SUPPLEMENTARY APPENDIX

Asymmetric dimethylarginine serum levels are associated with early mortality after allogeneic stem cell transplantation

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SUPPLEMENTAL METHODS

GVHD prophylaxis, diagnosis, and treatment

GVHD was clinically and histologically diagnosed and graded using standard criteria. Prophylaxis and treatment of GVHD was performed as reported previously.[1,2]

Assessment of serum levels of ADMA and endothelium-related serum factors

Serum samples were collected between 0 and 4 weeks before alloSCT and cryopreserved at −80°C. Serum ADMA levels were measured using the ADMA Xpress ELISA Kit® (Immundiagnostik AG, Bensheim, Germany) according to the manufacturer's instructions. For measurement of serum levels of soluble thrombomodulin (sCD141), interleukin-18 (IL-18), and IL-18-binding protein A (IL18BPa) commercial ELISA kits (DuoSet, R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions were applied. Serum levels of nitrates were assessed as described previously.[3] All measurements were performed centrally in Heidelberg. The concentration of free IL-18 was calculated on the basis of the concentrations of total IL-18 and total IL-18BPa as determined by ELISA applying the law of mass action, a 1:1 stoichiometry in the complex of IL-18 and IL-18BPa and a dissociation constant (Kd) of 400 pM.[4]

SNP analyses

Three single nucleotide polymorphisms (rs1137933, rs2297518, and rs2779248) in the inducible nitric oxide synthase (INOS) gene were selected based on their association to inflammatory diseases.[5,6] The polymorphisms rs1137933 (D385D) and rs2297518 (S608L) are located within coding region and tag an additional 23 SNPs (r^2 ≥0.8) based on 1000 genome database for CEU population. Genomic DNA from patients and controls were genotyped by using an allele-specific method as described previously.[7]

Additional statistical methods

Violation of proportional hazards assumption was tested for by cox.zph plots, which give an estimate of the time-dependent coefficient β(t). Since the proportional hazards assumption was violated for pre-transplant ADMA, a step function for β(t), i.e., different coefficients over different time intervals for pre-transplant ADMA were analyzed as suggested by Therneau et al[8] and the ADMA data set was divided into 3 epochs of the first year, year 2-3, and greater than 3 years.

The predictive performance of pre-transplant ADMA within the first year post-transplant or within the first year post-acute GVHD was assessed by calculating the prediction error (Brier score).[9]

Genotype frequencies of SNPs were tested in patients for deviation from Hardy-Weinberg equilibrium using Pearson's χ^2 test. The effects of INOS polymorphisms, using dominant and recessive models, were determined on OS and NRM in the first year post-transplant. Maximally selected rank statistics were performed for ADMA with regard to OS in the first year post alloSCT in order to stratify patients in high and low ADMA groups in the context of the INOS polymorphisms.

All statistical analyses were carried out in statistical software R, version 3.4.3, together with the R packages 'survival', version 2.41-3, 'cmprsk', version 2.2-7, 'maxstat', version 0.7-25, 'pec' version 2.5.4, 'DescTools' version 0.99.24.

