SUPPLEMENTARY APPENDIX

High pre-transplant serum nitrate levels predict risk of acute steroid-refractory graft-*versus*-host disease in the absence of statin therapy

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Steroid-refractory GVHD

Steroid-refractory GVHD was defined as histologically confirmed disease not responding to standard prednisone therapy (2x1 mg/kg body weight, for intestinal GVHD combined with mycofenolate mofetil 2x1 g/d) and requiring second-line salvage immunosuppressive therapy which was generally pentostatin [1].

The decision to apply pentostatin was made clinically. Generally, pentostatin was applied if patients did not respond to immunosuppressive induction therapy within 7 days, or if patients rapidly deteriorated within the first 3 days, or if patients lost response within the first 4 weeks in the presence of the immunosuppressive induction regimen. In addition, 3 patients died from their GVHD without salvage therapy applicable. One patient received infliximab instead of pentostatin. Supportive care was applied according to local standard operating procedures. Notably, routine administration of pravastatin to all patients undergoing allo-SCT from day -1 until withdrawal of CNI was introduced as standard of care in January 2010 in order to minimize CNI-associated vascular morbidity [2, 3]. We chose pravastatin because it is not metabolized by the CYP system and is approved in the United States as well as in Germany for combination treatment with cyclosporine A in the post-transplant setting [4]. Furthermore, pravastatin was shown to be safely tolerated during allogeneic stem cell transplantation [5].

Nitrate assays

Determination of nitrate in serum was performed using the Griess Reagent System. Ten microliters of serum and 70 µl MilliQ water were mixed and incubated in a microtiter plate (Greiner, 655101, Bahlingen, Germany) for 2 hours with 10 µl nitrate reductase (Sigma-Aldrich, No N7265, 4U/ml) and 10 µl of 0.88 mM β-NADPH (Sigma-Aldrich, No N1630) at room temperature in darkness. After a subsequent incubation of 10 minutes each 5 µl glutamate dehydrogenase (Sigma-Aldrich, No G4387), 5 µl 2 M ammonium chloride (Sigma-Aldrich, No A9434) and 5 µl 0.8 M alpha-ketoglutaric acid (Sigma-Aldrich, No K3752), the

final Griess Reagent including 0.1% NED (Sigma-Aldrich, No N9125) and 1% sulfanilamide (Sigma-Aldrich, No S9251) was added to each sample. After an additional incubation time of 15 minutes 200 µl of each well were pipetted in a microtiterfilter plate (MultiScreen® HTS Millipore MSGVN2250, Darmstadt, Germany) and centrifuged with a microtiter plate centrifuge (3220 g_{max}, 2 minutes) in a photometer microtiter plate (Greiner 655101) to remove particles/precipitates which can disturb the photometric measurement. Absorbance was measured on a microplate reader (SpectraMax Plus 384, Molecular Devices, USA) at a wavelength of 550 nm. Double measurements were performed for each sample. For external calibration sodium nitrate (Sigma-Aldrich, No S 8170) was diluted in Milli Q water covering a concentration range of 0 - 24 µM nitrate. Extraction and diazotization were performed similarly. Serum samples containing more than 24 µM were diluted with Milli Q water and re-measured. Normal nitrate concentration range in serum of patients prior to alloSCT was found to be between 8.5 and 51.8 µM (mean 30.1 ±10.8 µM). Serum nitrate levels in a cohort of 49 normal blood donors ranged between 13.2 and 114.0 µM, with a median of 26.5 µM. We therefore chose a cut-off of 26.5 µM to categorize patients into high and low pre-transplant nitrate cohorts.

Statistical Analyses

The cut-off for nitrates was chosen based on the maximally selected rank statistics with regard to refractory GVHD incidence among patients without statins. The R package maxstat 0.7-17 was used and yielded three maxima between 19 and 35 µM at 22, 26 and 30 µM. Because the median of the normal subject cohort was at 26.5 µM and this most likely reflects a situation not influenced by chemotherapy treatment, we choose 26.5 µM as a cut-off. The cut-off for ANG2 at 1,000 pg/mL was also based on maximally selected rank statistics and was previously published by our group [6].

