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Clinical laboratory markers of inflammation as determinants of chronic graft-versus-host disease activity and NIH global severity

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Abstract

Chronic graft versus host disease (cGVHD) remains a 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 analyzed clinical markers of inflammation in the sera of patients with established cGVHD and correlated those with definitions of disease activity. 189 adults with cGVHD (33% moderate and 66% severe according to NIH global scoring) were consecutively enrolled onto 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 (PST) for their cGVHD. 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 diseased

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defined by NIH global score. This study identified common laboratory indicators of inflammation that can serve as markers of cGVHD activity and severity.

Keywords

chronic graft versus host disease; inflammation; activity; CRP; platelets

Introduction

Chronic graft versus host disease (cGVHD) is the leading cause of non-relapse morbidity and mortality in survivors after allogeneic hematopoietic stem cell transplantation (HSCT), but it is also associated with lower malignancy relapse rate.[1-3] Typical manifestations 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.[4] It may often appear similar to systemic autoimmune diseases such as systemic sclerosis (SSc) or Sjogren's syndome. Despite recent progress in cGVHD severity staging[5] there are no reliable clinical measures of disease activity to differentiate active inflammation from residual tissue damage in order to better guide disease monitoring and clinical decision.

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.[6-8] Acute-phase responses (APR) are systemic reactions that reflect organ site inflammation in acute and chronic diseases.[9] 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 antiinflammatory cytokines such as IL-10, as is seen in APR.[6-7,9-13] A number of acute phase reactants have well established roles in monitoring clinical outcomes for systemic inflammatory and autoimmune diseases.[9,14] 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.[15] 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.[16-17] 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.[18-19] However, the utility of markers of inflammation in the evaluation of cGVHD is not known. We hypothesize that incorporation of laboratory markers of inflammation in the assessments of cGVHD patients may convey information about disease activity and severity. Identification of patterns of inflammatory markers in patients with clinically diagnosed cGVHD could improve the clinical diagnosis and disease monitoring. The objectives of this study were to determine the associations between a set of clinical laboratory parameters of inflammation and definitions of cGVHD activity and severity in a cohort of patients enriched for severe, long-standing

and mostly heavily treated disease. We also developed a prognostic model using laboratory and clinical factors to inform clinical judgments of cGVHD activity and severity.

Patients and methods

Patient population

Between October 2004 and August 2010, 217 patients were enrolled in the NCI protocol "Natural History Study of Clinical and Biological Factors Determining Outcomes in Chronic Graft-Versus-Host Disease" (clinicaltrials.gov identifier: NCT00331968). Patients referred from outside of the NIH (National Institutes of Health; N=175) or from the NIH (N=42), 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. Clinical assessments and laboratory data were recorded at the time of the visit using the pre-defined data collection forms. Nine subjects were determined to be ineligible due to absence of diagnostic criteria for chronic GVHD.[20] Two enrollees were diagnosed with late acute GVHD without evidence of chronic GVHD. Seventeen pediatric patients were also excluded due to age limitations that did not allow us to conduct all protocol driven procedures as in the adult patients (e.g. pulmonary function tests (PFT's), Schirmer's tear test and quality of life questionnaires). The final analytic sample was comprised of 189 adult participants. Six of seventy-seven patients with elevated CRP (>0.8 mg/dL) had documented infection (positive blood cultures and acute sinusitis). 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. 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.

Measures

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.[21] 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). This scale is based on the clinician's subjective impression of cGVHD change over the past month based on patient's symptoms and overall clinical history over the previous month.[22]

Disease severity—Was defined by: a) Global NIH scoring: 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. The genital area was scored in females only[20]; 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[23]; d) using the physical component summary (PCS) scale, drawn from the SF-36 v.2, a well validated measure of self-assessed health[24]; 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[25]; g) Percentage of skin body surface area (BSA) affected by: erythema, moveable sclerotic skin manifestations, and non-moveable skin changes and fasciitis.[22]

Laboratory assessments

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.

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,[26] 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.

