's global assessment of change over the previous month and for 34 patients data were missing. Median NIH average score was 1.09 [0.14-2.14]. The median Lee symptom score was 34 [1-83]. Median PCS score using norm-based scoring (Physical) was 34.75 [11.11-58.4]. Schirmer’s tear test median score was 3 [0-29.5]. Oral mucositis rating scale median score was 9 [0-60]. Six (3%) patients had more than 50 % of BSA (body surface area) affected by erythema, and 23 (12%) manifested 50% BSA sclerotic changes (moveable and/or non-moveable).

Table 5. Clinical, demographic and transplant characteristics (N=189)

Characteristic	N (%)		N (%)	Transplant related	N (%)
Age		**KPS**		**Donor**	
< 40	58 (31)	≤80	115 (61)	Related	130 (69)
40≤x<60	110 (58)	80-100	70 (37)	Unrelated	59 (31)
≥60	21 (11)	unknown	4 (2)	**Stem cell Source**	
Gender		**FEV1**		Bone Marrow	35 (18.5)
Male	99 (52)	<57	47 (25)	Peripheral Blood	153 (81)
Female	90 (48)	>57	139 (73)	Cord Blood	1 (0.5)
Disease		unknown	3 (2)	**HLA Matched**	
ALL/AML/MDS	78 (41)	**Creatinine**		Yes	156 (83)
CML	30 (16)	≤1.2	142 (75)	No	29 (15)
CLL	14 (8)	>1.2	47 (25)	Unknown	4 (2)
Lymphoma	42 (22)	**Bilirubin total**		**Myeloablative conditioning**	
Multiple Myeloma	15 (8)	≤1	179 (95)	Yes	102 (54)
Aplastic Anemia/PNH	6 (3)	>1	10 (5)	No	86 (45.5)
Other	4 (2)	**LDH**		Unknown	1 (0.5)
CMV status at transplant		≤226	123 (65)	**Acute GVHD**	
Positive	59 (31)	>226	66 (35)	Yes	120 (63)
Negative	50 (27)			No	69 (37)
Unknown	80 (42)				

Table 6. Chronic graft versus host characteristics (N=189)

Characteristic	N (%)		N (%)
Time from transplant to enrollment		**Intensity of immunosuppression***	
<1 year	30 (16)	None/Mild	49 (26)
1-2 years	23 (12)	Moderate	71 (37)
2-3 years	41 (22)	High	69 (37)
3-5 years	44 (23)	**Activity by Therapeutic Intent****	
>5 years	51 (27)	Active	71 (38)
cGVHD Onset		Non Active	84 (44)
Progressive	80 (42)	Unknown	34 (18)
Quiescent	41 (22)	**NIH global score*****	
De Novo	67 (35.5)	Mild	2 (1)
Unknown	1 (0.5)	Moderate	62 (33)
Classification of cGVHD		Severe	125 (66)
Classic	166 (88)	**Number of organs involved**	
Overlap	23 (12)	1-3	47 (25)
Number of prior systemic treatments		4-6	123 (65)

< 2	19 (10)	7-8	19 (10)
2-3	72 (38)	**Platelet count (K/µL)**	
4-5	61 (32)	< 100,000	13 (7)
>5	35 (19)	>100,000	176 (93)
Unknown	2 (1)		

*** Mild chronic GVHD involves only 1 or 2 organs or sites with no clinically significant functional impairment (max score 1). Moderate involves at least 1 organ or site with clinically significant but no major disability (max score 2) or 3 or more organs or sites with no clinically significant functional impairment (max score 1). A lung score of 1 is also moderate chronic GVHD. Severe chronic GVHD indicates major disability caused by cGVHD (score 3). A lung score of 2 or 3 is also classified as severe cGVHD; * None/Mild=single agent prednisone<0.5 mg/kg/day; Moderate=single agent prednisone≥0.5mg/kg/day and/or any single agent/modality; High: 2 or more agents/modalities±prednisone≥0.5 mg/kg/day; ** Active: 1) increase systemic therapy because cGVHD is worse; 2) substitute systemic therapy due to lack of response; and 3) withdraw systemic therapy due to lack of response. Non-active: 1) decrease systemic therapy because cGVHD is better; 2) not change current systemic therapy because cGVHD is stable; 3) alter systemic therapy due to its toxicity. Other: either did not receive any immunosuppressive therapy or did not meet any of criteria.

NIH global score and organ involvement are shown in Table 7.

Table 7. NIH cGVHD Scores

NIH Global Score	N (%)		
1 = mild	2 (1)		
2 = moderate	62 (33)		
3 = severe	125 (66)		
NIH Organ Score	**N**		**N**
Skin		**Liver**	
0 = none	42 (22)	0 = none	91 (48)
1 = mild	30 (16)	1 = mild	64 (34)
2 = moderate	46 (24)	2 = moderate	34 (18)
3 = severe	71 (38)	3 = severe	0
Mouth		**Lungs**	
0 = none	59 (31)	0 = none	45 (24)
1 = mild	104 (55)	1 = mild	79 (42)
2 = moderate	23 (12)	2 = moderate	42 (22)
3 = severe	3 (1.5)	3 = severe	23 (12)
Eyes		**Joints and Fascia**	
0 = none	33 (17)	0 = none	75 (40)
1 = mild	66 (35)	1 = mild	40 (21)
2 = moderate	69 (36)	2 = moderate	55 (29)
3 = severe	21 (11)	3 = severe	19 (10)
GI Tract		**Genital (female only n= 90)**	
0 = none	107 (57)	0 = none	46 (51)
1 = mild	62 (33)	1 = mild	13 (14)
2 = moderate	14 (7)	2 = moderate	13 (14)
3 = severe	6 (3)	3 = severe	18 (9)

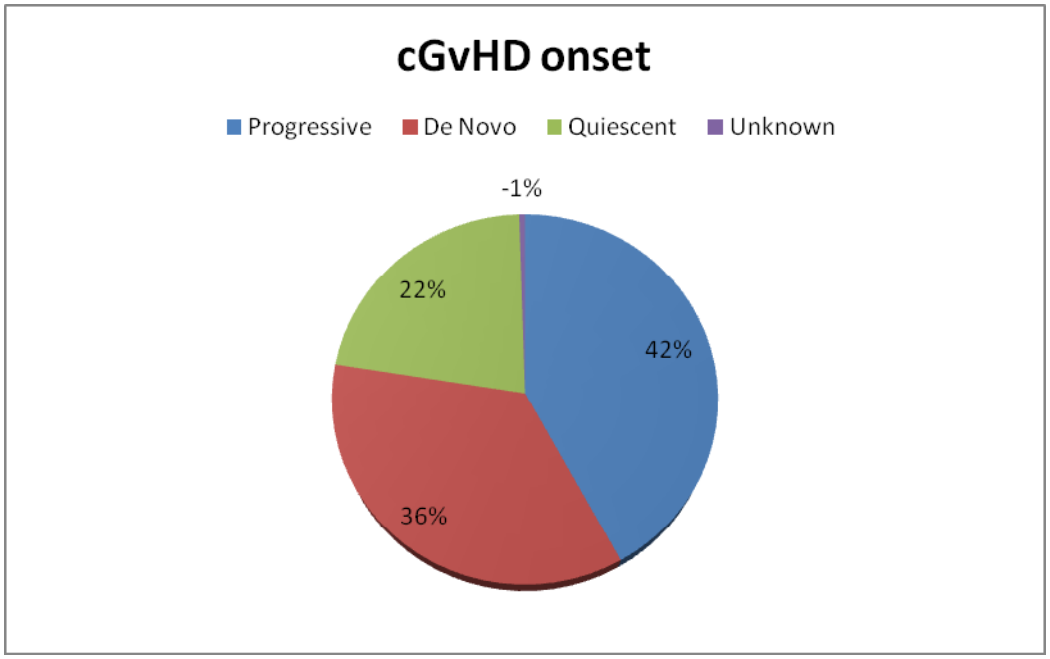


Figure 10. Types of cGVHD onset

Median age in the control group was 53 [37-67] and median time from transplant to enrollment was 23 months [4-127].

Three of seventeen controls were receiving "moderate intensity" of systemic immunosuppressive therapy (protocol planned tapering of GVHD prophylaxis) at the time of study enrollment. One was receiving cyclosporine A 100 mg daily, one cyclosporine A 125 mg daily and one tacrolimus 1 mg daily.

5.2. Comparison of laboratory parameters in patients with chronic GVHD and control group

Laboratory parameters in patients with cGVHD and controls are shown in Table 8.

Compared to non-cGVHD controls, patients with cGVHD had significantly higher CRP, WBC (white blood count), ANC (absolute neutrophil count) and platelet count and lower hemoglobin, albumin and TP values (Table 8.) In the univariate analyses only weak to moderately strong ($0.3 < |r| < 0.5$) correlations were found between laboratory parameters and continuous outcomes of BSA (body surface area) sclerotic changes (moveable and non-no moveable) and NIH average scores.

Among categorical outcomes higher C4 levels were associated with lower Clinician global assessment of change, (e.g. cGVHD worsened; p=0.0011).

Table 8. Laboratory parameters assessed and comparison to non GVHD controls

Laboratory parameter	Median (range)		p-value*	Reference range
	cGVHD Patients (N=189)	Non cGVHD HSCT Controls (N=17)		
CRP	0.65 (0.02-15.4)	0.30 (0.07-1.50)	0.028	0-0.8 (mg/dL)
WBC	6.98 (1.96-31.3)	4.98 (2.48-9.29)	0.0012	4.23-9.07(K/ µL)
ANC	4.14 (0.86-	2.30 (1.19-5.08)	0.0001	1.78-5.38(K/

	26.32)			µL)
Platelets	247 (33-648)	197 (68-286)	0.013	161-347 (K/ µL)
HGB	12.7 (8.2-17.1)	13.8 (9.9-16.2)	0.022	13.7-17.7 (g/dL)
Albumin	3.6 (1.9-4.8)	4.1 (3.2-4.7)	<0.000 1	3.7-4.7 (g/dL)
TP	6.2 (3.9-8.9)	6.60 (5.1-8)	0.041	6.4-8.2 (g/dL)
ALC	1.27 (0.11-7.55)	1.69 (0.57-3.85)	0.13	1.32-3.57(K/ µL)
AEC	0.09(0-3.47)	0.15 (0.02-0.37)	0.24	0.04-0.54(K/ µL)
Ferritin	387 (8-6426)	218 (34-1466)	0.27	18-370 (mcg/L)
β_2-microglobulin	2.2 (0.9-22.9)	2.2 (1-8)	0.72	0.9-1.7 (mg/L)
ESR	16 (2-123)	12 (2-72)	0.14	0-25 (mm/hr)
IgG	650 (98-3380)	793 (589-854)	0.63	642-1730 (mg/dL)
IgM	51.5 (7-424)			34-342 (mg/dL)
IgA	59 (10-647)			91-499 (mg/dL)
C3 comp	132 (64-216)			90-180 (mg/dL)

C4 comp	27 (13-74)			10-40 (mg/dL)
Comp Total	130 (9-228)			55-145(CAEU)
PTH	44.3 (29-448)			16-87 (pg/mL)

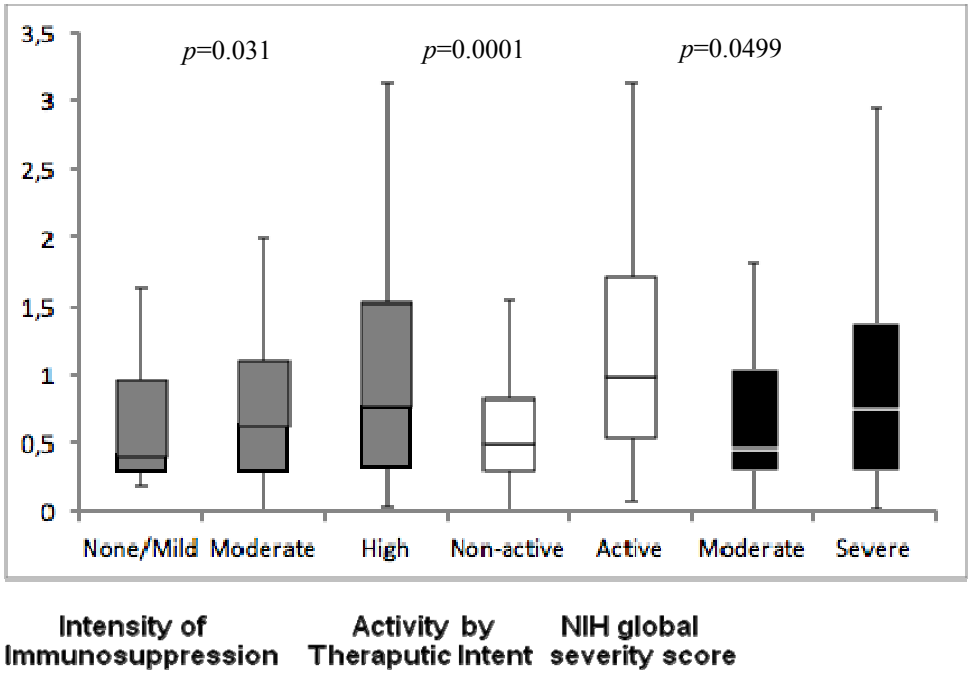
* as determined by Wilcoxon rank sum test; significant if p<0.05.

