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Donor and Recipient Plasma Follistatin Levels are Associated with Acute Graft-versus-Host Disease in Blood and Marrow Transplant Clinical Trials Network 0402

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Abstract

Follistatin is an angiogenic factor elevated in the circulation after allogeneic hematopoietic cell transplantation (HCT). Elevations in follistatin plasma concentrations are associated with the onset of and poor survival after acute graft versus host disease (aGVHD). Using data from the Blood and Marrow Transplant Clinical Trials Network 0402 study (n=247), we sought to further quantify the longitudinal associations between plasma follistatin levels in transplant recipients, as well as baseline HCT donor follistatin levels, and allogeneic HCT outcomes. Higher recipient baseline follistatin levels were predictive of development of aGVHD (P=0.04). High donor follistatin levels were also associated with the incidence of aGVHD (P<0.01). Elevated follistatin levels on day 28 were associated with the onset of grade II–IV aGVHD prior to day 28, higher one-year non-relapse mortality, (NRM), and lower overall survival (OS). In multivariate analyses, individuals with follistatin levels >1088 pg/mL at day 28 had a four-fold increased risk for NRM (RR=4.3, 95% CI 1.9–9.9, P<0.01) and a nearly three-fold increased overall risk for mortality (RR=2.8, 95% CI 1.5–5.2, P<0.01). Given the multiple roles of follistatin in tissue inflammation and repair, and

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Conflict of Interest

The authors do not have any relevant conflicts of interest to disclose.

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the confirmation that this biomarker is predictive of important HCT outcomes, the pathobiology of these relationships need further study.

Introduction

Although hematopoietic cell transplantation (HCT) is a curative therapy for multiple life-threatening benign and malignant conditions [1], it is associated with significant morbidity and mortality [2–6]. Many studies have sought to identify biomarkers that are predictive of outcomes following allogeneic HCT [7–10]. A recent analysis of circulating angiogenic factors in individuals undergoing allogeneic HCT revealed associations between multiple angiogenic factors and clinical outcomes in individuals with acute graft versus host disease (aGVHD) [11]. Notably, plasma follistatin was elevated at the onset of aGVHD, and follistatin levels >2000 pg/mL at day 28 after initiation of systemic therapy for aGVHD was independently associated with poor survival.

The source of circulating follistatin in aGVHD is not yet known, nor is its potential role in regulating post-HCT inflammation and tissue repair. It is expressed in many tissues including endothelial cells, skeletal muscle, pituitary gland, and brain, and functions in tissue inflammation and repair. In migrating endothelial cells it is an angiogenic factor that is required for wound healing [12, 13]. It binds to and antagonizes activin-A, a member of the transforming growth factor (TGF)-β family of growth factors involved in inflammation, fibrosis, and cellular proliferation [14, 15]. Follistatin also antagonizes myostatin, a negative regulator of muscle growth and also a member of the TGF-β superfamily [16, 17]. It has been studied in the setting of inflammatory conditions such as sepsis, type 2 diabetes mellitus, polycystic ovary syndrome, and nonalcoholic fatty liver disease [18–24]. It has also been shown to promote adipogenesis in females [21]. In addition to endothelial expression, recent evidence suggests that another source of circulating follistatin is the liver, expressed in states of energy deprivation, such as exercise or fasting [25–27].

Given the previously identified associations between elevated follistatin at the onset of aGVHD and its association with subsequent survival, as well as the known roles of follistatin in angiogenesis and inflammatory processes, we sought to characterize longitudinal associations between both donor and recipient follistatin levels and allogeneic HCT outcomes. We aim with this study to identify whether follistatin measured at baseline in both the donor and the recipient, prior to any transplant related toxicities, as well as at time points following HCT can predict HCT outcomes such as GVHD and survival. Specifically, we hypothesized that patients with elevated pre-HCT follistatin levels would be at increased risk of aGVHD secondary to an increased baseline inflammatory state. We further hypothesized that given the role of follistatin in inflammation, we would observe an association between donor follistatin and subsequent development of aGVHD in the recipient.