Results

Patient Characteristics

Median age was 48 years [18-70 years] and 48% of patients were female. 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]. 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 affected by erythema, and 23 (12%) manifested 50% BSA sclerotic changes (moveable and/or non-moveable). Clinical, demographic and cGVHD-related characteristics are summarized in Table 1.

Association between laboratory parameters and chronic GVHD

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 2). 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 sclerotic changes (moveable and non-no moveable) and NIH average scores (data not shown). Among categorical outcomes higher C4 levels were associated with lower Clinician global assessment of change, (e.g. cGVHD worsened; p=0.0011).

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 1). Univariate analyses of laboratory parameters and categorical outcomes intensity of immunosuppression, active vs. non-active disease and NIH global severity are shown in Table 3.

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 GVHD outcomes (data not shown).

Models determining chronic GVHD activity and severity

The following categorical outcomes were developed with a multivariable model: intensity of immunosuppression, active vs. non-active disease based on therapeutic intent and the 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.

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 4).

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 4). Using this model the equation for predicting disease activity was established (Table 5). 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.

To improve specificity in the initial model we developed an alternative model in which only the laboratory parameters of CRP, albumin, platelets, C3 and C4 complement were included.

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 6).

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 4). Using this model the equation for predicting disease severity was established (Table 5). 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)[20], 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

Survival

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 2). Patients with active disease had decreased survival compared to patients with non active disease (p=0.057). 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) 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. The difference between active vs. non-active disease was not significant in the multivariable analysis.

Discussion

Chronic GVHD is the most severe late effect of therapy in survivors who undergo allogeneic HSCT.[27] It affects numerous organs, often requiring a comprehensive multidisciplinary approach and prolonged immunosuppressive therapy for a median duration of 2.5-3 years.[28] 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.[29] 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.[30-31] 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.[32] 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)[22] 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.[19] Values greater than 1 mg/dL (10 mg/L) reflect clinically significant inflammation.[33-34] Values between 0.3-1mg/dL (3-10 mg/L) indicate “low grade inflammation” described in various chronic diseases.[35] 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.[17,36] The role of CRP in cGVHD is suggested by few reports.[37-38] 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.[35] 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.

C3 deposits can be found in the skin,[39] and in glomerular membranes in patients with cGVHD and nephrotic syndrome with normal C3 and C4 serum levels.[40-41] Elevated complement and complement activation by autoantibodies is one of the possible mechanism of endothelial damage and fibrosis in SSc patients.[42] 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.[9] Higher C3 levels were associated with most severe (sclerotic) changes skin ($p<0.0001$) and joint/fascia involvement ($p<0.0001$). Low platelet counts (<100 K/μL) at diagnosis are predictive for higher risk of non-relapse mortality[43-44] and thrombocytopenia in cGVHD patients is among the strongest predictors of poor survival across many studies.[45] Low platelets were not prognostic for survival in this cohort, possibly due to only 7% of patients with platelets <100 K/μL or long time from cGVHD diagnosis to enrollment (median 23 months). In contrast, higher platelet counts were associated with more active and severe disease in this cohort. Inflammation is one of the causes of reactive thrombocytosis, mediated by IL-6, a strong stimulator of platelet production.[46-47] 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.[48-49] In this study higher platelets were associated with most severe skin ($p=0.0016$) and joint/fascia involvement ($p=0.0001$).

Most of the laboratory markers of inflammation that we tested are driven by cytokines that have been shown to be over expressed in cGVHD patients.[6,9] In this study, there were no statistically significant associations between the cytokines measured and cGVHD outcomes (data not shown). This could be the result of the difference in temporal relationship between disease onset and measurement of cytokines in this study as compared to others, or cytokine predominance in the tissue rather than in the blood.[50] The median time from cGVHD diagnosis to enrollment onto our study was 23 months, while in other studies cytokine analyses were performed less than one year after transplant.[6-7]

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.

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 (Supplementary Table 1).

