SUPPLEMENTARY APPENDIX

Pre-transplant testosterone and outcome of men after allogeneic stem cell transplantation

Aleksandar Radujkovic,[1] Lambros Kordelas,[2] Julia Krzykalla,[3] Axel Benner,[3] David Schult,[1] Joshua Majer-Lauterbach,[1] Dietrich W. Beelen,[2] Carsten Müller-Tidow,[1] Christian Kasperk,[4] Peter Dreger[1] and Thomas Luft[1]

[1]Department of Internal Medicine V, University of Heidelberg, Heidelberg; [2]Department of Bone Marrow Transplantation, University Hospital, Essen; [3]Division of Biostatistics, German Cancer Research Center, Heidelberg and [4]Department of Internal Medicine I, University of Heidelberg, Heidelberg, Germany

 doi:10.3324/haematol.2019.220293

Received: February 23, 2019.

Accepted: July 10, 2019.

Pre-published: July 11, 2019.

Correspondence: *THOMAS LUFT* - thomas.luft@med.uni-heidelberg.de

SUPPLEMENTARY INFORMATION

METHODS

Assessment of total testosterone serum levels
Total testosterone serum levels were quantified using an in-house ^{3}H radioimmunoassay (RIA) after extraction and chromatographic separation. Briefly, testosterone was extracted from 500 µL serum with isooctane/dichloromethane (2:1 v/v) followed by stepwise polarity-based chromatography on celite mini-columns (750 mg) using a solvent gradient of ethyl acetate in isooctane and propylene glycol as stationary phase. The RIA was performed using a highly specific antibody (clone 53/1/pool), activated dextran-coated charcoal count separation and an assay-specific standard curve. Recovery was at least 60% and the lower limit of detection was 1 pg per 100 µL elate (corresponding to 2 ng testosterone per 100 mL of serum). Inter- and intra-assay variations were <15% and <7%, respectively. Results were calculated applying the RIACalc software (Pharmacia, Wallac, Finland). The measurements were carried out using accredited laboratory methods (certified according to ISO 15189 by Germany's national accreditation body).
For pre-transplant serum levels of suppressor of tumorigenicity-2 (ST2) a commercial ELISA kit was applied (DuoSet, R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions and as described previously.[1]

Transplantation procedure, GVHD prophylaxis and supportive care
Allogeneic stem cell transplantation (alloSCT) was performed according to local standard operating procedures. Graft-*versus*-host disease (GVHD) prophylaxis and supportive care were performed as previously described.[2] Acute GVHD was clinically and histologically diagnosed and graded using standard criteria and as reported previously.[3]

Supplemental statistical methods
Categorical data of patient characteristics between cohorts were compared using the $\chi 2$ test. Continuous variables were compared applying the Mann-Whitney *U* test and the Kruskal-Wallis test. The relationship between pre-transplant testosterone serum levels and BMI and CRP, an ST2 MIG was assessed by Spearman's rank correlation coefficients.
Distributions of survival times were estimated by using the method of Kaplan and Meier. The cumulative incidences of acute GVHD grade 3-4 were calculated with death without acute GVHD grade 3-4 as competing event. The effect of testosterone on the hazard of acute GVHD grade 3-4 was investigated using cause-specific hazards models. Follow-up times were calculated by the reverse Kaplan-Meier estimate.[4]
Since acute GVHD and its treatment are major contributors to post-transplant mortality, outcome was also assessed after acute GVHD (i.e. from the date of onset of acute GVHD).
For pre-transplant testosterone as continuous variable, "full models" with the covariates disease stage, conditioning intensity, patient age, donor type, recipient/donor sex match and stem cell source were fitted for the endpoints overall survival (OS) and progression-free survival (PFS). In the models with the endpoints non-relapse mortality (NRM), time to relapse, OS and PFS after acute GVHD covariates were restricted to disease stage and patient age due to the smaller number of events in the training cohort ("slim models"). In addition, in the training cohort, pre-transplant testosterone was also analyzed in multivariable models with the endpoints OS and PFS including patient age, levels of C-reactive protein (CRP), body-mass index (BMI), Karnofsky performance status (KPS) and specific comorbidities as confounding variables. The major findings were replicated in an independent cohort of patients allografted for acute myeloid leukemia (AML) at the University Hospital Essen (confirmation cohort, n=168).
Next, for illustration of the testosterone effect on all endpoints and in order to provide guidance for possible interventions in the setting of alloSCT, an optimal cut-off determination with respect to OS according to the method of Lausen and Schumacher[5] was performed in the training set. Finally, this optimal pre-transplant testosterone cut-off value was tested in multivariable models in the confirmation cohort.
Calculations were done using the statistical software environment R, version 3.4.1, together with the R packages 'survival', version 2.41-3, 'maxstat', version 0.7-25, 'prodlim', version 1.6.1, and 'riskRegression', version 1.4.3. HR and CHR were estimated with 95% confidence interval (CI). All statistical tests were two-sided. P-values of $P < 0.05$ were considered statistically significant.