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Methods

Subjects and Quantification of Follistatin Levels

Plasma samples and clinical data were obtained from the Blood and Marrow Transplant Clinical Trials Network (BMT CTN) 0402 study (ClinicalTrials.gov registration # NCT00406393), a phase III randomized study comparing sirolimus and tacrolimus (TAC/SIRO) vs. tacrolimus and methotrexate (TAC/MTX) as GVHD prophylaxis following myeloablative, human leukocyte antigen-matched related peripheral blood stem cell transplantation in individuals with hematologic malignancies. Clinical and demographic data were collected prospectively for HCT donors and recipients. Plasma samples were collected from donors (n=84) prior to granulocyte colony stimulating factor administration. Recipient samples were obtained prior to the preparative regimen (n=213), and then at day 28 (n=222) and day 100 (n=206) post-HCT. We measured circulating follistatin levels from cryopreserved plasma samples using MILLIPLEX (Millipore, Billerica, MA) magnetic bead array, which identifies follistatin isoforms 288aa, 300aa and 315aa, and performed all analyses in duplicate.

All subjects provided informed consent for inclusion in the BMT CTN 0402 study. The study protocol was approved by applicable institutional review boards at participating centers.

Statistical Analysis

Statistical comparisons of follistatin levels at different time points and between donors and recipients were completed using Wilcoxon rank sum tests due to the data not being normally distributed. Differences in continuous variables across categories were calculated using the Kruskal-Wallis rank-sum test. Cox regression was used to determine the independent effects of follistatin levels and clinical factors on non-relapse mortality (NRM), GVHD-free, relapse-free survival (GRFS), and overall survival (OS). Clinical factors considered in regression models were: recipient age and sex, disease, conditioning, year of transplant, GVHD prophylaxis, CMV serostatus and time-dependent onset of GVHD. Correlations between continuous variables were determined by Spearman's correlation coefficient. Exploratory analyses to determine the optimal cutpoint for plasma concentrations of follistatin associated with the development of grade II–IV aGVHD (>436 pg/mL at pre-HCT baseline), NRM (>1088 pg/mL at day 28), and OS (>1073 pg/mL at day 28), were determined by recursive partitioning [28]. Plasma concentrations above those cutpoints are referred to as "high" follistatin levels with respect those clinical outcomes throughout the manuscript. Since the follistatin plasma concentration associated with NRM and OS were similar, 1088 pg/mL was selected as the optimal cutpoint for regression analyses. All analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC). All reported p-values are 2-sided.

Results

Patient and transplant characteristics are presented in Table 1. Of the 247 recipients included in the study, the median age at the time of transplant was 44 years and 51% were male. The

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most common diagnoses were acute myeloid leukemia (AML) (45%) and acute lymphoblastic leukemia (ALL) (40%).

Donor and Recipient Characteristics

Donor and recipient characteristics did not correlate with baseline follistatin levels. Specifically, donor and recipient age (P=0.73 and 0.99, respectively) and sex (P=0.76 and 0.36, respectively) did not show a significant association with baseline follistatin levels. Recipient body mass index (BMI) (P=0.26), diabetes (P=0.43), smoking (P=0.51), hematologic disease (P=0.18) and CMV serostatus (P=0.75) were not significantly associated with follistatin levels. Detailed donor characteristics, including BMI, diabetes, and smoking, were not available for analysis.