5.2.1. APR values and activity and severity of cGVHD

Patients with active disease had higher values of CRP (p=0.0001), C3 (p=0.0003), C4 (p=0.0004) and platelets (p=0.012) as well as lower levels of albumin (p=0.044). Similarly, patients with severe NIH global score had higher values of CRP (p=0.0499), C3 (p=0.0017) and platelets (p=0.0028) compared to patients with moderate disease (Figure 11 A-E).

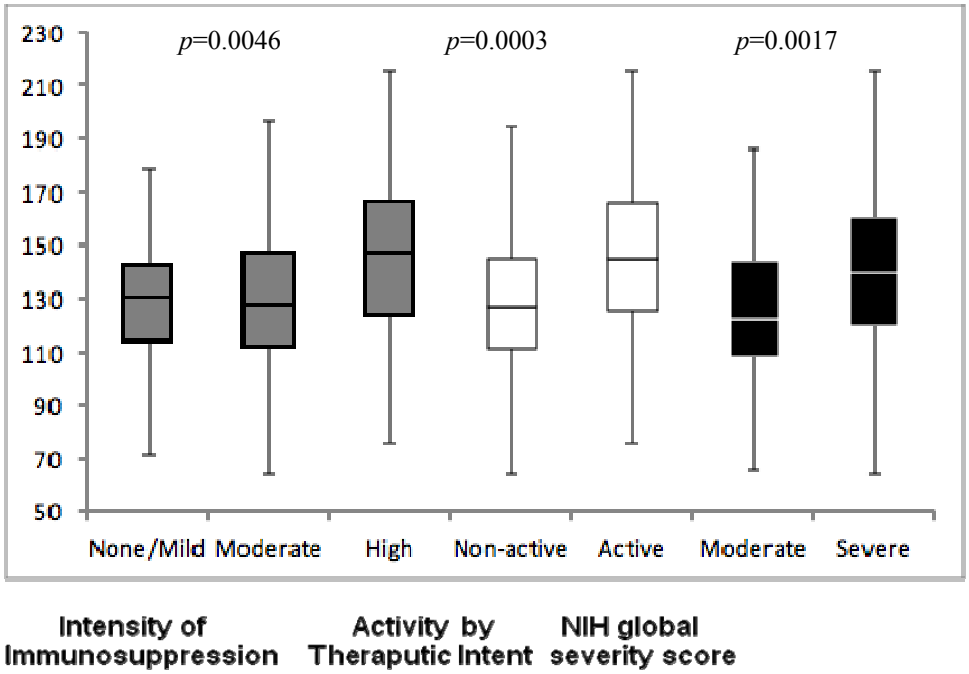
A

CRP mg/dl



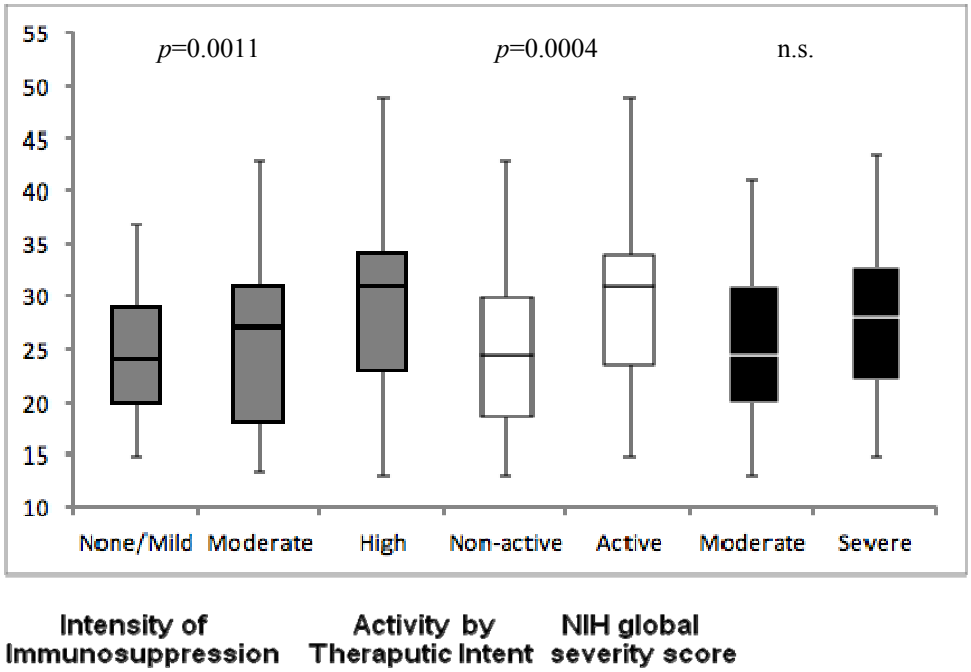
B

C3 mg/dl



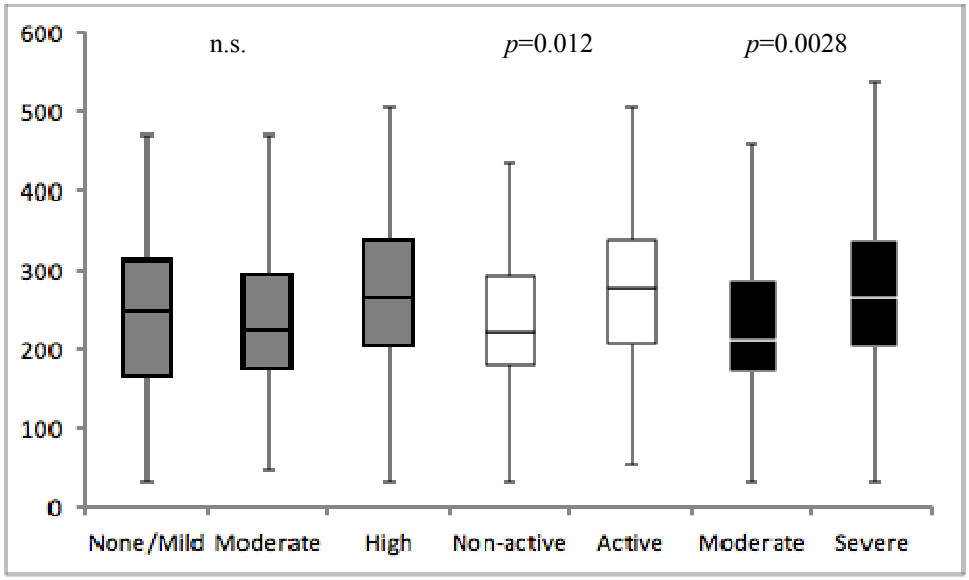
C

C4 mg/dl



D

Platelets K/µL



E

Albumin g/dl

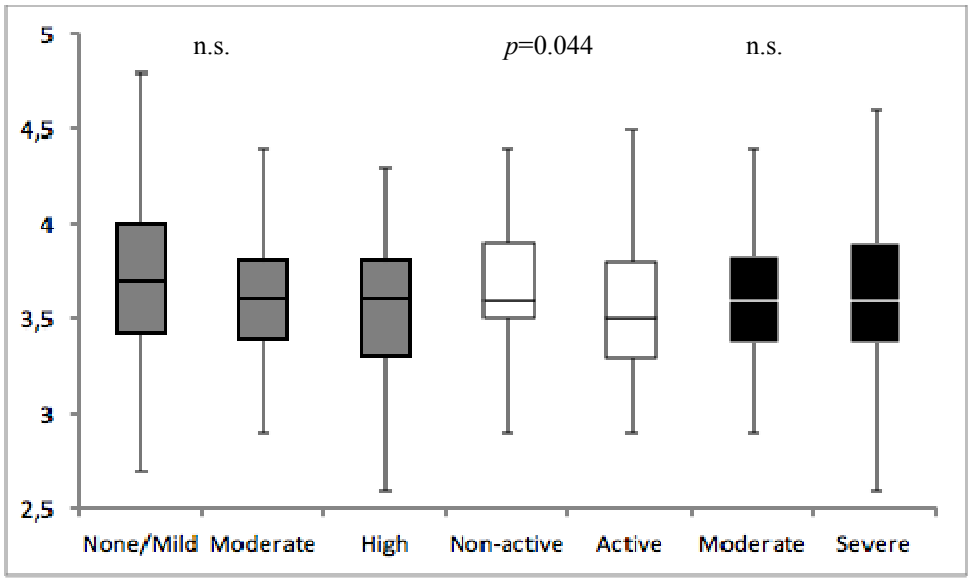


Figure 11. Association between cGVHD activity/severity definitions and laboratory parameters

Association between cGVHD activity/severity definitions and laboratory parameters presented as medians, 25th and 75th percentile and 1.5IQR (interquartile range) of the lower quartile (q1-1.5xIQR), and the 1.5IQR of the upper quartile (q3+1.5xIQR) for intensity of immunosuppression (gray), cGVHD activity (white) and cGVHD severity (black).

Figure illustrates higher CRP (A) values in patients with higher immunosuppression and in those with active and severe disease. (B) Figure illustrates higher C4 values in patients with higher immunosuppression or with active and severe disease. (C) Figure illustrates higher C3 values in patients with higher immunosuppression and with active disease. (D) Figure illustrates higher platelets values in active and severe disease. (E) Figure illustrates lower albumin levels in active disease; n.s. = not statistically significant.

5.3. Univariate analyses of laboratory parameters and categorical outcomes intensity of immunosuppression, active vs. non-active disease and NIH global severity

This data are shown in Table 9.