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. 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.[28] 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.[30,51]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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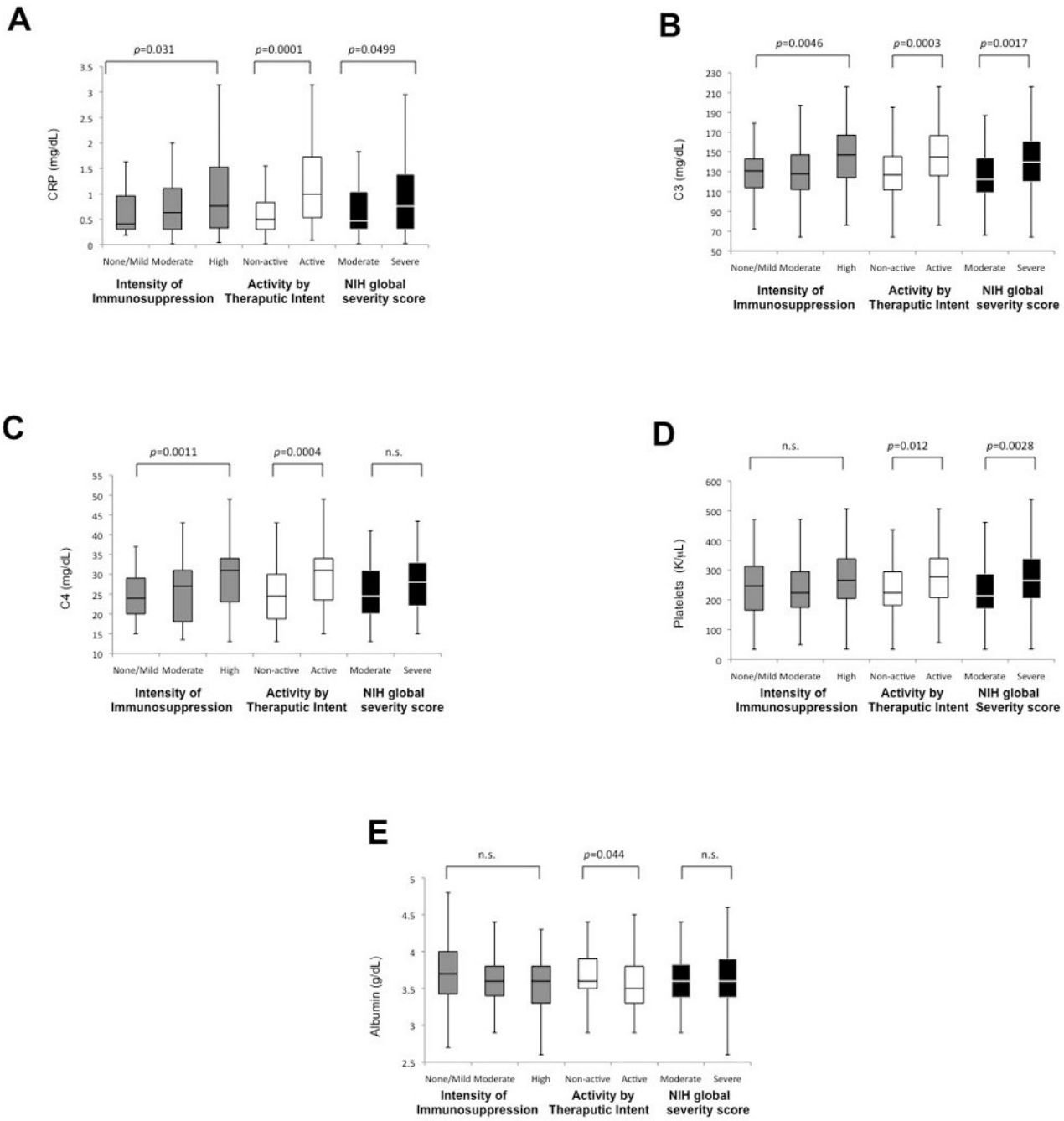

Figure 1.

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 (q1-1.5×IQR), and the 1.5IQR of the upper quartile (q3+1.5×IQR) for intensity of immunosuppression (gray), cGVHD activity (white) and cGVHD severity (black).