Literature:

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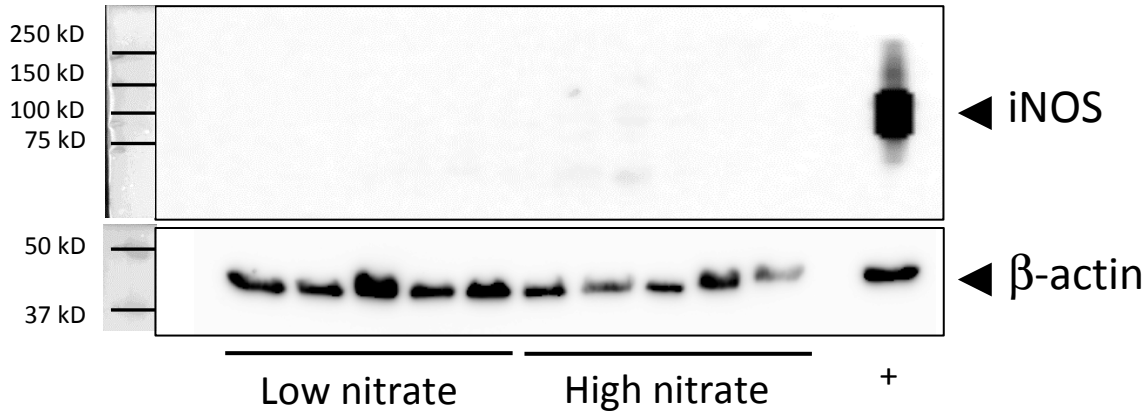
Supplemental Table 1. Patient characteristics separated by high and low serum nitrates. P-values were calculated using the Mann Withney Test, the Fisher or χ^2-test.

	Low Nitrates **(≤26.5 µM) N=248**	High Nitrates **(>26.5 µM) N=169**	p-value
Median age at SCT (years, range)	54 (19-70)	51(17-70)	p=0.04
Sex			p=0.03
Female	90 (36%)	80 (47%)	
Male	158 (64%)	89 (53%)	
Donor			p=0.99
RD	81 (33%)	56 (33%)	
MUD	109 (44%)	74 (44%)	
MMUD	58 (23%)	39 (23%)	
Sex mismatch (Donor-Recipient)			p=0.59
Male-Male, Female-Female	143 (58%)	95 (58%)	
Male-Female	59 (24%)	47 (26%)	
Female-Male	46(18%)	27 (19%)	
Disease			
AML, MDS	112 (45%)	58 (34%)	p=0.03
ALL	25 (10%)	13 (8%)	p=0.48
Lymphoma, CLL	59 (24%)	46 (27%)	p=0.49
MPS	19 (8%)	15 (9%)	p=0.71
MM, Amyloidosis	31 (12%)	35 (21%)	p=0.03
AA, PNH	2 (1%)	2 (1%)	p=1.00
Disease Score before SCT			p=0.03
0	78 (33%)	37 (24%)	
1	31 (13%)	34 (22%)	
2	129 (54%)	85 (54%)	
NA	10	13	
Stem cell source			p=0.98
Peripheral stem cells	229 (92%)	181 (93%)	
Bone marrow stem cells	19 (8%)	67 (7%)	
Conditioning			p=0.64
RIC	181 (73%)	119 (72%)	
MAC	67 (27%)	50 (28%)	
GVHD site			
Skin	70 (29%)	43 (23%)	p=0.43
Gut	59 (23%)	42 (27%)	p=0.81
Liver	16 (6%)	8 (5%)	p=0.52
GVHD grade			p=0.55
No	138 (56%)	97 (57%)	
Grade 1-2 sensitive	67 (27%)	43 (26%)	
Grade 3-4 sensitive	25 (10%)	12 (7%)	
Grade 3-4 refractory	18 (7%)	17 (10%)	

Abbreviations:

AA: aplastic anemia, ALL: acute lymphoblastic leukemia, AML: acute myelogenous leukemia, CLL: chronic lymphocytic leukemia, MAC: myeloablative conditioning, MM: multiple myeloma, MMUD: mismatched unrelated donor, MPS: myeloproliferative syndrome, MUD: matched unrelated donor, NA: not available; NRM: non-relapse mortality, PD: progressive disease, PNH: paroxysmal nocturnal hemoglobinuria, RD: related donor, RIC: reduced intensity conditioning, SCT: stem cell transplantation.

Supplemental Figure 1



Suppl. Figure 1

INOS expression was compared between patients with high (n=6) and low (n=5) nitrates by western blot analysis of protein lysates of perip heral blood mononuclear cells. No correlation was observed between serum nitrates and iNOS.
A mouse macrophage + IFNγ/LPS lysate(BD Transduction Laboratories) was used as positive control (+). By using synthetic peptides, the epitope recognized by this mouse monoclonal anti-iNOSantibody (http://www.rndsystems.com/Products/MAB9502)has been mapped to amino acids 781-798 of human iNOS. The corresponding sequence of mouse iNOS is identical.