Table 9. Univariate associations between laboratory parameters and categorical outcomes

Parameter (units) median, [range]	Intensity of immunosuppression				Activity by therapeutic intent			NIH global severity stage		
	none/mild (n=49)	moderate (n=71)	high (n=69)	p-value*	non-active (n=84)	active (n=71)	p-value*	moderate (n=62)	severe (n=125)	p-value*
CRP (mg/dL)	0.42 [0.19-4.43]	0.63 [0.02-15.4]	0.77 [0.04 - 6.92]	0.031	0.49 [0.02 - 7.84]	1.00 [0.09 - 15.4]	0.0001	0.49 [0.02 - 7.84]	0.76 [0.03-15.4]	0.0499
C3 (mg/dL)	129 [66-179]	128 [64-210]	147 [76-216] p=0.0038#	0.0046	126 [64-210]	145 [76-216]	0.0003	122 [66-187]	139 [64-216]	0.0017
C4 (mg/dL)	24 [15-37]	27 [13-61]	31 [13-74] p=0.001#	0.0011	24 [13-74]	31 [15-68]	0.0004	25 [13-55]	28 [15-74.1]	0.09
Comp total (CAE U)	121 [9-180]	136 [69-228]	136 [21-207]	0.032	128 [9-198]	135 [21-228]	0.19	127 [9-197]	136 [48-228]	0.17
IgG (mg/dL)	887 [200-3380] p=0.0003‡	570 [98-2190]	580 [142-2050] p=0.0007#	0.002	608 [139-3080]	599 [98-2190]	0.58	675 [139-3080]	602 [98-3380]	0.29

IgM (mg/dL)	109 [10-413] p=0.0011‡	41 [7-424]	42.5 [10-257] p<0.0001#	0.0003	41 [10-413]	47 [7-424]	0.76	42 [10-413]	59 [7-424]	0.63
IgA (mg/dL)	81 [11-647] p=0.0014‡	54 [10-388]	39.5 [10-258] p<0.0001#	<0.0001	55 [10-388]	51 [10-647]	0.61	60 [10-388]	58 [10-647]	0.49
TP (g/dL)	6.6 [5.1-8.9]	6.1 [4.2-8.8]	6.2 [3.9-7.7] p=0.0044#	0.013	6.2 [4.7-8.9]	6.3 [3.9-8.8]	0.24	6.2 [3.9-8.9]	6.30 [4.7-8.8]	0.2
HGB (g/dL)	13.3 [10.7-17.1] p<0.0001‡	12.5 [8.2-16.1]	12.3 [8.9-16.2] p=0.0002#	0.0006	12.5 [8.2-16.6]	12.5 [8.8-16.2]	0.8	12.9 [8.2-17.1]	12.5 [8.8-16.2]	0.28
ALC (K/µL)	1.63 [0.34-7.55]	1.22 [0.11-5.00]	1.00 [0.15-5.30] p=0.0046 #	0.011	1.19 [0.11-6.88]	1.21 [0.15-5.30]	0.58	1.33 [0.11-6.88]	1.19 [0.15-7.55]	0.36
β-2-microglobulin (mg/L)	2.10[1.1-6.9]	2.20[0.9-22.9]	2.60[1.2-5]	0.046	2.1 [0.9-8]	2.5 [1-6.7]	0.16	2.5 [1.1-6.9]	2.2 [0.9-22.9]	0.25
Platelets (K/µL)	244 [33-471]	224 [49-539]	266 [34-648]	0.07	223 [33-465]	278 [56-648]	0.012	214 [33-461]	265 [34-648]	0.0028
Albumin (g/dL)	3.7 [2.5-4.8]	3.6 [2.1-4.4]	3.6 [1.9-4.3]	0.082	3.6 [2.9-4.6]	3.5 [1.9-4.5]	0.044	3.6 [1.9-4.8]	3.6 [2.1-4.6]	0.95
Ferritin (mcg/L)	200 [32-6426]	464 [23-5401]	421 [8-5961]	0.73	374 [21-6426]	448 [8-5961]	0.7	358 [21-6426]	437 [8-5961]	0.89

ESR (mm/hr)	12 [2-91]	21 [2-123]	18 [2-80]	0.15	15 [2-123]	21 [2-95]	0.063	21 [2-123]	16 [2-116]	0.63
WBC (K/μL)	6.31 [2.27-14.1]	7.14 [1.96-27.85]	7.14 [2.47 - 31.3]	0.65	6.62 [2.27 - 19.40]	7.62 [1.96 - 31.3]	0.076	6.4 [1.96 - 14.1]	7.37 [2.65-31.3]	0.1
ANC (K/μL)	3.58 [1-10.3] p=0.0039‡	5.27 [0.86-26.32]	3.99 [1.05 - 18.3]	0.32	4.13 [1-18.3]	5.09 [0.86 - 26.3]	0.43	3.79 [0.86 - 12.2]	4.45 [1.05-26.3]	0.08
AEC (K/μL)	0.12 [0-0.97]	0.07 [0-1.26]	0.09 [0-3.47]	0.3	0.08 [0-1.26]	0.1 [0-3.47]	0.78	0.1 [0-3.47]	0.08 [0-3.26	0.14
PTH (pg/mL)	41.6 [4.8-161]	44.6 [5.7-448]	6.2 [3.9-7.7]	0.76	45 [2.9-448]	39 [6.8-256]	0.72	41 3.6-273]	45 [2.9-448]	0.37

* p-values for parameters across ordered intensity of immunosuppression were determined by Jonckheere-Terpstra test for trend, while those for therapeutic intent and NIH global severity were determined by Wilcoxon rank sum test. Across 'intensity of immunosuppression' categories parameters were compared between the two groups at a time using a Wilcoxon rank sum test.; If $p<0.005$ consider the difference to be significant while if $0.005 < p < 0.05$, this indicates a strong trend (bold). ‡None/mild significantly different from moderate, #None/mild significantly different from high. Moderate and high never differed significantly ($p>0.005$ in all cases).

A statistically significant association was found between higher levels of CRP (p=0.0002), C3 (p<0.0001) and platelets (p=0.0001) and more severe joint/fascia involvement (NIH score 3). Similarly, higher levels of CRP (p=0.0004), C3 (p<0.0001) and platelets (p=0.0016) were associated with more severe skin involvement (NIH score 3).

No statistically significant association was found between ferritin, ESR, WBC, ANC, absolute eosinophil count and parathyroid hormone and clinical activity or severity outcomes.

Serum cytokines (MCP1, IL-1RA, IL-6, and TNFRII) were measured in an exploratory analysis on a subset of 107 patients and there were no statistically significant association with cGVHD outcomes.

5.4. Multivariable model determining chronic GVHD activity and severity

The following categorical outcomes were developed with a multivariable model:

1. Intensity of immunosuppression, (none/mild vs. moderate vs. high)

2. Active vs. non-active disease based on therapeutic intent

3. NIH global score (moderate vs. severe)

Continuous outcomes: Lee total score, SF36 physical, Schirmer's tear test, OMRS, BSA erythema, non-moveable sclerosis/fasciitis and NIH average score were excluded from further analyses due to correlation coefficients with laboratory parameters of <0.40. Clinician's global assessment and BSA moveable sclerotic changes were not found to be related to any laboratory markers in the final analysis, so no models were developed related to these outcomes.

5.4.1. Intensity of immunosuppression (none/mild vs. moderate vs. high)

The following variables were included in the initial multivariable model: CRP, C3, C4, complement total, IgG, IgM, IgA, total protein, hemoglobin, absolute lymphocyte count (ALC), beta-2-microglobulin, number of prior treatments and stem cell source.

As expected, patients who were receiving high levels of immunosuppression had lower values of total protein, IgM, IgA, and received a greater number of prior treatments than patients who received moderate or low intensity immunosuppression, or who received low levels or no immunosuppression (Table 10.).

Table 10. Multivariable Cox proportional hazards model analysis of factors associated with GVHD activity and severity

Outcome	Parameter	Estimate	Standard error	p-value
Intensity of immunosuppression	TP	-0.2442	0.0681	0.0003
	#Prior Treatments	0.4303	0.082	<0.0001
	IgA	-0.0044	0.002	0.0278
	IgM	-0.0057	0.00197	0.0036
Active vs. Non-active disease	Albumin	-1.013	0.1927	<0.0001
	Platelets	0.00446	0.00205	0.0296
	CRP	0.2567	0.1266	0.0427
	#Prior Treatments	0.4996	0.1163	<0.0001
Global NIH severity	Platelets	0.00395	0.00171	0.021
	FEV1	-0.0251	0.0054	<0.0001
	#Prior Treatments	0.4991	0.1057	<0.0001

5.4.2. Clinician's therapeutic intention (active vs. non-active)

The following variables were included in the initial multivariable model: CRP, C3, C4, platelets, albumin, number of prior treatments, FEV1 (forced expiratory volume in the first second), Karnofsky performance status and TBI (total body irradiation) conditioning. Logistic regression analysis showed that patients with active disease received more prior systemic therapies, and had higher values of CRP and platelets as well as lower values of albumin compared to patients with inactive disease (Table 10.). Using this model the equation for predicting disease activity was established (Table 11.). Based on this rule, among those used to develop the rule, 71% of patients with active disease and 79 % of those with non-active disease would be correctly classified.

Table 11. Equations predicting cGVHD activity and severity

cGVHD	
active	398.05*albumin-1.74*platelets -194.40*number of prior treatments - 99.88*CRP <100
non-active	398.05*albumin -1.74*platelets -194.40* number of prior treatments - 99.88*CRP >100
severe	-1.026*platelets -129.65 * number of prior treatments + 6.52*FEV1 <-100
moderate	-1.026*platelets - 129.65*number of prior treatments + 6.52*FEV1 >-100

An alternative model included the laboratory parameters of CRP, albumin, platelets, C3 and C4 complement.

In this model, the thresholds for each parameter which provided the best classification to active/non-active disease were developed by individual logistic regression models. Each patient was then identified as to whether they were in the range associated with active disease by each of the 5 laboratory parameters. The total number of categories in which they would be classified as active was determined. The following describes the levels of the parameters which were associated with active disease: CRP>0.7 mg/dL, C3>140 mg/dL, C4>28 mg/dL, platelets>250 K/μL and albumin <3.6 g/dL. If 0-3 parameters fit these criteria, the chance of cGVHD to be active is 69%, and if all 5 parameters fit these criteria the chances of cGVHD to be active is 80%. If none of the parameters fits these criteria the chances of disease to be non-active is 100% (Table 12.).

12. Prediction of the cGVHD activity based on 5 laboratory parameters

Parameter	Active (80%)	Non-active (100%)
CRP (mg/dL)	≥0.7[1]	≤0.7[1]
C3 (mg/dL	≥140	≤140
C4 (mg/dL)	≥28	≤28
Platelets (K/μL)	≥250	≤250
Albumin (g/dL)	≤3.6	≥3.6

[1]Thresholds shown were determined by univariate logistic regression model analyses.

Additional analysis to compare the group of the patients who did not receive any systemic immunosuppression (N=39) and the group of patients who received mild, moderate or high immunosuppression (N=150) has been also evaluated. There was a statistically significant difference (or a strong trend) between these two groups in the following laboratory parameters: patients receiving immunosuppression had higher values of CRP ($p=0.048$), complement total ($p=0.0015$), ferritin ($p=0.022$), ESR ($p=0.018$), and absolute neutrophil count (0.0011), likely reflecting active disease. The same group of patients had lower values of absolute lymphocyte count ($p=0.0002$), IgG ($p<0.0001$), IgM ($p=0.0005$), IgA ($p<0.0001$), total protein ($p=0.0003$), hemoglobin ($p<0.0001$), and AEC ($p=0.016$), that is probably the result of systemic treatment (Table 13.)

Table 13. Comparison of laboratory markers between patients who received and who did not receive systemic immunosuppressive treatment (only significant values shown)

Parameter (units) median, [range]	Intensity of immunosuppression		p-value
	None (n=39)	Mild, Moderate or High (n=150)	
CRP (mg/dL)	0.37 [0.19-1.97]	0.67 [0.015-15.4]	0.048
Compl total (CAE U)	117 [9-178]	136 [21-228]	0.0015
IgG (mg/dL)	906 [200-3380]	575 [98-2190]	<0.0001
IgM (mg/dL)	109 [10-413]	43 [7-424]	0.0005
IgA (mg/dL)	87 [11-647]	50 [10-388]	<0.0001
TP (g/dL)	6.7 [5.3-8.9]	6.2 [3.9-8.8]	0.0003
HGB (g/dL)	13.3 [10.7-17.1]	12.4 [8.2-16.6]	<0.0001
ALC (K/μL)	1.817 [0.338-7.548]	1.120 [0.114-5.304]	0.0002
Ferritin (mcg/L)	170 [32-6426]	457 [8-5961]	0.022
ESR (mm/hr)	12 [2-91]	18 [2-123]	0.018
ANC (K/μL)	3.246 [1.001-10.293]	4.417 [0.862-26.320]	0.0011
AEC (K/μL)	0.122 [0.000-0.973]	0.08 [0.000-3.470]	0.016

Parameters were compared between the two groups using a Wilcoxon rank sum test.