RESULTS

Correlations of pre-transplant testosterone serum levels with additional patient characteristics in the training cohort

Pre-transplant testosterone serum levels were correlated to additional patient characteristics including: body-mass index (BMI), levels of C-reactive protein (CRP), Karnofsky performance status (KPS), and hematopoietic cell transplantation-specific comorbidity index (HCT-CI)[6] and specific comorbidities prior to alloSCT (presence of diabetes, cardiovascular disease and infection) as defined by Sorror et al.[6] Information on BMI, CRP, KPS, HCT-CI and information on specific comorbidities were only available for the training cohort (**Suppl. Table 4**). CRP levels were collected retrospectively by chart review to match the date of assessment of pre-transplant testosterone levels.

Pre-transplant testosterone levels were lower in obese patients of the training cohort (BMI ≥30 kg/m^2, *P*=0.028) (**Suppl. Figure 2A**). There was a trend towards lower pre-transplant testosterone levels in patients with lower Karnofsky performance status (KPS ≤80%), whereas testosterone levels were similar between the low hematopoietic cell transplantation-specific comorbidity index group (HCT-CI 0), intermediate (HCT-CI 1 to 2) and high risk (HCT-CI 3 or more) HCT-CI groups (**Suppl. Figure 2C** and **D**). No significant associations were found for CRP and presence of cardiovascular disease, diabetes or infection prior to alloSCT (**Suppl. Figure 2B, E-G**).

REFERNCES

1. Luft T, Benner A, Jodele S, et al. EASIX in patients with acute graft-versus-host disease: a retrospective cohort analysis. Lancet Haematol. 2017;4(9):e414-e423.

2. Radujkovic A, Kordelas L, Krzykalla J, et al. Pretransplant Vitamin D Deficiency Is Associated With Higher Relapse Rates in Patients Allografted for Myeloid Malignancies. J Clin Oncol. 2017;35(27):3143-3152.

3. Luft T, Dietrich S, Falk C, et al. Steroid-refractory GVHD: T-cell attack within a vulnerable endothelial system. Blood. 2011;118(6):1685-1692.

4. Schemper M, Smith TL. A note on quantifying follow-up in studies of failure time. Control Clin Trials. 1996;17(4):343-346.

5. Lausen B, Schumacher M. Maximally selected rank statistics. Biometrics 1992;48:73-85.

6. Sorror ML, Maris MB, Storb R, et al. Hematopoietic cell transplantation (HCT)-specific comorbidity index: a new tool for risk assessment before allogeneic HCT. Blood 2005; 106(8):2912-2919.

7. Gratwohl A, Stern M, Brand R, et al. Risk score for outcome after allogeneic hematopoietic stem cell transplantation: a retrospective analysis. Cancer. 2009;115(20):4715-4726.

8. Bacigalupo A, Ballen K, Rizzo D, et al. Defining the intensity of conditioning regimens: working definitions. Biol Blood Marrow Transplant. 2009;15(12):1628-1633.

9. Bornhäuser M, Kienast J, Trenschel R, et al. Reduced-intensity conditioning versus standard conditioning before allogeneic haemopoietic cell transplantation in patients with acute myeloid leukaemia in first complete remission: a prospective, open-label randomised phase 3 trial. Lancet Oncol. 2012;13(10):1035-1044.

Suppl. Table 1. Patient, disease and transplant characteristics of male patients of the discovery cohort and by diagnosis of AML.

	Discovery cohort (n=346)	Patients with non-AML (n=170)	Patients with AML (n=176)	*P*
Parameter				
Age [years] at alloSCT (median, IQR)	56 (48-63)	55 (47-60)	59 (50-64)	0.131
Disease, n (%)				<0.001
AML	176 (51)	0 (0)	176 (100)	
MDS, MPN	67 (19)	67 (39)	0 (0)	
Lymphoid disease[a]	75 (22)	75 (44)	0 (0)	
Multiple myeloma	28 (8)	28 (16)	0 (0)	
Disease stage before alloSCT[b], n (%)				0.004
Early	116 (34)	42 (26)	74 (42)	
Intermediate	78 (23)	46 (28)	32 (18)	
Late	143 (42)	74 (46)	69 (39)	
NA	9	8	1	
Conditioning[c], n (%)				<0.001
RIC	258 (75)	144 (85)	114 (65)	
MAC	88 (25)	26 (15)	62 (35)	
Stem cell source, n (%)				0.189
PB	324 (94)	156 (92)	168 (96)	
BM	22 (6)	14 (8)	8 (4)	
Donor, n (%)				0.271
Related	90 (26)	49 (29)	41 (23)	
Unrelated	256 (74)	121 (71)	135 (77)	
Recipient – donor sex match, n (%)				0.165
Matched	237 (69)	110 (65)	127 (72)	
Male – female	109 (31)	60 (35)	49 (28)	
Pre-transplant testosterone, ng/dL (median, IQR)	400 (269-584)	388 (279-533)	423 (256-611)	<0.001