Recipient Follistatin and aGVHD

Individuals developing grade II–IV aGVHD had nearly 50% higher baseline pre-HCT median follistatin levels (625 pg/ml, n=69) compared with those not developing aGVHD (420 pg/ml, n=144, P=0.04, Table 2, Figure 1). After adjusting for age at transplantation, individuals with pre-HCT baseline levels >436 pg/mL had a significantly increased risk of day 100 aGVHD (RR=1.8, 95% CI 1.1–3.0, =0.03, Figure 2A). Patients who developed grade II–IV aGVHD prior to day 28 post-HCT had 50% higher follistatin levels at the day 28 blood draw (749 vs. 500 pg/mL in patients with grade 0–I aGVHD, P=0.04). At day 100, follistatin levels were greater in those with prior aGVHD (693 pg/mL vs. 431 pg/mL, P<0.01). Organ-specific aGVHD data were not available for this study. There was no difference in baseline follistatin levels between individuals receiving TAC/SIRO compared to TAC/MTX (P=0.2). Day 28 follistatin levels were modestly, albeit significantly greater in the TAC/SIRO group compared with TAC/MTX (607 pg/mL vs. 494 pg/mL, P=0.02), but the groups were not significantly different at day 100 (P=0.5), nor were differences seen in multivariable analysis.

Donor Follistatin and aGVHD

Donor follistatin levels were over 2-fold higher than baseline levels in HCT recipients (median 1010 vs. 470 pg/ml, P<0.01, Figure 1, Table 2). Higher donor follistatin levels were associated with development of grade II–IV aGVHD compared with those with no aGVHD (1281 pg/mL [IQR 818–1267 pg/mL] vs. 1,049 pg/mL [IQR 992–1431 pg/mL], P<0.01). Cumulative incidence of GVHD was significantly higher among recipients when the donor follistatin level was above the optimal cutpoint of 1088 pg/ml (P<0.01, Figure 2B). Donor levels were also moderately correlated with day 28 follistatin levels in recipients (Spearman's rho=0.3, P=0.01).

Follistatin and NRM

In multivariable analysis, the risk of one-year NRM was over 4-fold higher among individuals with high day 28 follistatin levels >1088 pg/mL (n=32, 31%, 95% CI 15–47%, RR = 4.3, 95% CI 1.9–9.9, P<0.01) compared with those with lower levels (n=189, 8%, 95% CI 4–12%) (Table 3, PFigure 3A). Baseline, day 100, and donor follistatin levels were not associated with NRM (not shown); however, for every 1000 pg/mL increase in follistatin

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from baseline to day 28, the risk for NRM significantly increased (RR = 2.5, 95% CI 1.5–4.1, <0.01).

Follistatin and OS

Significantly poorer survival was observed among individuals with high day 28 follistatin >1088 pg/mL (56%, vs 81%, RR = 2.8, 95% CI 1.5–5.2, *P*<0.01) (Table 3). Baseline, day 100, and donor follistatin levels were not associated with OS (not shown), but as was observed for NRM, for every 1000 pg/mL increase in follistatin from baseline to day 28, the risk for mortality increased (RR = 1.7, 95% CI 1.2–2.5, *P*<0.01). We did not identify a significant association between follistatin levels and GRFS, chronic GVHD or relapse-related mortality for donor levels or at any of the recipient time points (not shown). Causes of death did not differ significantly between individuals with day 28 follistatin levels >1088 pg/mL vs. lower levels (Table 4). For individuals with day 28 levels >1088 pg/mL, 17% of deaths were from veno-occlusive disease (VOD) and 17% were from respiratory failure, whereas among those with levels ≤ 1088 pg/mL, 3% of deaths were from VOD and 3% were from respiratory failure, neither of which reached statistical significance.

From our previous study [11], follistatin levels of >2000 pg/mL had the strongest association with poor survival, thus regression analyses for one-year NRM and OS were also performed using levels >2000 pg/mL. At day 28, individuals with follistatin >2000 pg/mL (n=9) had a significantly higher risk of NRM (RR=6.1, 95% CI 2.1–18.1, *P*<0.01), and lower OS, although the latter did not meet statistical significance possibly due to few patients in this cohort with such marked elevations in follistatin (RR=2.6, 95% CI 0.9–7.4, *P*=0.08).