5.4.3. NIH global staging (moderate vs. severe)

The following variables were included in the initial multivariable model: CRP, C3, platelets, number of prior treatments, age (continuous), FEV1, Karnofsky performance status and myeloablative conditioning. Patients with severe disease had higher platelet counts, received more prior systemic treatments, and had lower values of FEV 1 (Table 10.) Using this model the equation for predicting disease severity was established (Table 11.) Based on this rule, among those used to develop the rule, 76% of patients with severe disease and 74% of those with moderate disease would be correctly classified.

Age, sex, donor type, cell source, conditioning regimen, Karnofsky performance status, time from transplant to enrollment, time from cGVHD diagnosis to enrollment, time from transplant to cGVHD diagnosis, gender match between recipient and donor, HLA (human leukocyte antigen) match, cGVHD classification (classic vs. overlap), cGVHD onset, eosinophil count (<0.5/>0.5 K/μL) and platelet count (<100/>100 K/μL) had no impact on disease activity or severity in any of the multivariate analyses.

5.5. Survival

Overall survival on study is shown in Figure 12.

Patients with active disease had decreased survival compared to patients with non active disease (p=0.057). Higher white blood count (adjusted p=0.029), higher absolute neutrophil count (adjusted p=0.05), lower lymphocyte count (adjusted p=0.057) and lower IgG (adjusted p=0.033) were shown to be associated with decreased survival in the univariate analysis (Figure 13. A-E).

In the Cox proportional hazards model, in addition to higher Karnofsky performance status (>= 80; p=0.0008; Hazard ratio=0.33; 95 CI: 0.17-0.63), lower NIH lung score (0-2; p<0.0001; Hazard ratio=6.52; 95% CI: 3.07-13.87) and higher FEV1 (>57; p=0.0028; Hazard ratio=0.35; 95% CI: 0.18-0.70) higher absolute lymphocyte count (>0.65; p=0.017; Hazard ratio=0.43 (95% CI: 0.22-0.86) was the only laboratory marker associated with better survival from the day the patient went on study (Figure 14. A-C). The difference between active vs. non-active disease was not significant in the multivariable analysis.

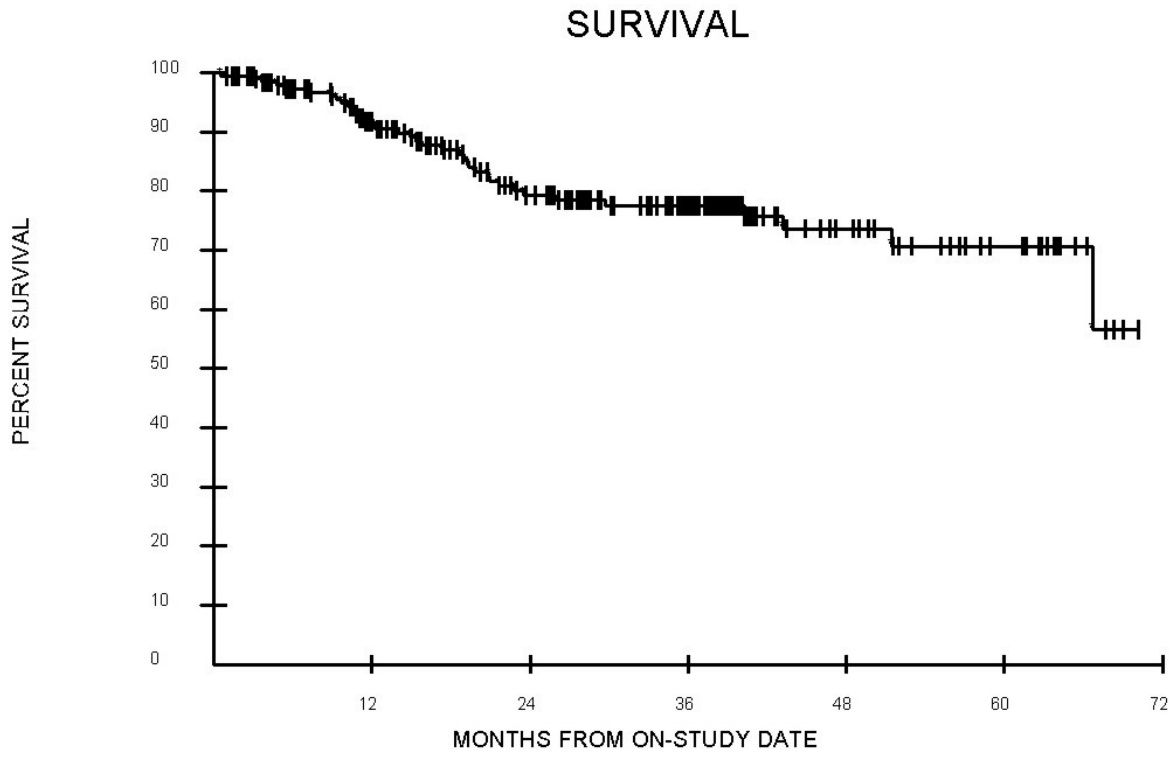
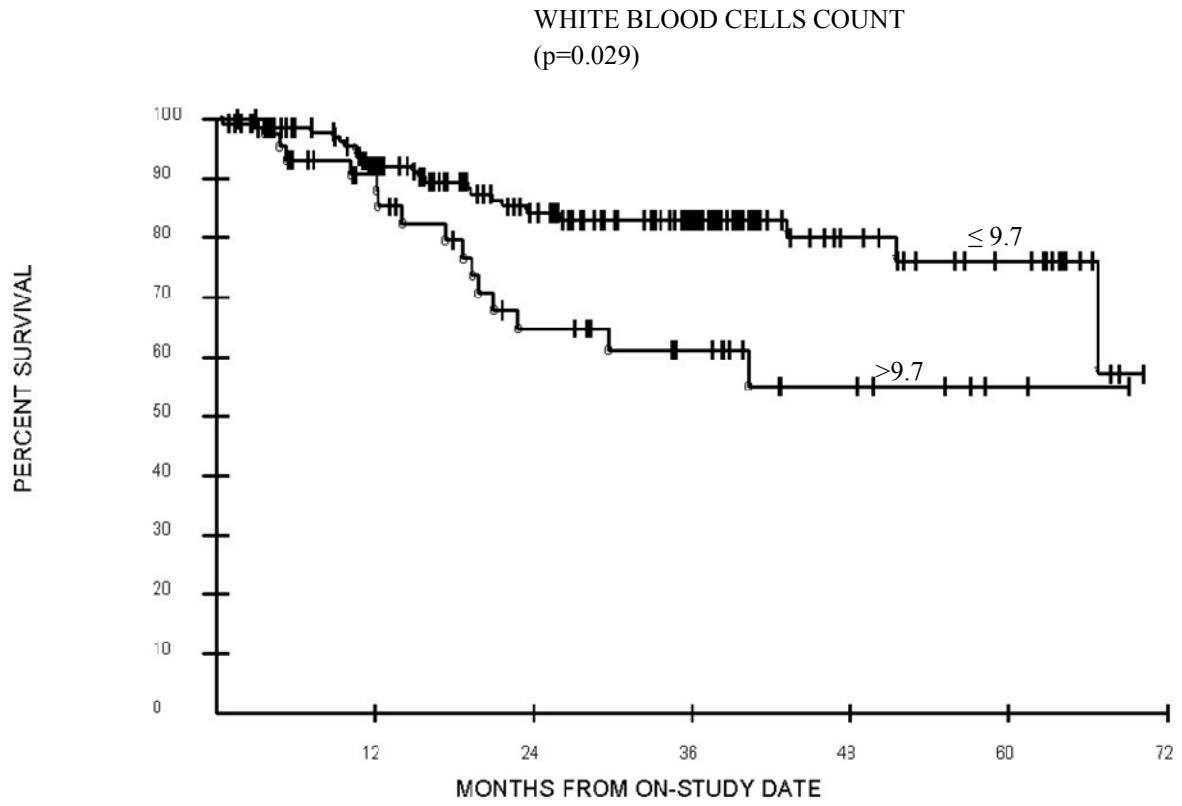
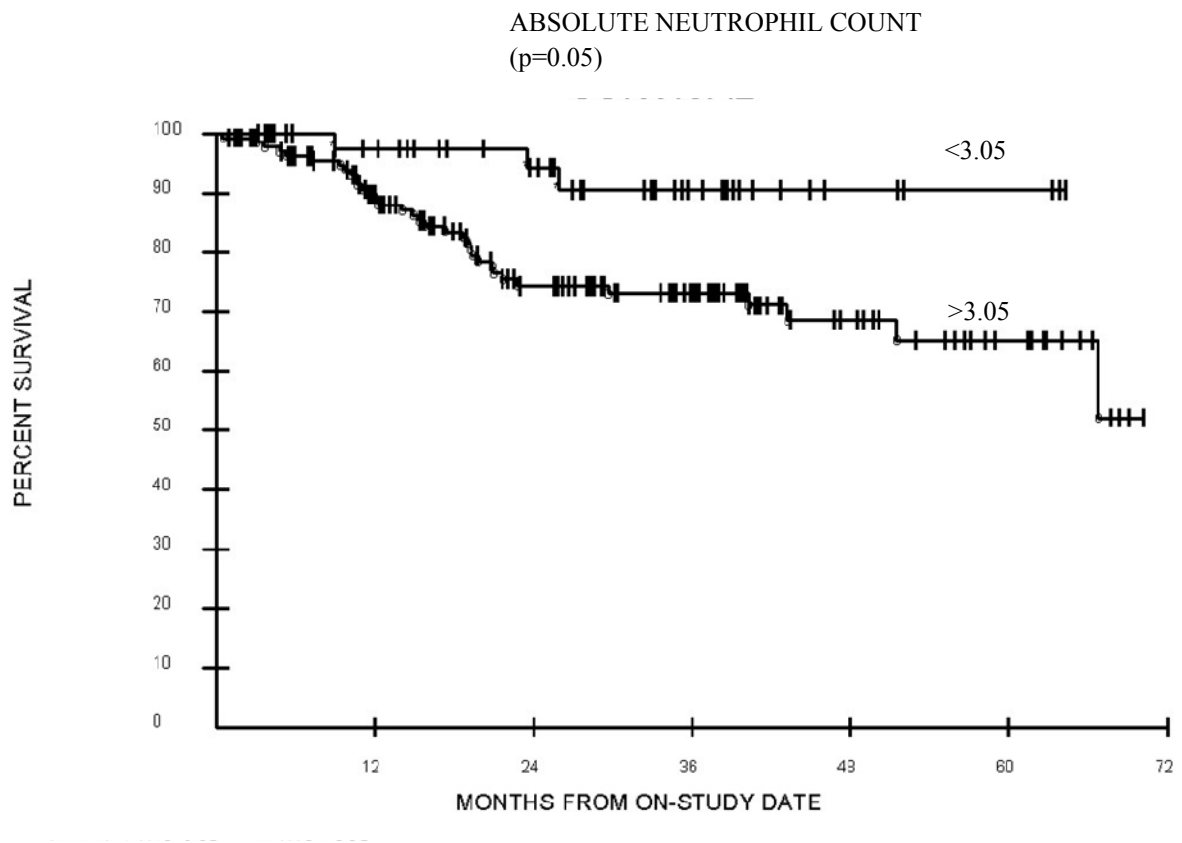


Figure 12. Overall survival on study

A.