Abbreviations: AlloSCT, allogeneic stem cell transplantation; AML, acute myeloid leukemia; BM, bone marrow; IQR, interquartile range; MAC, myeloablative conditioning; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm; NA, not available or not assessable; PB, peripheral blood; RIC, reduced intensity conditioning.

[a]Acute lymphoblastic leukemia, chronic lymphocytic leukemia, and lymphoma.

[b]According to Gratwohl et al.[7]

[c]According to Bacigalupo et al[8] and Bornhäuser et al.[9]

Suppl. Table 2. Multivariable analysis of the discovery cohort with the endpoints overall survival (OS), non-relapse mortality (NRM), and relapse following allogeneic stem cell transplantation – interaction between pre-transplant testosterone and the diagnosis AML (complete case analysis, n=337).

	OS		NRM		Relapse	
	HR 95%CI	***P***	**CHR 95%CI**	***P***	**CHR 95%CI**	***P***
Covariate						
Testosterone (per 100 ng/dL decrease)		0.078 (0.142)*		0.043 (0.047)*		0.305 (0.669)*
No AML	1.00 (0.90 -1.12)		0.98 (0.84 -1.15)		1.04 (0.90 -1.20)	
AML	1.12 (1.02 - 1.23)		1.24 (1.05 -1.46)		1.08 (0.97 -1.21)	
Diagnosis AML		0.026 (0.142)*		0.099 (0.047)*		0.026 (0.669)*
Testosterone† 250 ng/dL	1.76 (1.17 - 2.65)		1.75 (0.95 - 3.23)		1.84 (1.12 - 3.02)	
Testosterone† 500 ng/dL	1.34 (0.94 -1.92)		0.99 (0.57 - 1.71)		1.67 (1.09 - 2.57)	
Diagnosis AML						
No	Ref		Ref		Ref	
Yes	2.31 (1.16-4.60)	0.017	3.12 (1.10-8.82)	0.032	2.03 (0.88-1.11)	0.098
Disease stage‡						
Early	Ref		Ref		Ref	
Intermediate	1.58 (1.01-2.47)	0.047	1.30 (0.69-2.45)	0.421	1.54 (0.89-2.65)	0.122
Late	2.01 (1.34-3.03)	0.0008	1.46 (0.81-2.63)	0.204	2.20 (1.35-3.58)	0.002
Age at transplant (per 1 year)	1.01 (1.00-1.03)	0.062	1.03 (1.00-1.05)	0.059	1.01 (0.99-1.02)	0.435
Conditioning*						
MAC	Ref		Ref		Ref	
RIC	0.75 (0.52-1.11)	0.150	0.79 (0.43-1.43)	0.433	0.76 (0.48-1.20)	0.235
Donor						
Related donor	Ref		Ref		Ref	
Unrelated Donor	1.10 (0.77-1.58)	0.589	0.92 (0.52-1.62)	0.775	1.35 (0.89-2.06)	0.157
Recipient - donor sex match						
Matched	Ref		Ref		Ref	
Male recipient /female donor	1.00 (0.71-1.40)	0.990	1.18 (0.72-1.95)	0.507	0.94 (0.63-1.42)	0.778
Donor source						
PB	Ref		Ref		Ref	
BM	1.35 (0.72-2.54)	0.343	1.11 (0.44-2.83)	0.824	1.33 (0.61-2.89)	0.478

Number of events: OS, n=168; NRM, n=75; Relapse, n=117.

* Factor + interaction (interaction); † Levels of pre-transplant testosterone were chosen to reflect the first and third quartile of both cohorts. ‡ According to Gratwohl et al.[7] *According to Bacigalupo et al[8] and Bornhäuser et al.[9]

Abbreviations: AML, acute myeloid leukemia; BM: bone marrow; CHR: cause-specific hazard ration; CI, confidence interval; HR, hazard ratio; MAC, myeloablative conditioning; PB, peripheral blood; RIC, reduced intensity conditioning.