Discussion

This analysis adds to the previously demonstrated associations between elevated follistatin with the onset of aGVHD and with OS [11], and now demonstrates a potential predictive value of both donor and baseline recipient follistatin levels prior to the onset of aGVHD, as well as the association of follistatin with prognosis after allogeneic HCT based on measurements taken from pre-HCT baseline, day 28, and day 100 post-HCT. Recipients with higher baseline follistatin levels were more likely to experience aGVHD, potentially due to increased susceptibility secondary to tissue damage from previous therapies, leading to aGVHD or NRM. Follistatin can acutely enhance neovascularization following tissue damage as a part of normal tissue repair processes, but typically decreases after 5 days, as repair processes progress [29]; however, the regulation of follistatin levels and neovascularization after allogeneic HCT is unknown.

We also demonstrated in this BMT CTN 0402 cohort that donor follistatin levels were significantly higher than recipients. This finding leads to additional questions about the function of follistatin in healthy individuals. Follistatin has been shown to be greater among males, likely in part because of increased lean body mass, compared with females [30], and although a greater proportion of the donors were male (61%) compared with transplant recipients (51%), we did not observe significant differences in follistatin levels based on donor or recipient sex. Increased levels in donors could be explained by multiple factors, including greater muscle mass[30], obesity [21], or insulin resistance [31]; unfortunately, those

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data are not available for this donor population. Plasma follistatin levels in this cohort of donors is similar to levels described using multiplex cytokine array methodology for healthy, non-diabetic individuals (1366 ± 153 pg/mL), with reported levels in type 2 diabetics significantly higher (2159 ± 327 pg/mL) [32]. Further study of donor levels is warranted to confirm our findings.

Additional potential explanations for the difference in follistatin levels between donors and recipients may be related to the previous treatments and exposures experienced by the HCT recipients. It may be that cells responsible for follistatin production are reduced in response to cytoreductive therapies. Among recipients developing GVHD, follistatin levels were still far below donors, despite the association between higher follistatin levels and GVHD. The explanation for this is not clear. It is possible that differences may be due, in part, to differences in follistatin isoform expression in donors versus recipients, or other differences yet to be determined.

In this cohort, elevated day 28 follistatin levels were observed in patients who developed aGVHD prior to that time point. In BMT CTN 0402, blood draws were calendar-driven, not event-driven (as in BMT CTN 0302/0802), which makes broader conclusions regarding follistatin and the onset of aGVHD challenging. Given the multiple other pro-inflammatory processes that can occur after HCT, such as infection, veno-occlusive disease and thrombotic microangiopathy, it is possible that the effects of follistatin on aGVHD risk beyond day 28 were obscured because of other transplant-related complications.

The observed increased risk for NRM and decreased OS associated with follistatin levels support the potential role of follistatin in aGVHD, although GRFS did not differ based on follistatin levels at any time point. NRM and OS findings could also be related to sepsis and acute inflammation, which may precede death. Follistatin levels increased over time in allogeneic HCT recipients with aGVHD, likely reflecting ongoing endothelial damage prior to day 100 after HCT.

Although the findings from this study further the understanding of the role of follistatin in aGVHD and HCT outcomes, our study has limitations. It lacks detailed donor characteristics, such as BMI, chronic or acute illness data, smoking status and current medications, which may contribute to our understanding of elevations in donor follistatin levels. Similarly, the lack of detailed clinical data on recipients is a significant limitation; we have only limited information on recipients and what other transplant-related complications or illnesses they may have experienced at the time of follistatin measures. As well, the present study population was young and received TBI-based myeloablative conditioning with PBSC grafts, so findings cannot necessarily be extended to other HCT populations. However, despite these limitations, we have demonstrated new relationships between baseline donor and recipient follistatin and aGVHD, as well as with day 28 follistatin levels and aGVHD, OS and NRM. These findings support our present hypothesis that follistatin is involved in tissue damage and inflammation and is contributing to these adverse HCT outcomes.