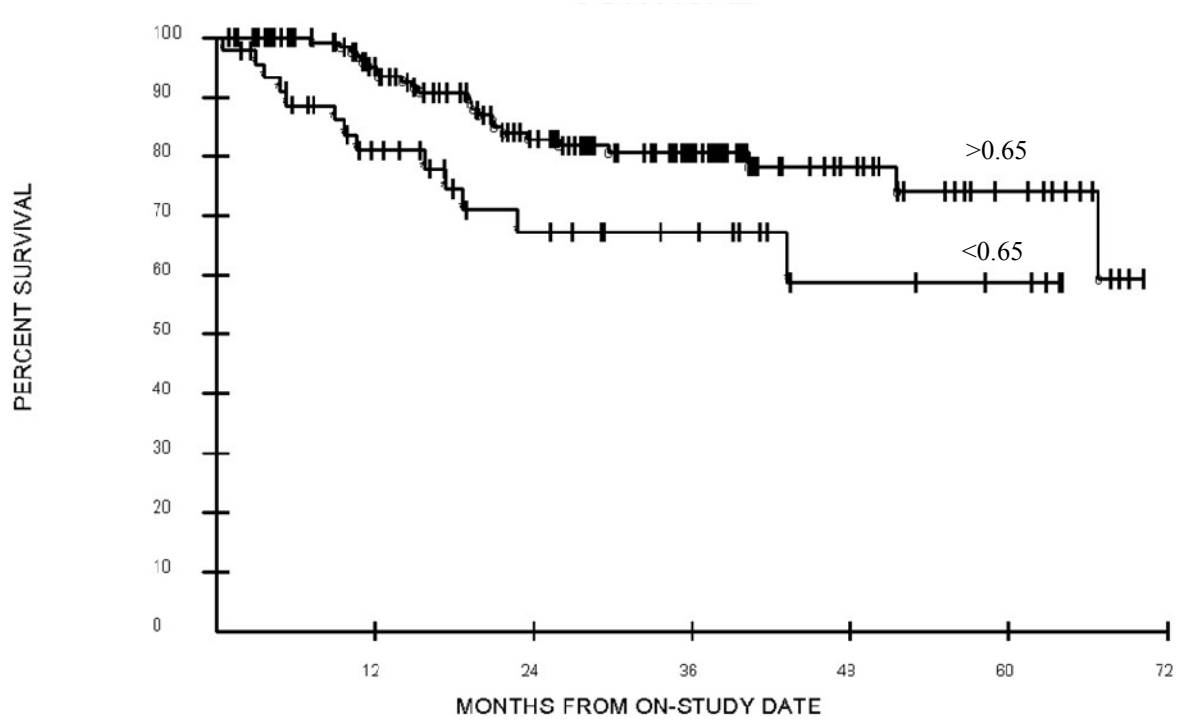

WHITE BLOOD CELLS COUNT
(p=0.029)
PERCENT SURVIVAL
100
90
80
70
60
50
40
30
20
10
0
≤ 9.7
>9.7
12
24
36
48
60
72
MONTHS FROM ON-STUDY DATE

B.

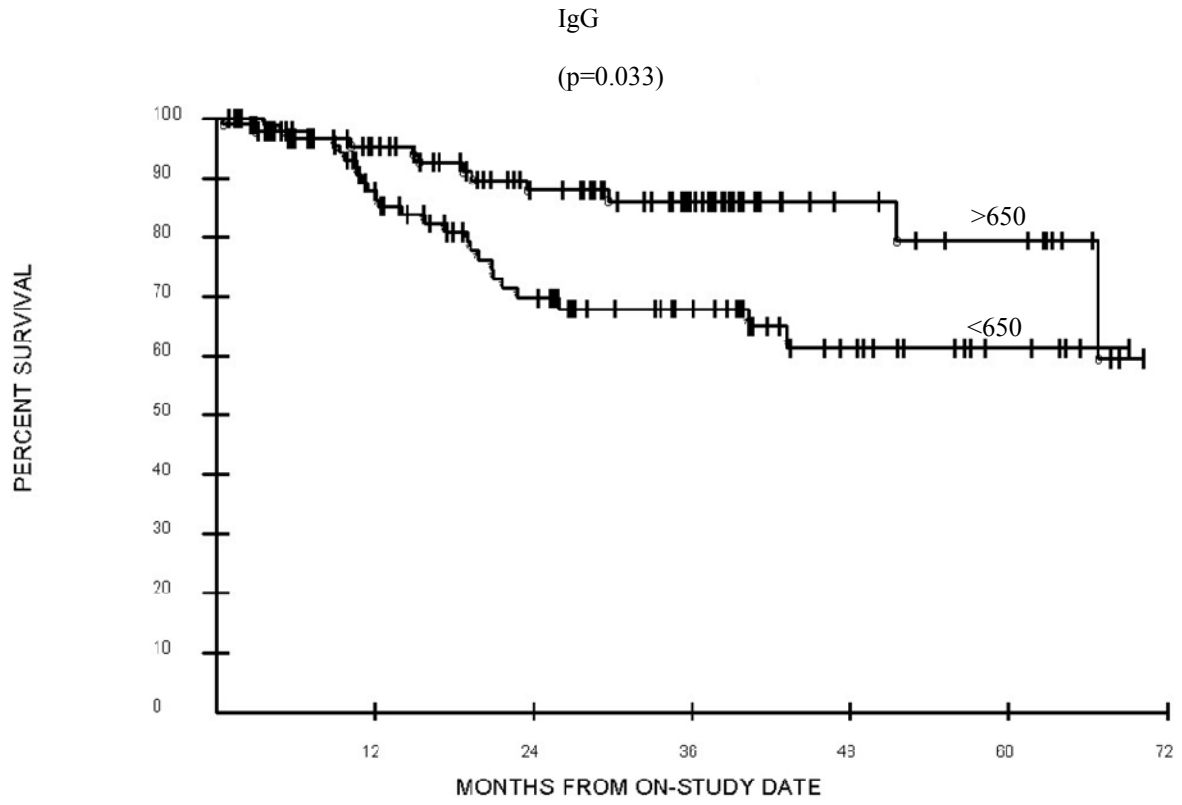

ABSOLUTE NEUTROPHIL COUNT
(p=0.05)
<3.05
>3.05
PERCENT SURVIVAL
100
90
80
70
60
50
40
30
20
10
0
12
24
36
48
60
72
MONTHS FROM ON-STUDY DATE

C.

ABSOLUTE LYMPHOCYTE COUNT
(p=0.057)



D.


IgG
(p=0.033)
PERCENT SURVIVAL
100
90
80
70
60
50
40
30
20
10
0
>650
<650
12
24
36
48
60
72
MONTHS FROM ON-STUDY DATE

E.

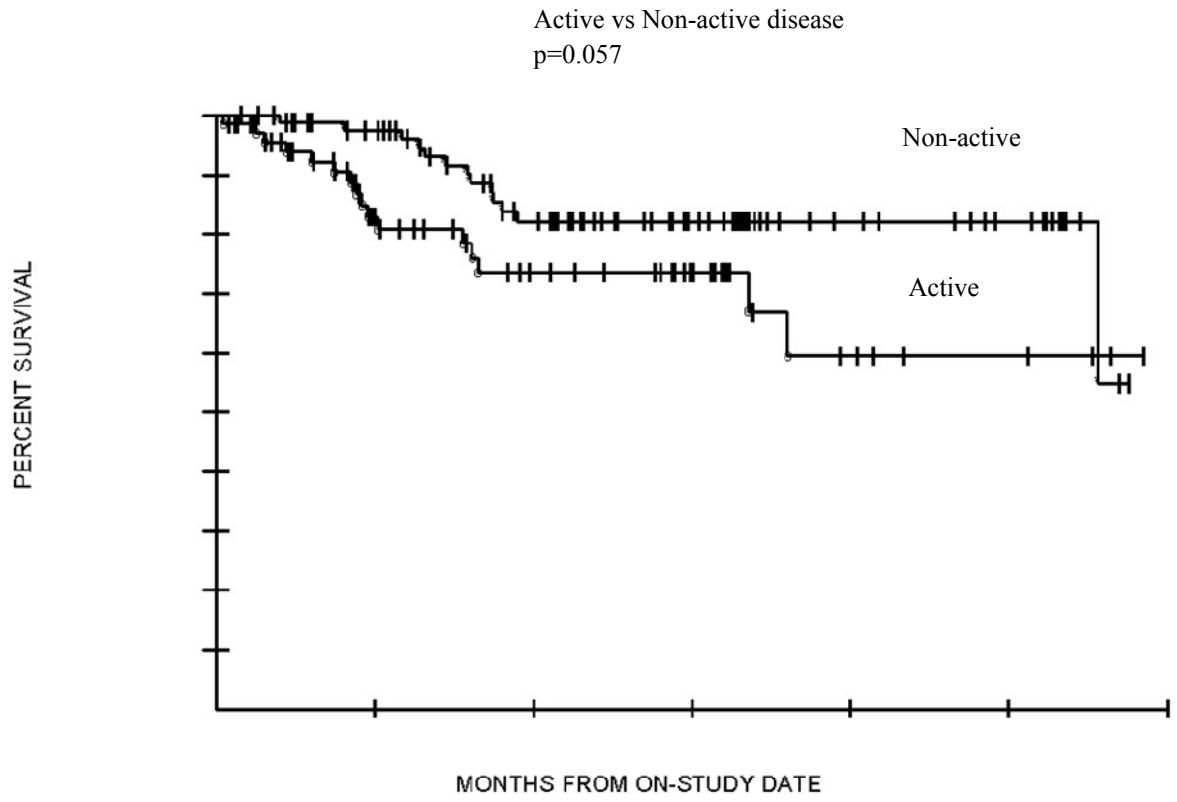
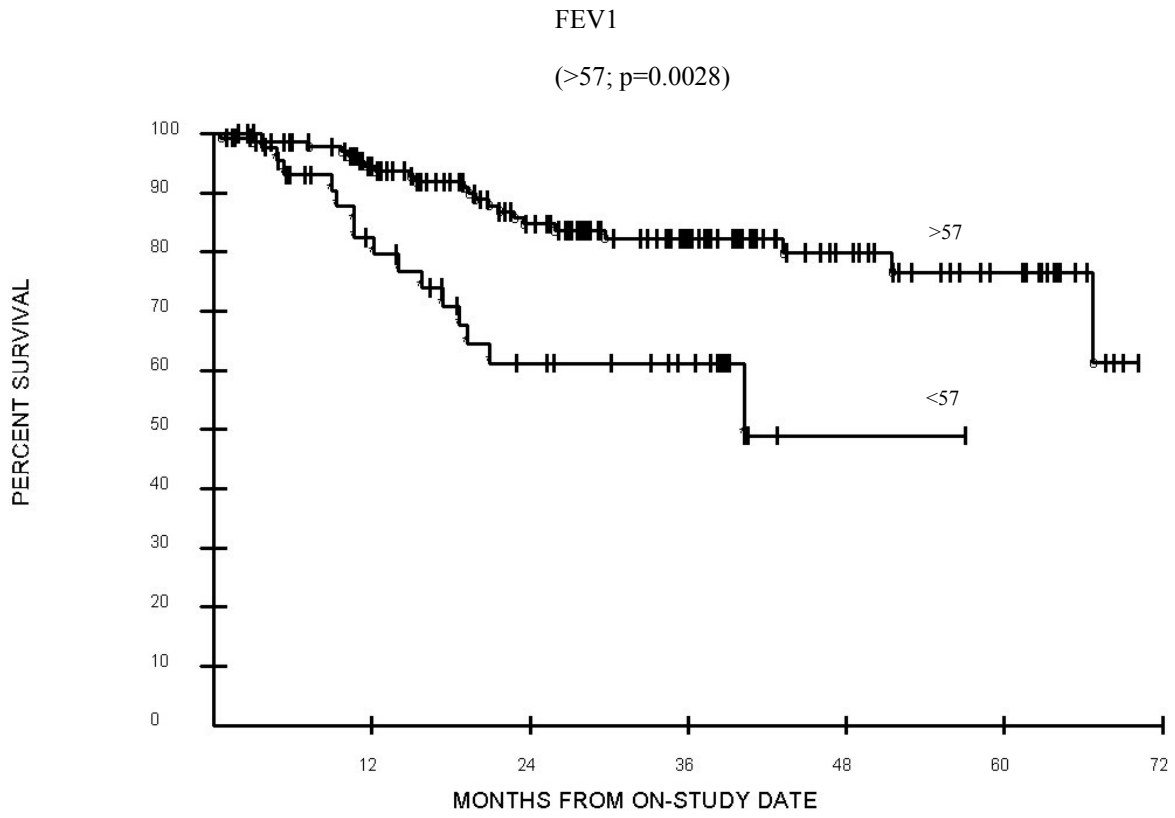
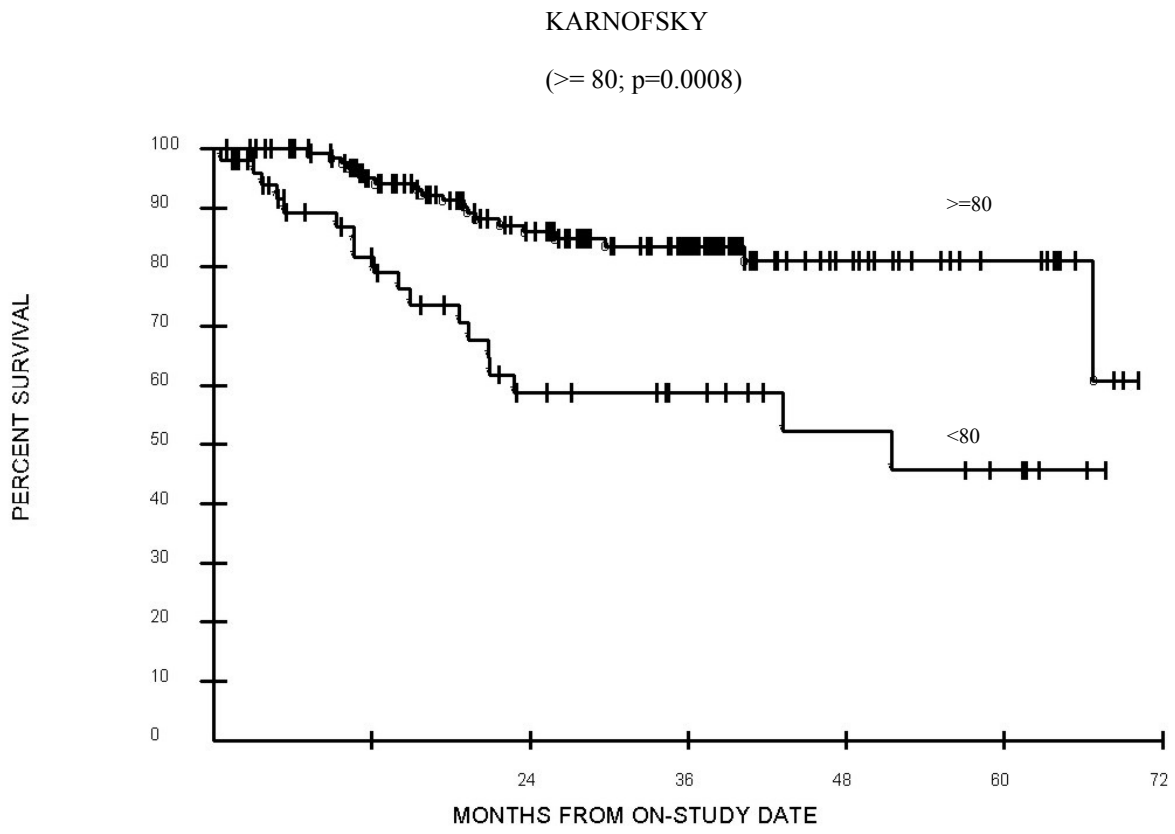