Suppl. Table 3. Univariable analysis of pre-transplant testosterone in male non-AML and AML patients of the discovery cohort.

	Patients with non-AML (n=170)		**Patients with AML (n=176)**	
	Testosterone per 100 ng/dL decrease		**Testosterone per 100 ng/dL decrease**	
	HR 95%CI	***P***	**HR 95%CI**	***P***
Endpoint				
OS	1.05 (0.94-1.1 8)	0.355	1.16 (1.05-1.28)	0.002
PFS	1.06 (0.96-1.19)	0.239	1.18 (1.08-1.28)	0.0005
OS after acute GVHD	-		1.19 (1.01-1.41)	0.040
PFS after acute GVHD	-		1.22 (1.04-1.43)	0.015
	CHR 95%CI	***P***	**CHR 95%CI**	***P***
NRM	1.03 (0.88-1.20)	0.705	1.28 (1.09-1.52)	0.003
Relapse	1.10 (0.94-1.27)	0.215	1.12 (1.01-1.25)	0.033
NRM after acute GVHD	-		1.45 (1.11-1.89)	0.005
Relapse after acute GVHD	-		1.06 (0.86-1.32)	0.552

Abbreviations: AML, acute myeloid leukemia; CHR, cause-specific hazard ratio; CI, confidence interval; GVHD, graft-versus-host disease; HR, hazard ratio; OS, overall survival; PFS, progression-free survival; NRM, non-relapse mortality.

Suppl. Table 4. Additional patient characteristics of male AML patients of the training cohort.

	Training cohort n=176
Parameter	
Karnofsky performance status, n (%)	
>80%	129 (80)
≤80%	33 (20)
Unknown	14
Pre-transplant CRP [mg/L] (median, IQR)	8.2 (2.9-17.4)
Pre-transplant CRP, n (%)	
≤5 mg/L	62 (37)
>5 mg/L	104 (63)
Unknown	10
Pre-transplant BMI [kg/m^2], (median, IQR)	25.7 (23.7-27.6)
Pre-transplant BMI, n (%)	
<18.5 kg/m^2	1 (1)
18.5-29.9 kg/m^2	148 (84)
≥30 kg/m^2	27 (15)
HCT-CI*, n (%)	
0	35 (20)
1+2	62 (36)
≥3	77 (44)
Unknown	2
Diabetes mellitus[a], n (%)	
Present	7 (4)
Absent	167 (96)
Unknown	2
Cardiovascular disease[b], n (%)	
Present	42 (24)
Absent	132 (76)
Unknown	2
Infection[c], n (%)	
Present	24 (14)
Absent	150 (86)
Unknown	2

Abbreviations: BMI, body-mass index; CRP, C-reactive protein; HCT-CI, hematopoietic cell transplantation-specific comorbidity index; IQR, interquartile range.
*According to Sorror et al.[6]
[a]Requiring treatment with insulin or oral hypoglycemic agents but not diet alone.
[b]Congestive heart failure, myocardial infarction, or EF <50%, one or more vessel-coronary artery stenosis requiring medical treatment, stent, or bypass.
[c]Infection by date of HCT-CI assessment requiring continuation of antimicrobial treatment after day 0.

Suppl. Table 5. Multivariable analysis of the training cohort with the endpoints overall survival (OS) and progression-free survival (PFS) after allogeneic stem cell transplantation considering pre-transplant testosterone as continuous variable (complete case analysis, n=154).

	OS		PFS	
	HR 95% CI	***P***	**HR 95% CI**	***P***
Covariate				
Testosterone (per 100 ng/dL decrease)	1.14 (1.01-1.29)	0.035	1.12 (1.00-1.25)	0.045
Age (per 10-year increase)	1.19 (0.95-1.48)	0.130	1.26 (1.02-1.54)	0.028
Karnofsky performance status				
>80%	Ref		Ref	
≤80%	1.34 (0.74-2.41)	0.332	1.23 (0.71-2.13)	0.469
BMI				
<30 kg/m^2	Ref		Ref	
≥30 kg/m^2	0.52 (0.24-1.10)	0.088	0.69 (0.35-1.35)	0.280
CRP				
≤5 mg/L	Ref		Ref	
>5 mg/L	1.77 (1.06-2.97)	0.030	1.40 (0.89-2.20)	0.144
Diabetes mellitus[a]				
Absent	Ref		Ref	
Present	2.40 (0.75-7.66)	0.140	1.53 (0.49-4.81)	0.470
Cardiovascular disease[b]				
Absent	Ref		Ref	
Present	1.01 (0.58-1.75)	0.973	0.98 (0.59-1.62)	0.935
Infection[c]				
Absent	Ref		Ref	
Present	1.40 (0.70-2.77)	0.342	1.34 (0.72-2.49)	0.360

Number of events: OS, n=75; PFS, n=90.
Abbreviations: BMI, body mass index; CRP, C-reactive protein.
[a]Requiring treatment with insulin or oral hypoglycemic agents but not diet alone.
[b]Congestive heart failure, myocardial infarction, or EF <50%, one or more vessel-coronary artery stenosis requiring medical treatment, stent, or bypass.
[c]Infection by date of HCT-CI assessment requiring continuation of antimicrobial treatment after day 0.