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In conclusion, we have identified an association between baseline donor and recipient circulating plasma follistatin levels with the subsequent development of aGVHD, and we have further shown that day 28 follistatin is increased among individuals experiencing early aGVHD and mortality after allogeneic HCT. Whereas our previous work successfully demonstrated the utility of follistatin as a marker for aGVHD treatment response and survival [11], this study demonstrates its utility as a prospective biomarker for aGVHD risk. Our findings are complementary to previous work examining biomarkers in GVHD [33] and support the use of biomarkers to predict onset and treatment response of certain post-HCT complications. Given the multiple roles of follistatin in angiogenesis, inflammation, and metabolism, it remains unclear whether elevated follistatin levels are in response to ongoing tissue damage and inflammation, or whether follistatin is antagonizing other angiogenic or inflammatory factors that are perpetuating tissue injury; however, our findings of the relationships between both donor and baseline recipient follistatin levels and aGVHD are supportive of a causative role in inflammation and tissue damage. Future clinical studies aimed at elucidating the role of follistatin would require event-based measures in the recipients and more comprehensive clinical data for donors and recipients. Mechanistic studies designed to understand the longitudinal role of circulating follistatin following HCT, differences between elevated donor and recipient follistatin levels, as well as the other related pro- and anti-angiogenic factors that may be contributing to aGVHD and post-HCT survival, are warranted in order to develop novel strategies that mitigate the risk of NRM in allogeneic HCT. Follistatin, and other factors, have the potential to be used clinically in risk prediction models to most successfully identify patients at highest risk for developing aGVHD and to predict those who are most likely to fail initial therapy or require more aggressive treatment.

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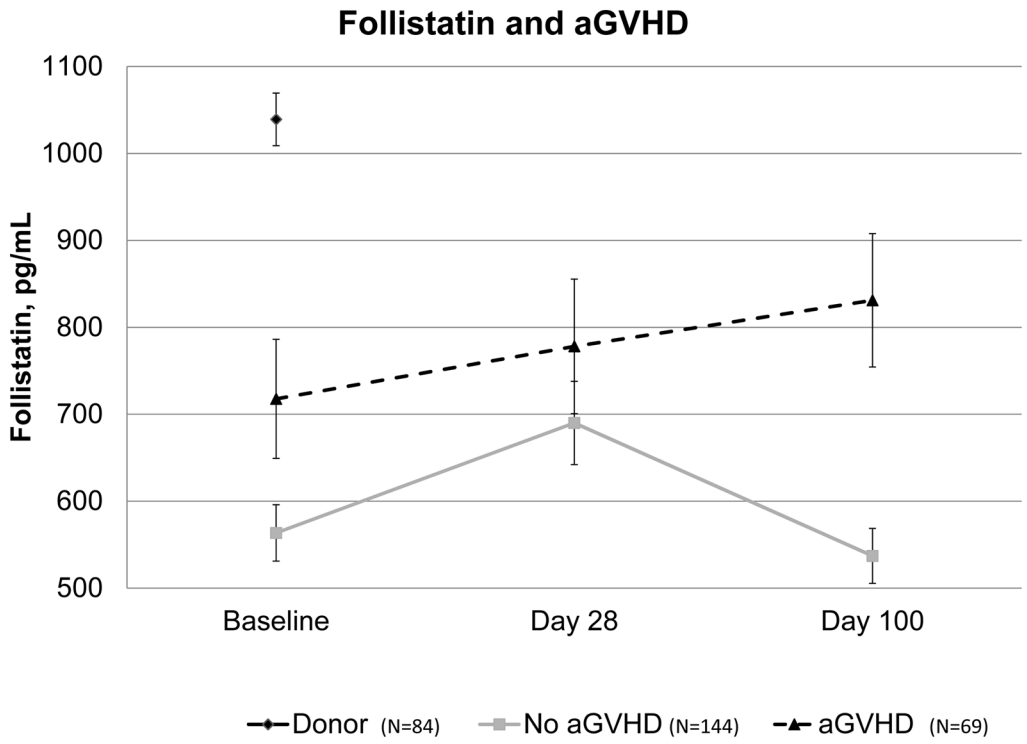

Figure 1.