Figure 13. Survival from study enrollment according to various laboratory parameters A. white blood cells count, B. absolute neutrophil count, C. absolute lymphocyte count, D. IgG, E. Active vs Non-active disease

A.


FEV1
(>57; p=0.0028)
PERCENT SURVIVAL
100
90
80
70
60
50
40
30
20
10
0
>57
<57
12
24
36
48
60
72
MONTHS FROM ON-STUDY DATE

B.


KARNOFSKY
(>= 80; p=0.0008)
PERCENT SURVIVAL
100
90
80
70
60
50
40
30
20
10
0
>=80
<80
24
36
48
60
72
MONTHS FROM ON-STUDY DATE

C.

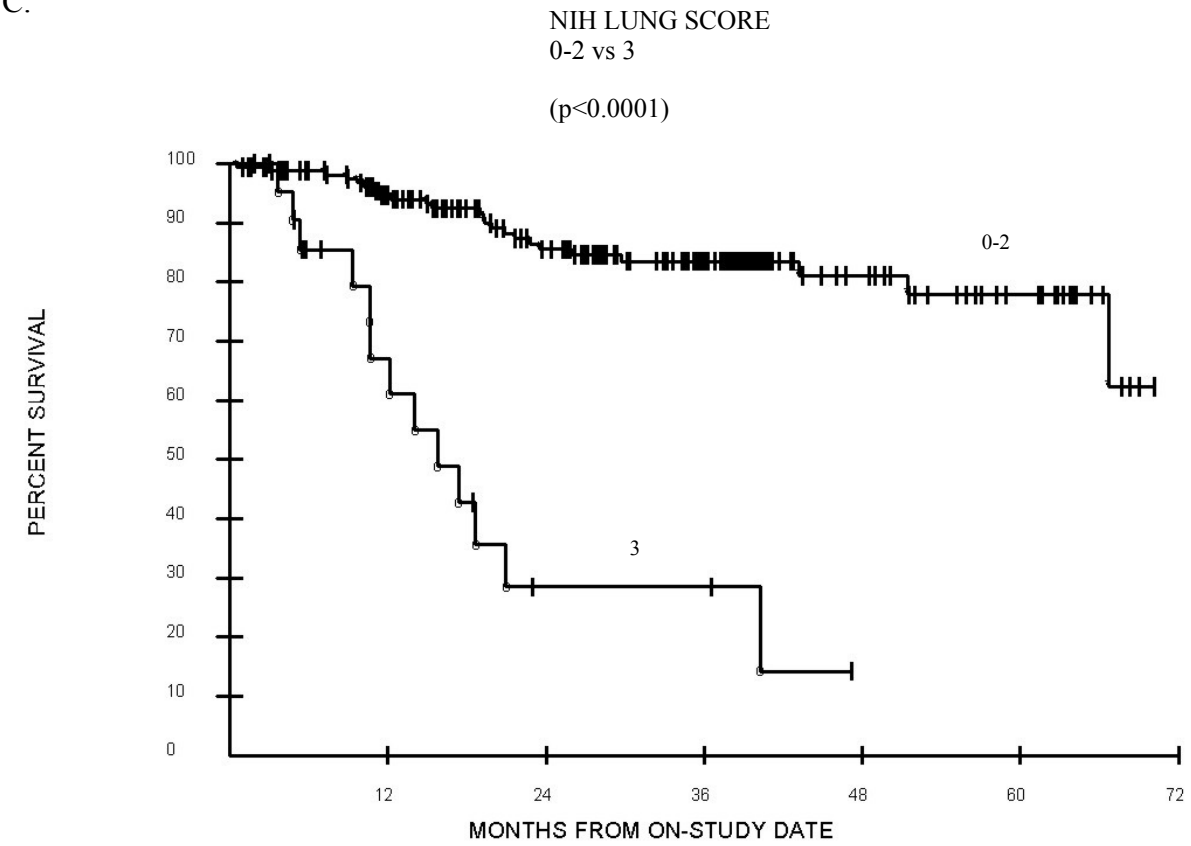


Figure 14. Association between demographic/clinical outcomes (A. FEV1, B. Karnofsky and C. NIH lung score) and survival

6. Discussion

Chronic GVHD is the most severe late effect of therapy in survivors who undergo allogeneic HSCT. [155] It affects numerous organs, often requiring a comprehensive multidisciplinary approach and prolonged immunosuppressive therapy for a median duration of 2.5-3 years. [156] The pathophysiology of cGVHD remains poorly understood, and the current mainstays of treatment are global immunosuppression rather than selective targeting of the key mechanisms of the disease. [157] First-line treatment with steroids with or without calcineurin inhibitor is successful in only about one-half of cases and there is no standard second-line treatment. [111,158] The decision whether to initiate, intensify, or taper immunosuppressive therapy is typically based on the clinician's assessment of disease activity and severity. While suppression of disease activity is desirable to control symptoms and prevent irreversible damage, excessive immunosuppression of inactive cGVHD could be only harmful without resulting improvement in cGVHD manifestations. [159] In spite of advances in cGVHD staging based on NIH consensus criteria, there are no defined clinical measures to differentiate cGVHD disease activity (by definition, reversible manifestations of the disease) [149] vs. damage to guide clinical therapy decisions or monitor outcomes. We performed this study in a referral cohort of cGVHD patients highly enriched for those with established, severe and heavily previously treated disease. All patients were evaluated in depth and at the single time-point in their disease trajectory and the sera samples were well annotated using a multidimensional battery of cGVHD descriptors.

This study identified a number of laboratory indicators of inflammation (CRP, WBC, ANC, platelets and albumin) differing between patients with primarily established, moderate or severe cGVHD and non-cGVHD transplanted controls, suggesting ongoing tissue inflammation in the patient cohort. We also identified several laboratory markers associated with the clinicians' assessment of disease activity or severity.

CRP is the best known acute phase serum protein which is widely used as a marker of intensity of inflammatory process and shows strong interactions with the complement system.[139] Values greater than 1 mg/dL (10 mg/L) reflect clinically significant inflammation. [123,125] Values between 0.3-1mg/dL (3-10 mg/L) indicate "low grade inflammation" described in various chronic diseases. [160] The role of CRP and other routinely used clinical laboratory indicators of inflammation are unknown in the setting of cGVHD in contrast to their well established role in other inflammatory conditions and autoimmune disease such as rheumatoid arthritis, SLE or Crohn's disease. [126,161]

The role of CRP in cGVHD is suggested by few reports. [162,163] In the study performed by Rovo et al. recipients had a decreased kidney function and higher liver function tests, except for bilirubin and higher TSH independent of presence or absence of cGVHD no difference existed between laboratory markers of inflammation between recipients without cGVHD and healthy donors. Patients with ongoing cGVHD had higher CRP (p=0.002) and vWF (p=0.002) values than patients without cGVHD and healthy donors. In addition patients with cGVHD had significantly lower albumin values (p=0.021). [163]

Our study demonstrated higher levels of CRP in sera of patients with active and severe disease compared to patients with non-active or moderate disease. The median CRP was 0.65 mg/dL (6.5 mg/L), which is in the range of minor CRP elevation (0.3-1 mg/dL), described as "low grade inflammation" in chronic inflammatory conditions that differs from acute inflammation caused by infection not only in magnitude but also by absence of the classic clinical signs of infection. [160] In this study all patients underwent detailed clinical evaluations, and only a small minority had active infections (3%) and most of them had concurrent active cGVHD, emphasizing the need for interpreting laboratory markers in such cases with caution and strictly in the context of all other clinical information.

Because active infections may influence CRP levels, we gave special attention to this during the data analysis. Microbiologic evaluation was not part of the routine clinical testing. However, during the detailed clinical evaluations we did look thoroughly for signs of infection and in case of any suspicion for an active infection further clinical and laboratory testing were pursued including an infectious disease consult. Six of seventy-seven patients with elevated CRP (>0.8 mg/dL) had documented infection (positive blood cultures and acute sinusitis). Three patients had positive blood cultures. The first patient reported occasional chills and his temperature was 37°C. He had Streptococcus bovis isolated from his blood culture and also Comamonas testosterone and Agrobacterium radiobacter isolated from his wound culture. His CRP was 2 mg/dL. The second patient reported temperatures up to 38°C two days before enrollment. His temperature at enrollment was normal. He had Staphylococcus epidermidis isolated from his blood cultures. He has also had Staphylococcus aureus isolated from his lung biopsy specimen. His CRP was 3.14 mg/dL. The third patient had CMV PCR positive blood (41 900 copies) with normal body temperature and no other signs of infection. CMV PCR testing is a standard part of this protocol. His CRP was 2.7 mg/dL. One patient had thumb paronychia with isolated Staphylococcus aureus and no other signs of infection. His CRP was 5.15 mg/dL. Two patients had acute sinusitis and their CRP

values were 8.01 and 15.4 mg/dL. One patient with normal CRP (0.55 mg/dL) had E.coli and Staphylococcus coagulase negative isolated from her blood cultures. Median CRP in this group was 4.15 mg/dL [2-15.4]. Because of the small number of patients (3% of the whole cohort) and because of the co-existence of active cGVHD in five patients they were kept in the study.