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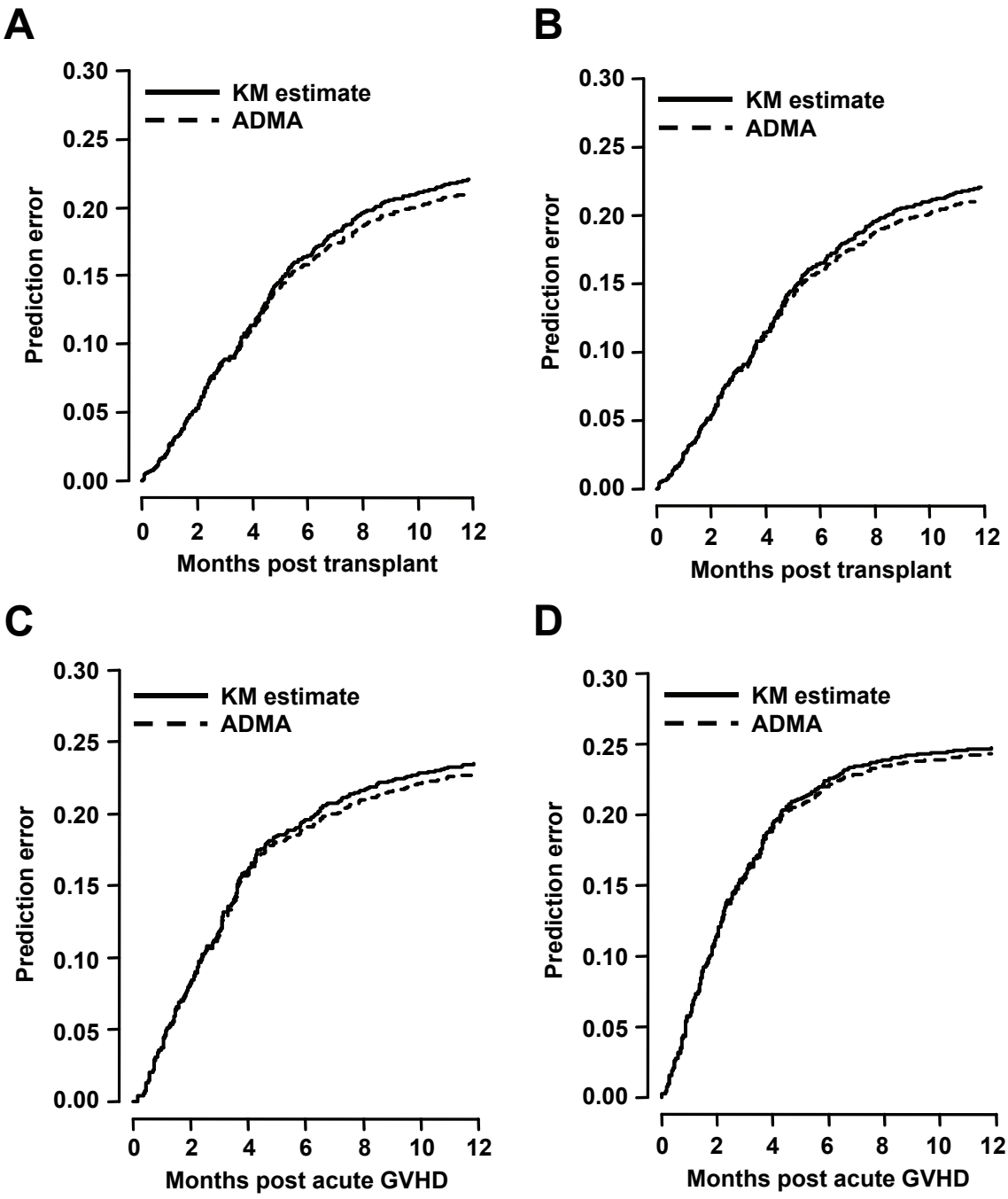
SUPPLEMENTAL DATA

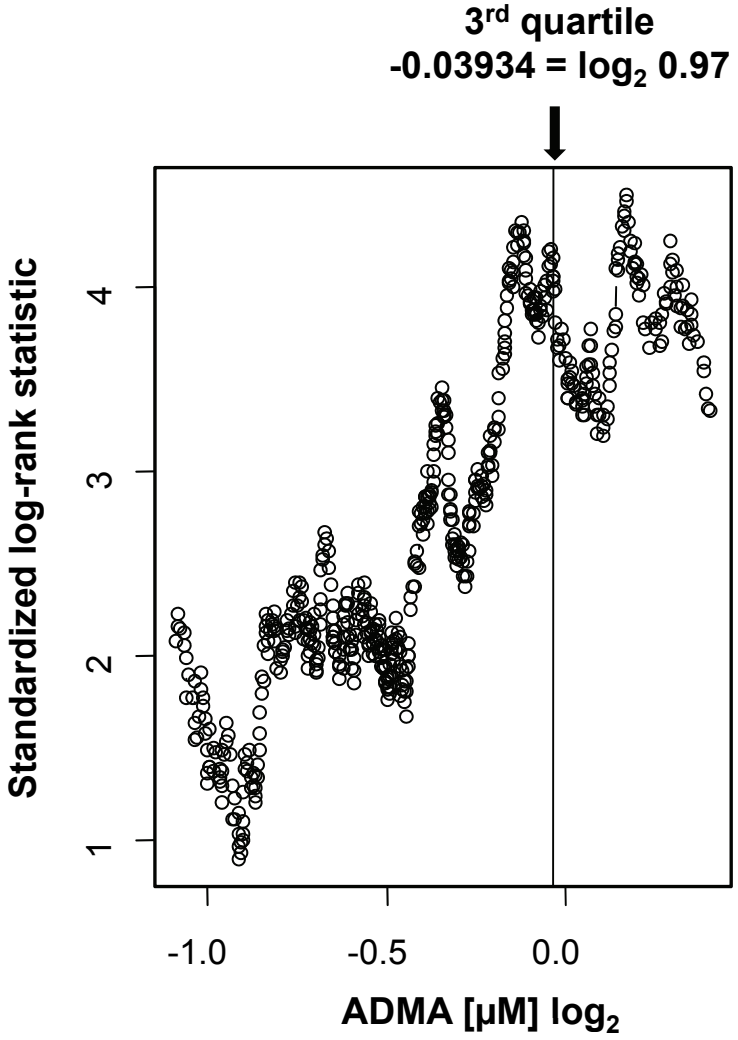
Supplemental Figure 1



Supplemental Figure 1. Prediction performance of the univariable pre-transplant asymmetric dimethylarginine (ADMA) models with regard to overall survival (OS) and progression-free survival (PFS) in the first year post-transplant and the first year after onset of acute graft-*versus*-host disease (GVHD).