Mean (±SEM) follistatin levels for donors and recipients at baseline (pre-HCT), and days 28 and 100 post-HCT, based on acute GVHD status. Black diamond represents donor baseline level (N=84), black triangle with hashed black line is recipients who developed acute GVHD (N=69), gray square with solid gray line is recipients who did not develop acute GVHD (N=144). Abbreviations: GVHD, graft versus host disease; HCT, hematopoietic cell transplantation; SEM, standard error of the mean.

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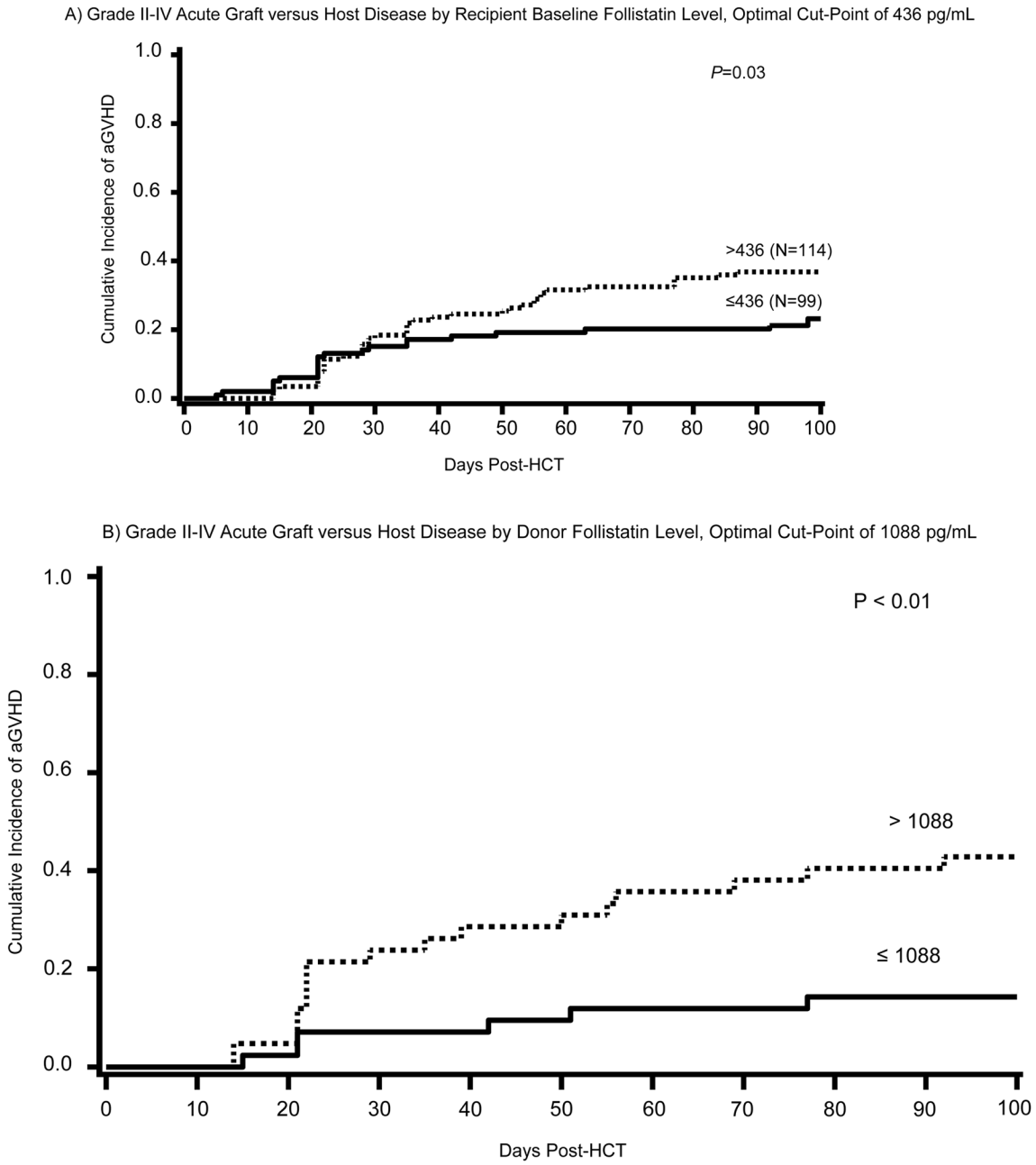