In a study performed by Uguccioni et al. no significant increase in SAA or CRP was found in chronic GVHD in contrast to patients with aGVHD and graft rejection who were transplanted for β-thalassemia. The different acute phase response in acute GVHD and rejection compared with chronic GVHD suggests different immunopathogenic mechanisms are responsible. [164]

Complement activation is increasingly recognized as a major contributor to vascular inflammation.

C3 deposits can be found in the skin, [165] and in glomerular membranes in patients with cGVHD and nephrotic syndrome with normal C3 and C4 serum levels. [166,167] Elevated complement and complement activation by autoantibodies is one of the possible mechanism of endothelial damage and fibrosis in SSc patients. [168] In our study higher levels of C3 and C4 were associated with active disease, most likely as response to increased inflammatory cytokines such as IL-6. [116] Higher C3 levels were associated with most severe (sclerotic) changes skin ($p<0.0001$) and joint/fascia involvement ($p<0.0001$).

It was mentioned earlier that thrombocytopenia in cGVHD patients is among the most consistent and strongest poor survival predictors in many cGVHD studies. [83] One of the earliest studies which showed that cGVHD patients with low platelet counts had the worst survival and that thrombocytopenia may reflect more severe cGVHD was study by First and colleagues published in 1985. [94] They found that among 65 patients who had full engraftment after alloHSCT, and who survived at least 60 days after transplantation, 24 (37%) developed isolated thrombocytopenia, 9 (14%) with transient and 15 (23%) with chronic thrombocytopenia (defined as platelet count remaining below 100,000/μL through day +120). The transient syndrome was not associated with adverse outcome, but patients with chronic thrombocytopenia had increased mortality and an increased risk of having severe acute and chronic graft versus host disease. Although bleeding complications in that study contributed directly to death in just two patients with chronic thrombocytopenia, there was a significantly higher mortality among cGVHD patients with chronic thrombocytopenia than in cGVHD patients with only transient or no thrombocytopenia. The authors concluded that observed low platelet count may be a marker for a more severe form of cGVHD. [94] A few years later

Sullivan et al studied 179 patients with extensive cGVHD, and found that those with platelets below 100,000/μL had increased mortality. [92] Another important work was published in 1989 by Anasetti et al. [86] They assessed mechanisms of persistent thrombocytopenia in 20 patients who were between 60 and 649 days (median 90) after alloHSCT; among them 17 had isolated thrombocytopenia, 10 aGVHD and 6 cGVHD. [86] Platelet survival studies demonstrated that platelets persisted in the circulation for a shorter period of time in patients with GVHD, and in all studied patients a direct relationship between platelet survival and platelet count was observed. Moreover, platelet autoantibodies were found in five of six patients with acute or chronic GVHD, and in none of six patients without GVHD. [86] The investigators concluded that persistent thrombocytopenia after HSCT is most often due to increased platelet destruction mediated by multiple mechanisms, that immune deregulation accompanying GVHD may produce autoimmune thrombocytopenia, and that increased mortality of cGVHD patients with thrombocytopenia may be result of underlying immunodeficiency and immune deregulation. [86] Akpek et al defined 3 risk factors at diagnosis of cGVHD that were significantly associated with increased non-relapse mortality: platelets less than 100,000/μL, more than 50% body surface area skin involvement and progressive type of cGVHD onset. [87] Study of Przepiorka et al validated risk stratification by platelet count in 116 alloHSCT patients. [90] Long term progression-free survival was 31% for patients without cGVHD, 51% for not thrombocytopenic cGVHD patients and just 16% for patients with cGVHD and platelets less than 100,000/μL. [90] Another large multicenter study published in 2003 with a total of 1105 cGVHD patients from 4 different cohorts showed that thrombocytopenia was uniformly associated with increased risk of mortality across all cohorts. [96] Arora et al studied 159 cGVHD patients to identify predictors of response and long-term mortality. [169] In multivariate analysis age older than 20 years, progressive onset of cGVHD, gastrointestinal tract involvement and platelets less than 100,000/μL were associated with increased mortality. [169] Pavletic et al described several independent prognostic risk factors for cGVHD incidence and severity comparing bone marrow (75 patients) and peripheral blood alloHSCT (87 patients) recipients, suggesting that stem cell source may influence not just the incidence of cGVHD but also its characteristics. [84] Negative predictive factors for survival at 3 years after cGVHD diagnosis in allo-PBSCT patients were platelets less than 100,000/μL and history of aGVHD of the liver, and only thrombocytopenia remained predictive for poor survival in allo-BMT group. [84] Another work of Arora et al published in 2007 analyzed clinical presentation and response to treatment in 170 patients with cGVHD; 123 after transplant from an unrelated

donor and 47 from umbilical cord blood. [89] In both cohorts thrombocytopenia and not achieving remission at 2 months were independently associated with increased mortality. [89]

In spite of such well known association of thrombocytopenia with negative survival of cGVHD patients, in this study low platelets were not prognostic for survival, possibly due to only 7% of patients with platelets <100 K/μL or because of long time from cGVHD diagnosis to enrollment (median 23 months). Surprisingly, higher platelet counts were associated with more active and severe cGVHD in this cohort.

Platelets play important roles in hemostasis, thrombosis, inflammation, and vascular injury, and interaction of inflammation and hemostasis is described in many different settings. Inflammation is one of the causes of reactive thrombocytosis, mediated by IL-6, a strong stimulator of platelet production. [170] Also, data suggests that platelets express an intrinsic capacity to interact with and trigger both classical and alternative pathways of complement. Under pathologic conditions, complement activation on/by platelets may contribute to thrombosis and thrombocytopenia. [171] In addition to that, platelets can contribute to pathogenesis of fibrosis as they are important source of growth factors such as TGF-β and PDGF, which stimulate fibrosis and vascular thickening. [172,173] Indeed, in this study higher platelets were associated with most severe skin (p=0.0016) and joint/fascia involvement (p=0.0001). Moreover, it was recently found that active thrombopoiesis, measured by the absolute immature platelet number in the blood, was associated with worse severity and activity of chronic GVHD, especially skin and joints/fascia manifestations, supporting hypothesis that ongoing inflammation in cGVHD stimulates increased thrombopoiesis. [174]

Eosinophilia was infrequently observed in this patient population (n=14), and did not came up significant in any analysis done in this study. Eosinophilia has been identified as a forerunner to the development of both aGVHD and cGVHD [175, 176] and some studies have further shown an association with eosinophilia and favorable outcomes following allo-HSCT [177] and lower grade cGVHD [178]. One retrospective study reported eosinophilia as a favorable prognostic factor for survival in patients with cGVHD [179] and another did not find any correlation [180,181] None of these studies used NIH criteria and eosinophilia was identified at the time of cGVHD diagnosis, which differs from this current patient population. Eosinophilia in our patient population did not correlate with any specific clinical manifestations or laboratory parameter.

Although cytokines (MCP1, IL-1RA, IL-6, and TNFRII) measured in this study on a subset of 107 patients did not have statistically significant association with GVHD outcomes their role in cGVHD is very important as reported in many studies as potential targeted therapy. For

instance, very recent study published by Zeiser et al showed impressive results in cGVHD treatment with JAK1/2 inhibitor ruxolitinib. [182] JAK1/2 signaling has been shown to be crucial in various steps leading to inflammation and tissue damage in GVHD. A critical event involved in T cell activation, lineage commitment and survival is signaling through the common gamma chain, a constituent of the receptor complexes for six different interleukins: IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. [183] Common gamma chain signaling occurs via JAK1 and we were recently able to identify the common gamma chain as a potent therapeutic target in aGVHD and cGVHD. [184] In this study ruxolitinib was given as salvage-treatment in patients suffering from steroid refractory cGVHD. Investigators observed high response rates (>80%) and 6-month survival rates, although the patients were heavily pretreated and all had moderate or severe form of cGVHD. [182]

Olivieri et al. have reported several reports of treating steroid-refractory cGVHD with imatinib that has anti-PDGFR activity. After 6 months, intention-to-treat analysis of 39 patients who received imatinib, regardless of the duration of treatment, revealed 14 partial responses (PR), 4 minor responses (MR) with relevant steroid sparing (46%) according to Couriel criteria, and 20 ≥ PR (51.3%), as per the National Institutes of Health (NIH) criteria and NIH severity score changes. Monitoring of PDGF-R antibodies showed a significant decrease in PDGF-R stimulatory activity in 7 responders, whereas it remained high in 4 nonresponders. [185]

Imatinib mesylate represents a novel targeted approachto the management of sclerotic GVHD through inhibition of specific signaling pathways implicated in skin fibrosis. Imatinib has inhibitory activity against PDGFR. Elevated PDGF and its receptor have been found in the skin and bronchoalveolar lavage fluid in patients with systemic sclerosis. [186,187]

Stimulatory PDGF receptor antibodies have been described in patients with systemic sclerosis and extensive cGVHD, suggesting a direct mechanistic link to skin fibrosis via the PDGF pathway [63] Recent pilot phase II prospective study by Baird et al showed interesting results treating steroid refractory, sclerotic cGVHD with joint involvement. Fourteen (of 20 total) patients were assessable for primary response, improvement in joint ROM deficit, at 6 months. Primary outcome criteria for partial response was met in 5 of 14 (36%), stable disease in 7 of 14 (50%), and progressive disease in 2 of 14 (14%) patients. [188]

Finally, we have clinically defined and validated by correlations with markers of tissue inflammation the definitions of cGVHD activity and severity, which could prove useful and feasible for clinical management and outcomes in trials. Of interest, distinct parameters were

associated with survival vs. disease activity. Higher WBC and ANC were associated with decreased survival, which could be a reflection of cytokines related to inflammation or a need for more systemic steroid therapy in patients with more severe cGVHD. By comparison, lower lymphocyte counts and IgG levels were also associated with decreased survival, and likely reflect higher burden of immunosuppression and more advanced cGVHD.

Prospective studies using the 2005 cGVHD Consensus criteria have shown that skin score, lung score and GI score each predict the risk of TRM. [189,190,191,192] Previous studies have identified several factors associated with worse survival such as decreased performance status, thromcocytopenia at the time of diagnosis (<100,000/mcgL), multiple organ involvement, progressive onset, hyperbilirubinemia and a higher percentage of skin involvement at the time of diagnosis. [78,193,194,31,88,92,195]

In this study factors associated with worse survival were higher white blood count (p=0.029), higher absolute neutrophil count (p=0.05), lower lymphocyte count (p=0.057) and lower IgG (p=0.033), in the univariate analysis (Figure 13.). In the Cox proportional hazards model higher absolute lymphocyte count (>0.65; p=0.017; HR=0.43 (95% CI: 0.22-0.86), higher Karnofsky performance status (>=80; p=0.0008; HR=0.33; 95 CI: 0.17-0.63) and higher FEV1 (>57; p=0.0028; HR=0.35; 95% CI: 0.18-0.70) were associated associated with better survival (Figure 14.).

Iron overload is an adverse prognostic factor in patients undergoing allogeneic stem cell transplantation for thalassaemia [196]. Serum ferritin is an indicator for iron stores and elevated levels are associated with worse outcomes following transplantation. [197] Iron overload, primarily due to multiple red blood cell transfusions, is a relatively common complication in allo-HSCT recipients. Elevated pretransplant ferritin levels have been reported to increase the risk of non-relapse mortality following HSCT and lower incidence of acute and cGVHD. [145] Iron availability influences innate and acquired immune responses. Reduced CD8+ T-cell counts have been observed in patients with iron overload. [142] In our study cGVHD patients had higher ferritin levels, compared to control group but not statistically significant, and did not correlate with intensity of immunosuppression, disease activity or NIH global severity score.