(A) Figure illustrates higher CRP values in patients with higher immunosuppression and in those with active and severe disease. (B) Figure illustrates higher C3 values in patients with higher immunosuppression or with active and severe disease. (C) Figure illustrates higher

C4 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.

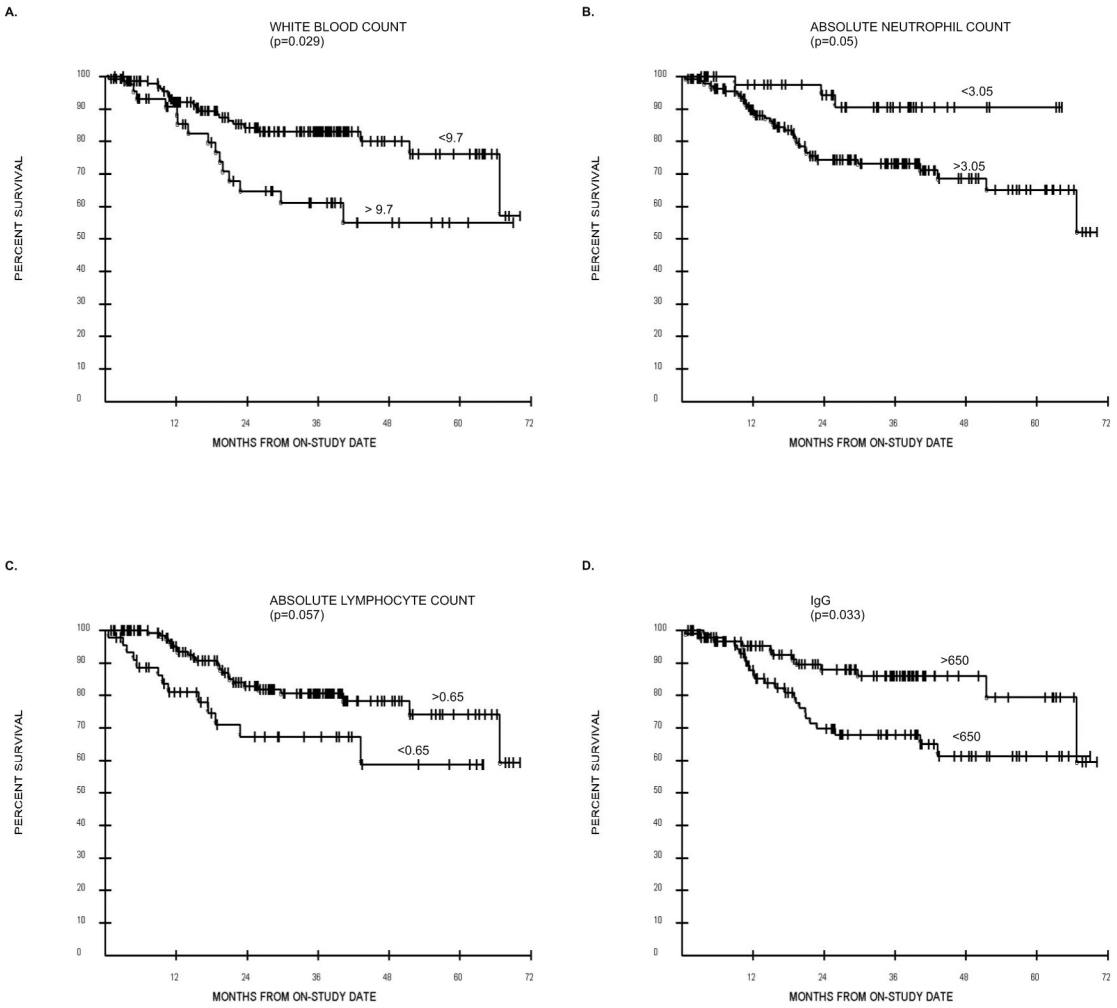

Figure 2. Survival from study enrollment according to various laboratory parameters

Table 1
Patient and cGVHD characteristics at study enrollment (N=189)