Figure 2.

Cumulative incidence of grade II–IV acute graft versus host disease, A) stratified by pre-HCT recipient baseline follistatin optimal cut-point, B) stratified by donor follistatin optimal cutpoint. Hashed line is follistatin level above the optimal cutpoint and the solid line is follistatin level less than or equal to the optimal cutpoint. Abbreviations: aGVHD, acute graft versus host disease; HCT, hematopoietic cell transplantation.

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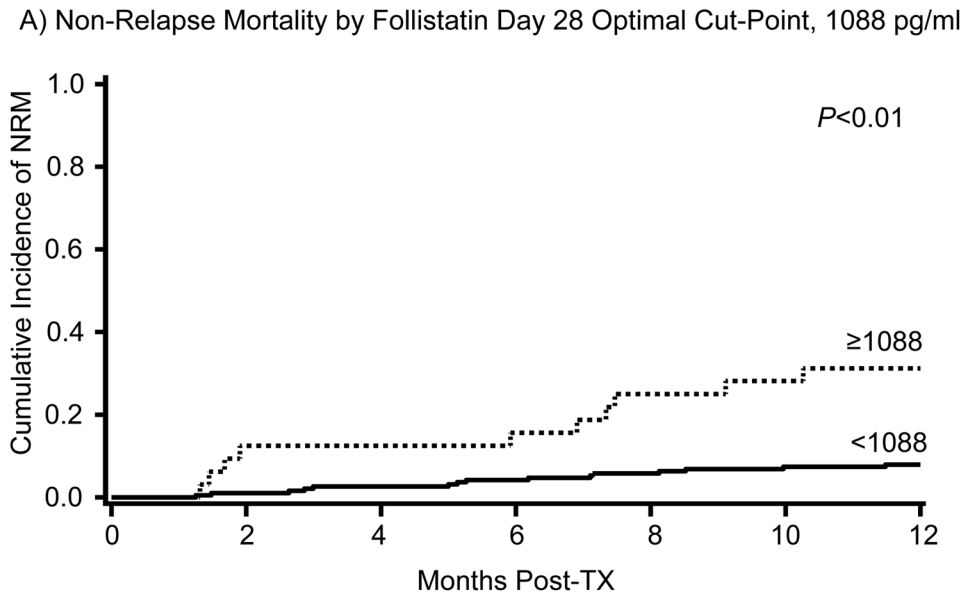

Figure 3.
Cumulative incidence of non-relapse mortality, stratified by day 28 optimal cut-point (1088 pg/mL).

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Table 1

Patient and transplant characteristics from BMT CTN 0402, with follistatin data from pre-HCT, or days 28 or 100 post-HCT.