In an additional analysis, patients receiving systemic immunosuppression, compared to ones who did not, had higher values of CRP, ferritin, and ANC, likely reflecting active disease and lower values of ALC and IgG, that is probably the result of treatment (Table 13.).

We developed a prognostic model and equations prediction for active and severe disease. Using this model the equation for predicting disease activity was established. Based on this rule, 71% of patients with active disease and 79 % of those with non-active disease would be correctly classified. Also, equation for predicting disease severity was made and based on developed equation, 76% of patients with severe disease and 74% of those with moderate disease would be correctly classified (Table 11.).

This present study has several potential limitations. First, its cross-sectional design does not allow longitudinal monitoring of identified markers to see if there is an improvement in responding patients. Second, due to the nature of referrals to the NIH, the study population is enriched for severe cases of cGVHD; therefore, further investigation is needed to determine if the factors identified are applicable to patients with newly diagnosed and untreated disease. Because of the nature of a cross-sectional study and because this represents a referral population, our population was enriched for refractory or persistent cGVHD manifestations. We identified a high incidence of lung, sclerotic skin and joint/fascia involvement. In the same manner, the time from transplant in our population (approximately one-half of patients were >3 years from transplant) is not representative of all time points in the cGVHD disease course, particularly the onset of cGVHD manifestations.

Lastly, cytokines of interest were studied only in sera and in a smaller number of patients limiting the ability for more detailed investigation of biological mechanisms of inflammation in cGVHD. The strengths of the study include the large prospectively acquired cohort of patients enriched for severe cGVHD and the systematic thorough characterization of cGVHD manifestations with laboratory correlates.

In summary, we identified a number of clinical laboratory marker candidates, which could serve as surrogate measures for disease activity. The findings of associations between laboratory markers of inflammation and clinical outcomes support using the cGVHD activity defined by clinician's intention and the NIH global severity as endpoints in clinical trials and practice. We also determined that laboratory factors predictive of survival differ from those predicting cGVHD activity, suggesting that active inflammation may not necessarily adversely impact long term prognosis if the cumulative damage from the disease and its treatments could be prevented. [156] Also, these results imply that disease activity may not be used as an adequate short term surrogate endpoint for survival outcomes.

Future longitudinal studies in more diverse cGVHD patient populations, particularly in conjunction with treatment trials will be integral to understand the mechanisms of these

observed laboratory changes and how they are implicated in cGVHD. Most importantly, the findings presented here may be ultimately relevant for characterizing and monitoring cGVHD disease activity and predicting of survival that may aid in the evaluation of future treatment strategies.[111, 198]

7. Conclusions

1. Patients with cGVHD had significantly higher CRP, WBC, ANC, platelet count and lower hemoglobin, albumin and total proteins values, compared to non-cGVHD controls.

2. Patients with active disease had higher values of CRP (p=0.0001), C3 (p=0.0003), C4 (p=0.0004) and platelets (p=0.012) as well as lower levels of albumin (p=0.044).

3. These clinical laboratory markers of inflammation could serve as surrogate measures for disease activity.

4. Patients with severe NIH global score had higher values of CRP (p=0.0499), C3 (p=0.0017) and platelets (p=0.0028) compared to patients with moderate disease.

5. Multivariable analyses showed:

a) patients with active disease received more prior systemic therapies, had higher values of CRP and platelets as well as lower values of albumin compared to patients with inactive disease.

b) Patients with severe disease had higher platelet counts, received more prior systemic treatments, and had lower values of FEV 1.

c) patients receiving immunosuppression had higher values of CRP, complement total, ferritin, and absolute neutrophil count, likely reflecting active disease. Also, they had lower values of absolute lymphocyte count, IgG, IgM, IgA, total protein, hemoglobin, and AEC, that is probably due to systemic treatment.

6. The chances of cGVHD to be active is 80% if CRP>0.7 mg/dL, C3>140 mg/dL, C4>28 mg/dL, platelets>250 K/μL and albumin <3.6 g/dL.

7. In the univariate analysis higher white blood count (p=0.029), higher absolute neutrophil count (adjusted p=0.05), lower lymphocyte count (p=0.057) and lower IgG (p=0.033) were shown to be associated with decreased survival.

8. In the Cox proportional hazards model higher absolute lymphocyte count (>0.65; p=0.017; HR=0.43 (95% CI: 0.22-0.86), higher Karnofsky performance status (>= 80; p=0.0008; HR=0.33; 95 CI: 0.17-0.63), lower NIH lung score (0-2; p<0.0001; Hazard ratio=6.52; 95% CI: 3.07-13.87) and higher FEV1 (>57; p=0.0028; HR=0.35; 95% CI: 0.18-0.70) were associated associated with better survival from the day the patient went on study.

9. Clinical laboratory markers of inflammation predictive of survival differ from those predicting cGVHD activity, suggesting that active inflammation may not necessarily adversely impact long term prognosis if the cumulative damage from the disease and its treatments could be prevented.

8. Summary

Chronic graft versus host disease (cGVHD) remains the major cause of non-relapse morbidity and mortality after allogeneic hematopoietic stem cell transplantation. Currently there are no accepted measures of cGVHD activity to aid in clinical management and disease staging. We performed this study in a cohort of cGVHD patients highly enriched for those with established, severe and previously heavily treated disease. All patients were evaluated, and at a single time-point in their disease trajectory, the sera samples were well-annotated using a multidimensional battery of cGVHD descriptors. We analyzed clinical markers of inflammation in the sera of patients with established cGVHD and correlated those with definitions of disease activity. 189 adult patients with cGVHD (33% moderate and 66% severe according to NIH global scoring) were consecutively enrolled into a cross-sectional prospective cGVHD natural history study. At the time of evaluation, 80% were receiving systemic immunosuppression and failed a median of 4 prior systemic therapies for their cGVHD. This study identified a number of laboratory indicators of inflammation differing between patients with primarily established, moderate, or severe cGVHD and non-cGVHD transplanted controls, suggesting ongoing tissue inflammation in the patient cohort. We also identified several laboratory markers associated with the clinician's assessment of disease activity or severity. Lower albumin ($p<0.0001$), higher CRP (C-reactive protein; $p=0.043$), higher platelets ($p=0.030$) and higher number of PST ($p<0.0001$) were associated with active disease defined as clinician's intention to intensify or alter systemic therapy due to the lack of response. Higher platelet count ($p=0.021$) and higher number of PST ($p<0.0001$) were associated with more severe disease as defined by NIH global score. In the Cox proportional hazards model, better Karnofsky performance status (>= 80; $p=0.0008$; Hazard ratio=0.33; 95 CI: 0.17-0.63), higher FEV1 (>57; $p=0.0028$; Hazard ratio=0.35; 95% CI: 0.18-0.70) and higher absolute lymphocyte count (>0.65; $p=0.017$; Hazard ratio=0.43 (95% CI: 0.22-0.86) were associated with better survival. We developed a prognostic model and prediction equations for active and severe disease. Using this model (Table 10.), the equation for predicting disease activity was established. Based on this model, 71% of patients with active disease and 79 % of those with non-active disease would be correctly classified. Also, the equation for predicting disease severity was made and based on the developed equation, 76% of patients with severe disease and 74% of those with moderate disease would be correctly classified. This study identified common laboratory indicators of inflammation that can serve as markers of cGVHD activity and severity.

9. Sažetak

Kronična reakcija davatelja protiv primatelja (cGVHD) ostaje glavni uzrok morbiditeta koji nije povezan s relapsom i mortaliteta nakon transplantacije alogeničnih krvotvornih matičnih stanica. Trenutno ne postoje prihvaćene mjere cGVHD aktivnosti koje pomažu u kliničkom upravljanju i stupnjevanju bolesti. Istraživanje je provedeno na kohorti bolesnika s cGVHD-om koja je uključivala velik broj onih s utvrđenom bolesti, ozbiljnim stupnjem bolesti te onih kod kojih je bolest prethodno višestruko liječena. Svi su bolesnici evaluirani te su u jednoj točki tijeka bolesti uzorci seruma temeljito opisani nizom multidimenzionalnih deskriptora cGVHD-a. Analizirani su klinički markeri upale u serumima bolesnika s utvrđenim cGVHD-om i korelirali ih s definicijama aktivnosti bolesti. 189 odraslih bolesnika sa cGVHD-om (33% umjereni i 66% teški oblik prema NIH globalnom skoringu) je konsekutivno upisano u cross-sectional prospektivno istraživanje prirodnog tijeka cGVHD-a. U vrijeme evaluacije, 80% je primalo sistemsku imunosupresiju te je medijan neuspješnih prethodnih sistemskih terapija za cGVHD bio 4. Ova je studija identificirala niz laboratorijskih pokazatelja upale koji se razlikuju među bolesnicima s primarno utvrđenim, umjerenim i teškim oblikom cGVHD-a i bolesnicima koji su transplantirani no nemaju cGVHD, što sugerira aktivnu upalu tkiva u kohorti bolesnika. Također smo identificirali nekoliko laboratorijskih markera povezanih s procjenom aktivnosti ili težine bolesti koju donosi kliničar. Niži albumin ($p<0.0001$), viši CRP (C-reaktivni protein; $p=0.043$), viši trombociti ($p=0.030$) i više vrijednosti PST-a ($p<0.0001$) povezani su s aktivnom bolešću koja se definira kao namjera kliničara da intenzivira ili mijenja sistemsku terapiju zbog nedostatka odgovora. Veći broj trombocita ($p=0.021$) i veća razina PST-a ($p<0.0001$) povezani su s težim oblikom bolesti prema NIH globalnom skoringu. U Coxovom regresijskom modelu, bolji Karnofskyjevom skalom izvedbenog statusa (Karnofsky Performance Status) (>= 80; $p=0.0008$; Omjer hazarda=0.33; 95 CI: 0.17-0.63), veći FEV1 (>57; $p=0.0028$; Omjer hazarda =0.35; 95% CI: 0.18-0.70) i viša apsolutna vrijednost limfocita (>0.65; $p=0.017$; Omjer hazarda=0.43 (95% CI: 0.22-0.86) su povezani s boljim preživljenjem. Razvijen je prognostički model i jednadžbe za predikciju za aktivne i teške oblike bolesti. Koristeći se ovim modelom (Tablica 10), utvrđena je jednadžba za predviđanje aktivnosti bolesti. Na temelju tog modela, pravilno je klasificirano da 71% bolesnika ima aktivnu bolest, a 79% neaktivnu bolest. Također je izrađena i jednadžba za predviđanje težine bolesti, na temelju koje je pravilno klasificirano da 76% bolesnika ima težak oblik bolesti, a 74% umjereni oblik bolesti. Ovim su istraživanjem

identificirani laboratorijski pokazatelji upale koji mogu služiti kao markeri za aktivnost i težinu cGVHD-a.

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11. Curriculum vitae

I was born in 1980 in Zagreb. In 2004, I obtained a medical degree from University of Zagreb, School of Medicine. I was a research fellow from 2006 to 2007 on the project of the Croatian Ministry of science, "Treatment of acute leukemia with allogeneic bone marrow transplantation" under the mentorship of Prof Labar. I enrolled into postgraduate program "Biomedicine and Health", University of Zagreb, Medical School in 2004. In 2007, I started the Internal Medicine residency at UHC Zagreb. Upon completion in 2013, I started working at the Division of Hematology, UHC Zagreb. From 2010 to 2011, I worked as a research fellow at the Experimental Transplantation and Immunology Branch, NCI, NIH, under the mentorship of Prof Pavletic. Since 2013, I have been working as associate investigator in the Research Cooperability Program of Croatian Ministry of science: "Clinical and biological factors determining severity and activity of cGVHD after allo-HSCT", and I coordinate the cGVHD multidisciplinary team. I am author of 15 scientific papers published in Current Contents journals, and I have presented numerous abstracts at national and international meetings. I received the American Society of Hematology abstract achievement award two times. My main scientific interests are allo-HSCT and cGVHD.