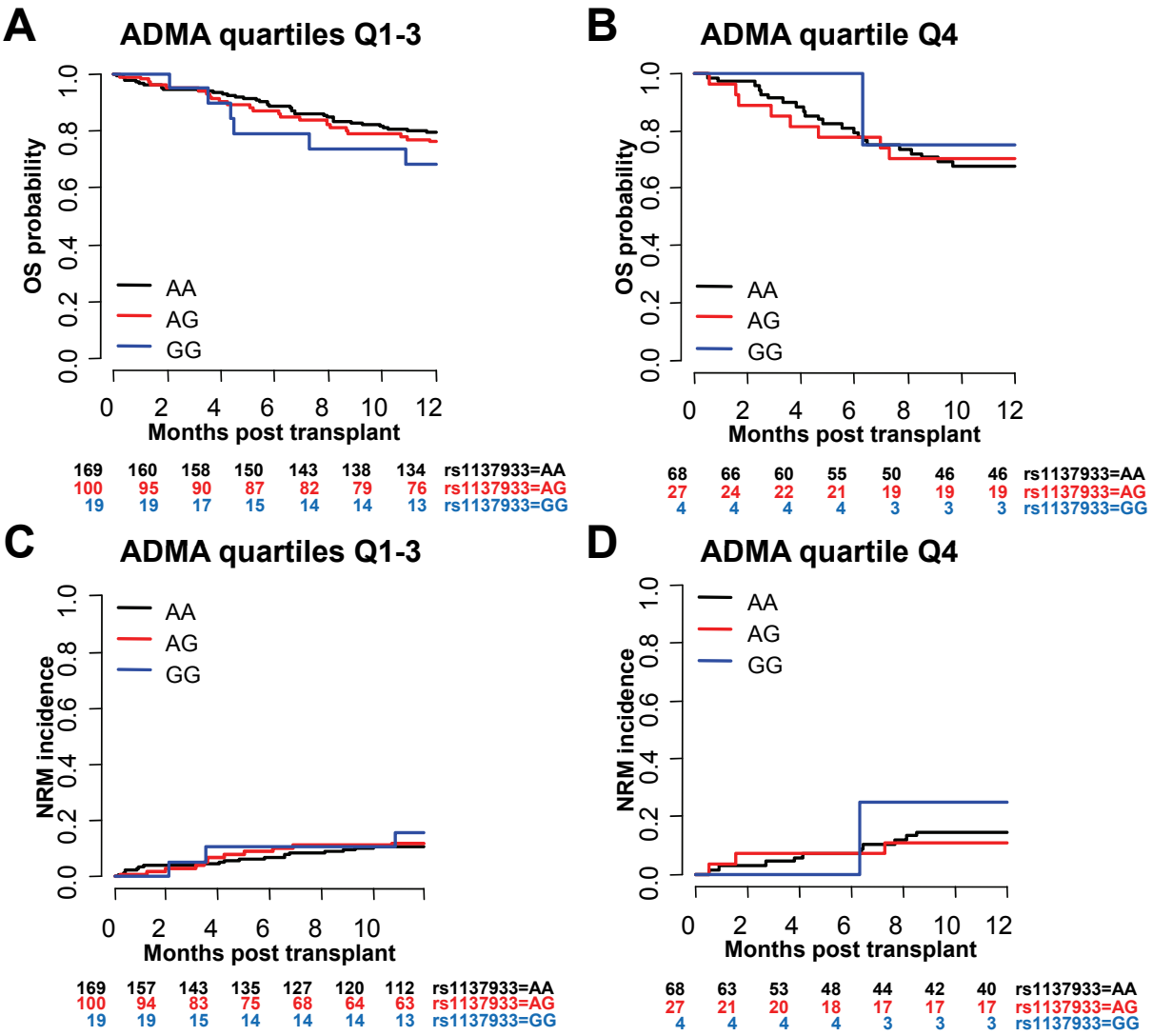
The prediction error curves representing the Brier score over time for the univariable pre-transplant ADMA models (dashed curves) with regard to OS and PFS in the first year post-transplant (A and B, respectively), and OS and PFS in the first year after onset of acute GVHD (C and D, respectively) show better, albeit slightly improved, performance in comparison to the respective Kaplan-Meier (KM) survival estimates (black curves).