Variable	Total Study Group
N	247
Patient Age (years)	
Median (range), (IQR)	44 (12–59), (35–50)
Donor Age (years)	
Median (range), (IQR)	44 (13–66), (34–50)
HLA-Matched PBSC Related Donor	247 (100%)
GVHD Prophylaxis	
TAC/MTX	126 (51%)
TAC/SIRO	121 (49%)
Patient Sex: Female	121 (49%)
Donor Sex: Female	97 (39%)
Disease	
ALL	98 (40%)
ABL	1 (0%)
AML	112 (45%)
CML	18 (7%)
MDS	18 (7%)
Disease Stage	
CR1	177 (72%)
CR2	34 (14%)
AP	3 (1%)
CP	15 (6%)
Not applicable *	18 (8%)
Conditioning	
Cyclophosphamide/TBI	198 (80%)
Etoposide/TBI	49 (20%)
CMV Serostatus: Positive	157 (64%)
Days to Onset of aGVHD in Patients with Day 28 Follistatin Data, Median (range), (IQR)	28.5 (5–227), (21–45.5)

* Patients not previously treated with chemotherapy.

Abbreviations: ABL, acute biphenotypic leukemia; aGVHD, acute graft versus host disease; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; AP, accelerated phase; CML, chronic myeloid leukemia; CMML, chronic myelomonocytic leukemia; CP, chronic phase; CMV, cytomegalovirus; CR, complete remission; HCT, hematopoietic cell transplantation; HLA, human leukocyte antigen; IQR, intra-quartile range; MDS, myelodysplastic syndrome; MTX, methotrexate; SIRO, sirolimus; TAC, tacrolimus; TBI, total body irradiation.

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Table 2

Baseline follistatin levels in individuals with no GVHD compared to those with grade II–IV GVHD.

Baseline Follistatin Levels			
	Donor	**No GVHD**	**Grade II–IV GVHD**
Median	1083	420	625
Mean	1084	564	718
IQR	843–1309	299–774	337–925

Abbreviations: GVHD, graft versus host disease; IQR, interquartile range.

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Table 3

Regression models for one-year non-relapse mortality and overall survival, based on day 28 follistatin levels.

		NRM				OS			
Factor	**N**	**1-Year Estimate (95% CI)**	***P***	**RR (95% CI)**	***P***	**1-Year Estimate (95% CI)**	***P***	**RR (95% CI)**	***P***
Follistatin			<0.01				<0.01		
≤1088	189	8% (4–12%)		1.0		81% (75–86%)		1.0	
>1088	32	31% (15–47%)		4.3 (1.9–9.9)	<0.01	56% (38–71%)		2.8 (1.5–5.2)	<0.01
Recipient Age			0.02				0.05		
<35	53	2% (0–6%)		1.0		89% (81–97%)		1.0	
35–49	120	13% (7–18%)		6.2 (0.9–43.8)	0.07	77% (70–84%)		2.0 (0.8–5.0)	0.11
≥50	48	19% (8–30%)		10.5 (1.4–76.5)	0.02	69% (56–82%)		3.1 (1.2–8.0)	0.02

Other factors considered in model: GVHD prophylaxis, conditioning, recipient sex, recipient/donor sex match, disease, CMV serostatus, year of transplant, time-dependent onset of GVHD

Abbreviations: GVHD, graft versus host disease; NRM, non-relapse mortality; OS, overall survival; RR, relative risk.

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Table 4

Causes of death based on day 28 follistatin levels.

	Day 28 follistatin ≤ 1088 pg/mL	Day 28 follistatin > 1088 pg/mL
Cause of Death	**N=60 deaths (%)**	**N=18 deaths (%)**
Acute GVHD	8 (13)	2 (11)
Chronic GVHD	9 (15)	3 (17)
Liver failure/VOD	2 (3)	3 (17)
Respiratory failure/ARDS	2 (3)	3 (17)
Infection	1 (2)	1 (6)
Relapse	37 (62)	6 (33)
Other	1 (2)	0

Abbreviations: ARDS, acute respiratory distress syndrome; GVHD, graft versus host disease; VOD, veno-occlusive disease.