Suppl. Table 6. Patient, disease and transplant characteristics of the pilot cohort of female AML patients (n=32).

Parameter	
Age [years] at alloSCT (median, IQR)	56 (46-61)
Disease stage before alloSCT[a], n (%)	
Early	20 (63)
Intermediate	2 (6)
Late	10 (31)
Conditioning[b], n (%)	
RIC	24 (77)
MAC	7 (23)
NA	1
Stem cell source, n (%)	
PB	28 (88)
BM	4 (12)
Donor, n (%)	
Related	8 (25)
Unrelated	24 (75)
Recipient – donor sex match, n (%)	
Matched	9 (28)
Female – male	23 (72)
Median pre-transplant testosterone, ng/dL (IQR)	17 (10-21)

Abbreviations: AlloSCT, allogeneic stem cell transplantation; AML, acute myeloid leukemia; BM, bone marrow; CI, confidence interval; IQR, interquartile range; MAC, myeloablative conditioning; NA, not available or not assessable; PB, peripheral blood; RIC, reduced intensity conditioning.
[a]According to Gratwohl et al.[7]
[b]According to Bacigalupo et al[8] and Bornhäuser et al.[9]

Suppl. Table 7. Univariable analysis of pre-transplant testosterone in the pilot cohort of female AML patients (n=32).

	Testosterone per 10 ng/dL decrease	
	HR (95% CI)	***P***
Endpoint		
OS	1.14 (0.75-1.72)	0.543
PFS	1.01 (0.70-1.45)	0.976
OS after acute GVHD	1.12 (0.61-2.08)	0.710
PFS after acute GVHD	1.19 (0.54-2.63)	0.661
	CHR (95% CI)	***P***
Relapse	1.06 (0.69-1.64)	0.790
NRM	0.84 (0.40-1.75)	0.642

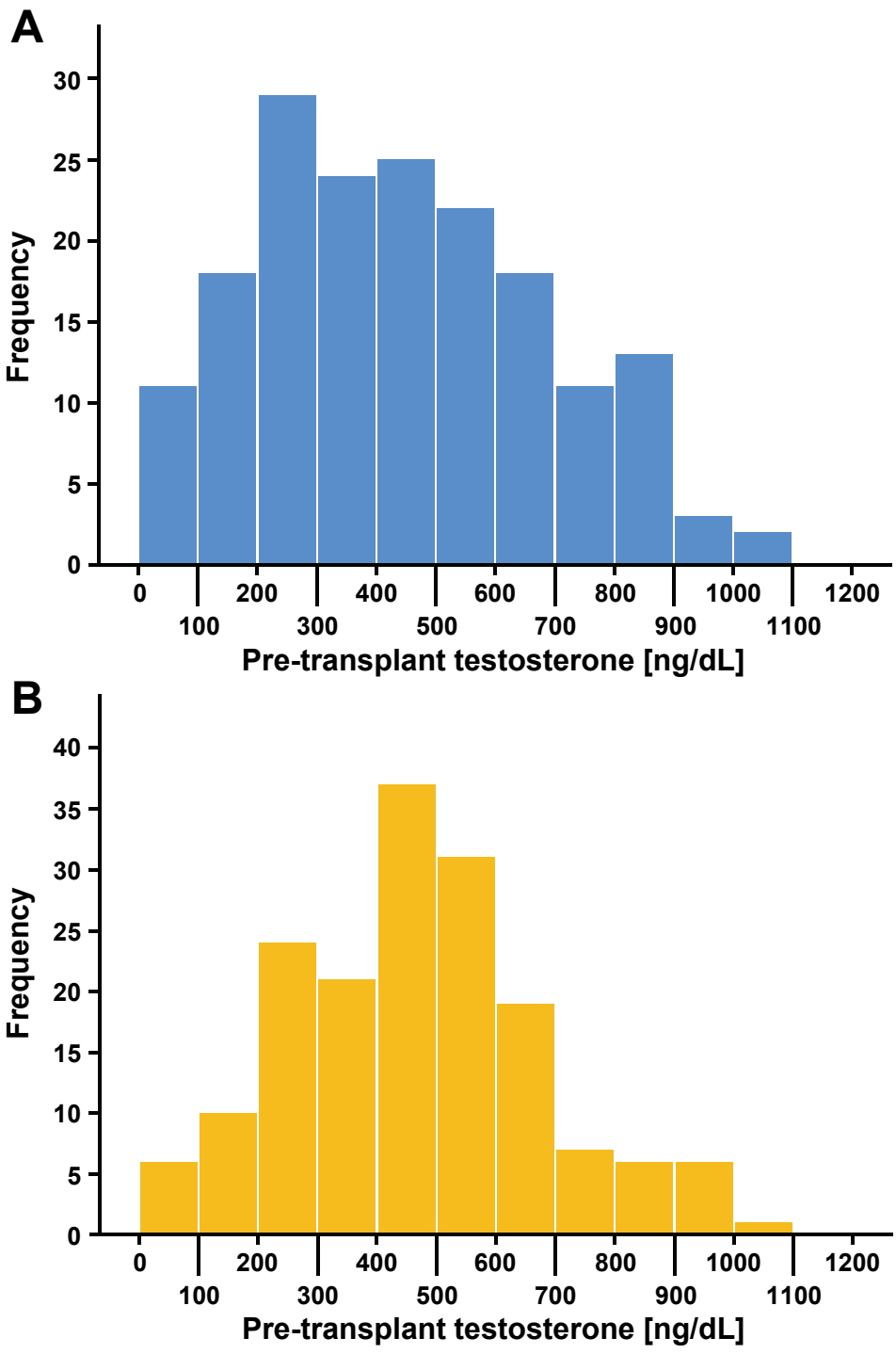
Abbreviations: AML, acute myeloid leukemia; CHR, cause-specific hazard ratio; CI, confidence interval; GVHD, graft-versus-host disease; HR, hazard ratio; OS, overall survival; PFS, progression-free survival; NRM, non-relapse mortality.