Patient Characteristics	N (%)
Age (median years, range)	48 (18-70)
Gender	
Male	99 (52)
Female	90 (48)
Disease	
AML/ALL/MDS	78 (41)
Lymphoma/CLL/MM	71 (38)
CML	30 (16)
AA/PNH	6 (3)
Non-malignant	4 (2)
Myeloablative regimen	102 (54)
Donor	
Related	130 (69)
Unrelated	59 (31)
HLA match	
Yes	156 (83)
No	29 (15)
Unknown	4 (2)
Donor Source	
BM	35 (18.5)
PB	153 (81)
Cord	1 (0.5)
cGVHD presentation	
De novo	67 (35.5)
Quiescent	41 (22)
Progressive	80 (42)
Unknown	1 (0.5)
Global NIH cGVHD score[1]	
Mild	2 (1)
Moderate	62 (33)
Severe	125 (66)
Organs involved	
Eye	156 (83)
Skin	147 (78)
Lung	144 (76)
Joints/fascia	114 (60)
Mouth	130 (69)
Liver	98 (52)
GI	82 (43)

Patient Characteristics	N (%)
GU (females)	44 (49)
Intensity of immunosuppression[2]	
None/Mild	49 (26)
Moderate	71 (37)
High	69 (37)
Activity by therapeutic intent[3]	
Active	71 (38)
Non-active	84 (44)
Unknown (other)	34 (18)
Number of prior treatments	
< 2	19 (10)
2-3	72 (38)
4-5	61 (32)
>5	35 (19)
Unknown	2 (1)
Platelet count (μL)	
<100	13 (7)
>100	176 (93)

Abbreviations: AML, acute myeloid leukemia; ALL= acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; AA, aplastic anemia; PNH, paroxysmal nocturnal hemoglobinuria; HLA, human leukocyte antigen; BM, bone marrow; PB, peripheral blood; cGVHD, chronic graft versus host disease; 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.[5]

[2]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;

[3]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; GI, gastrointestinal tract; GU, genital tract.

Table 2
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 (0.86-26.32)	2.30 (1.19-5.08)	0.0001	1.78-5.38 (K/ 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)	<0.0001	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 (CAE U)
PTH	44.3 (29-448)			16-87 (pg/mL)

Abbreviations: CRP, C reactive protein; WBC, white blood count; ANC, absolute neutrophil count; HGB, hemoglobin; TP, total protein; ALC, absolute lymphocyte count; AEC, absolute eosinophil count; ESR, erythrocyte sedimentation rate; IgG, immunoglobulin G; IgM, immunoglobulin M; IgA, immunoglobulin A; C3, Complement component 3; C4, Complement component 4; Comp total, Complement total; PTH, parathyroid hormone;

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

Table 3

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)	moderae (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 (CAEU)	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
Ig G (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
Ig M (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
Ig A (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-microglob ulin (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

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Parameter (units) median, [range]	Intensity of immunosuppression				Activity by therapeutic intent			NIH global severity stage		
	none/mild (n=49)	**moderae (n=71)**	**high (n=69)**	**p-value***	**non-active (n=84)**	**active (n=71)**	**p-value***	**moderate (n=62)**	**severe (n=125)**	**p-value***
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

Abbreviations: CRP, C reactive protein; C3, complement component 3; C4, complement component 4; Comp Total, complement total; IgG, immunoglobulin G; IgM, immunoglobulin M; IgA, immunoglobulin A; TP, total protein; HGB, hemoglobin; ALC, absolute lymphocyte count; ESR, erythrocyte sedimentation rate; WBC, white blood count; ANC, absolute neutrophil count; AEC, absolute eosinophil count; PTH, parathyroid hormone;

*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).

Table 4

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

Abbreviations: TP, total protein;

#Prior Treatments, number of prior treatments; IgA, immunoglobulin A; IgM, immunoglobulin M; CRP, C reactive protein; FEV1, forced expiratory volume in the first second.

Table 5
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

Abbreviations: cGVHD, chronic graft versus host disease; CRP, C reactive protein; FEV1, forced expiratory volume in the first second.

Table 6
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

Abbreviations: CRP, C reactive protein; C3, Complement component 3; C4, Complement component 4;

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