Suppl. Table 8. Multivariable analysis of the training cohort with the endpoints overall survival (OS) and progression-free survival (PFS) after allogeneic stem cell transplantation considering optimized pre-transplant testosterone cut-off value (complete case analysis, n=154).

	OS		PFS	
	HR 95% CI	***P***	**HR 95% CI**	***P***
Covariate				
Testosterone				
≥250 ng/dL	Ref		Ref	
<250 ng/dL	2.00 (1.19-3.36)	0.009	2.00 (1.24-3.22)	0.005
Age (per 10-year increase)	1.18 (0.94-1.48)	0.151	1.24 (1.01-1.53)	0.040
Karnofsky performance status				
>80%	Ref		Ref	
≤80%	1.30 (0.72-2.36)	0.386	1.19 (0.68-2.08)	0.540
BMI				
<30 kg/m^2	Ref		Ref	
≥30 kg/m^2	0.55 (0.26-1.15)	0.113	0.69 (0.36-1.35)	0.279
CRP				
≤5 mg/L	Ref		Ref	
>5 mg/L	1.81 (1.08-3.02)	0.024	1.42 (0.90-2.23)	0.128
Diabetes mellitus[a]				
Absent	Ref		Ref	
Present	2.35 (0.75-7.41)	0.145	1.59 (0.51-4.96)	0.423
Cardiovascular disease[b]				
Absent	Ref		Ref	
Present	1.01 (0.58-1.74)	0.979	0.96 (0.58-1.59)	0.887
Infection[c]				
Absent	Ref		Ref	
Present	1.44 (0.73-2.87)	0.296	1.38 (0.74-2.57)	0.314

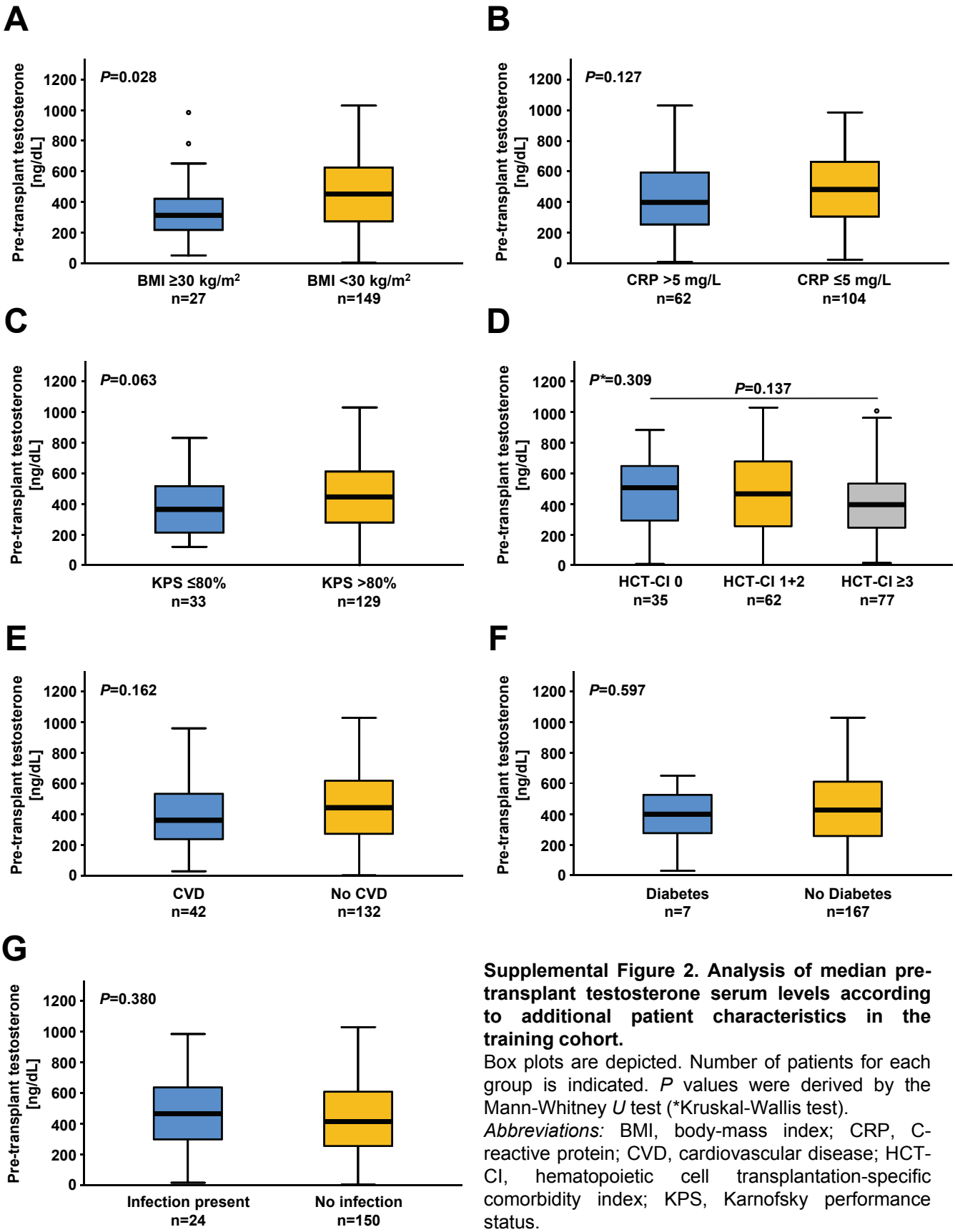
Number of events: OS, n=75; PFS, n=90.
Abbreviations: BMI, body mass index; CRP, C-reactive protein.
[a]Requiring treatment with insulin or oral hypoglycemic agents but not diet alone.
[b]Congestive heart failure, myocardial infarction, or EF <50%, one or more vessel-coronary artery stenosis requiring medical treatment, stent, or bypass.
[c]Infection by date of HCT-CI assessment requiring continuation of antimicrobial treatment after day 0.

Suppl. Figure 1.



Supplemental Figure 1. Histogram of the distribution of pre-transplant testosterone values in the training (A) and in the confirmation (B) cohort.

Suppl. Figure 2.

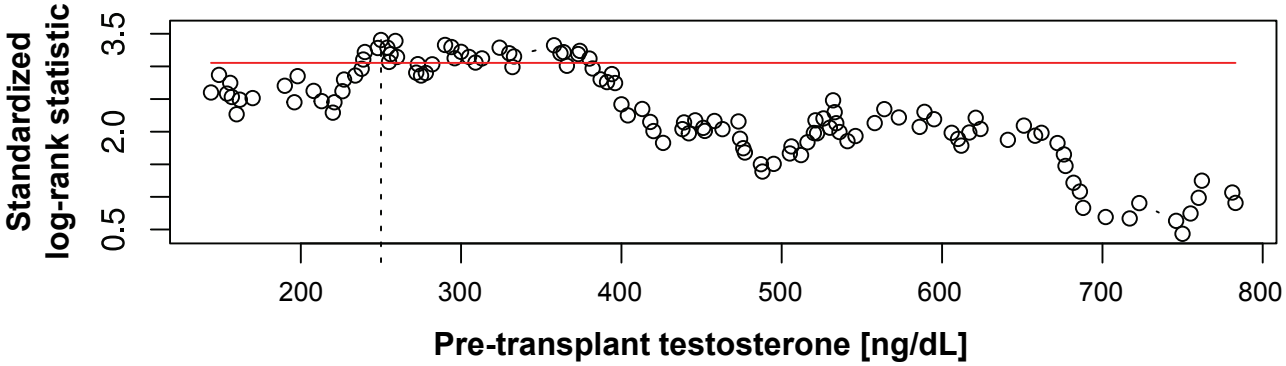


Supplemental Figure 2. Analysis of median pre-transplant testosterone serum levels according to additional patient characteristics in the training cohort.

Box plots are depicted. Number of patients for each group is indicated. *P* values were derived by the Mann-Whitney *U* test (*Kruskal-Wallis test).

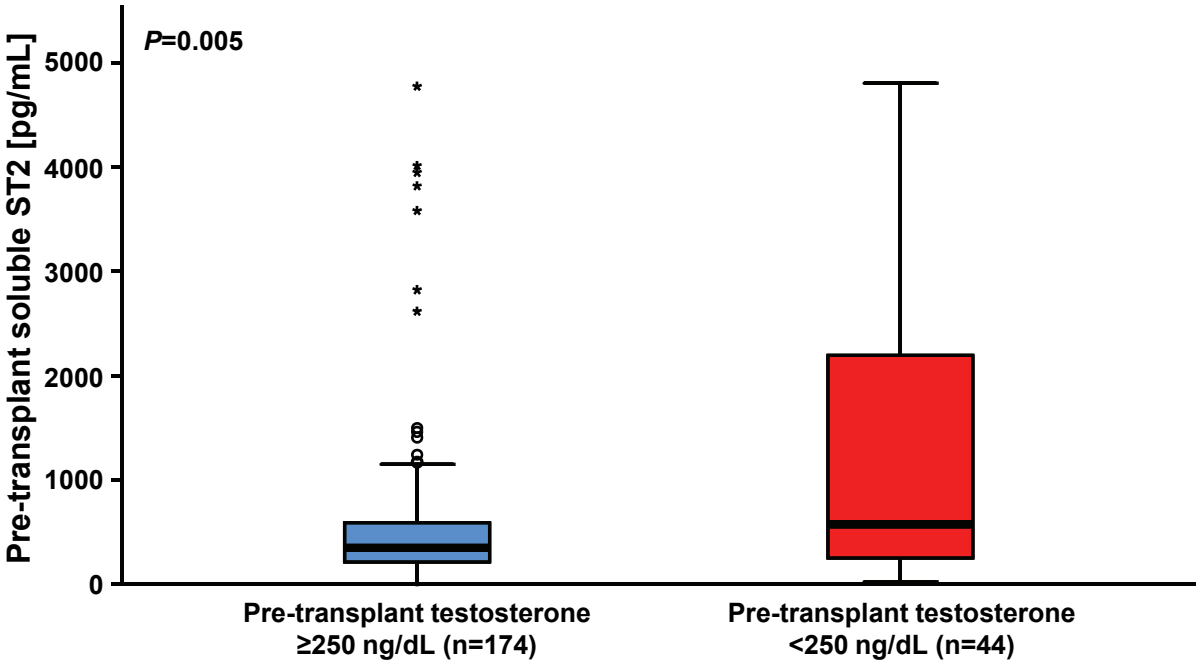
Abbreviations: BMI, body-mass index; CRP, C-reactive protein; CVD, cardiovascular disease; HCT-CI, hematopoietic cell transplantation-specific comorbidity index; KPS, Karnofsky performance status.

Suppl. Figure 3.



Supplemental Figure 3. Optimal cut-off of pre-transplant testosterone based on maximally selected log-rank statistics.
An optimal cut-off determination with regard to post-transplant overall survival was conducted in the training set. The value of 250 ng/dL (*P*=0.018), which is associated with mortality in non-transplant settings and also reflects the lower level of our center's reference range (250-1000 ng/dL) was chosen. The value of 250 ng/dL (corresponding to 8.7 nM) was used as cut-point to stratify patients of the training and confirmation cohort in low and high pre-transplant testosterone groups in order to illustrate the effect of pre-transplant testosterone status on outcome after alloSCT and after onset of acute GVHD.

Suppl. Figure 4.



Supplemental Figure 4. Pre-transplant testosterone status and pre-transplant serum levels of suppressor of tumorigenicity-2 (ST2).
For this analysis, patients of the training and the confirmation cohort were combined. The median pre-transplant ST2 serum levels was significantly higher in patients with low (<250 ng/dL) pre-transplant testosterone status as compared to patients of the high (≥250 ng/dL) pre-transplant testosterone group.
Box plots are depicted. Number of patients for each group is indicated. *P* value was derived by the Mann-Whitney *U* test.