Supplemental Figure 2



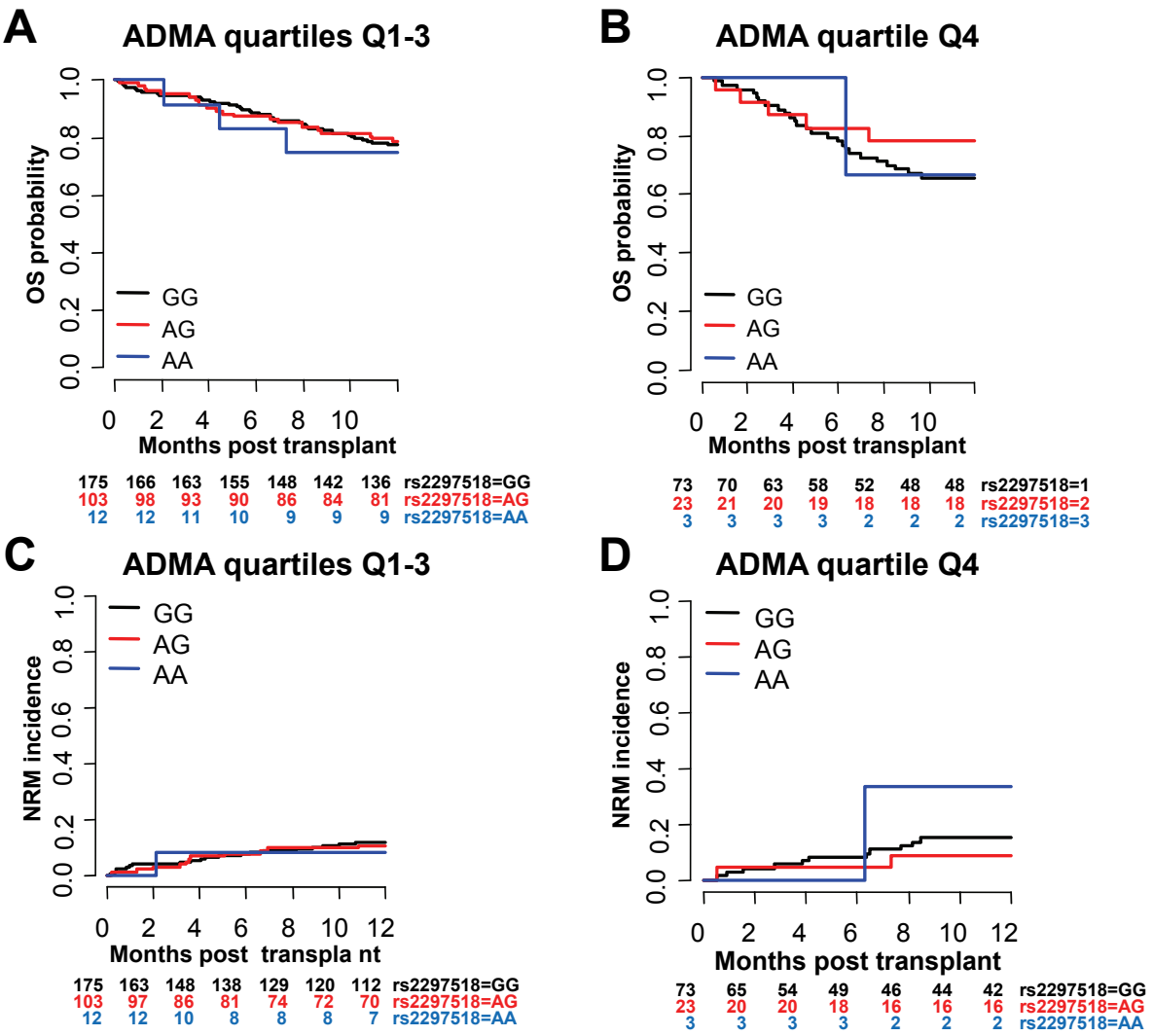
Supplemental Figure 2. Optimal cut-off of pre-transplant asymmetric dimethylarginine (ADMA) with regard to overall survival (OS) in the first post-transplant year based on maximally selected log-rank statistics.
In order to evaluate the effect of ADMA in the context of inducible nitric oxide synthase (INOS) gene polymorphisms, an optimal cut-off determination with regard to OS in the first post-transplant year was conducted yielding multiple cut-points (maxima).
The maximum at 0.97 µM ($=2^{-0.03934}$) which also represents the upper quartile of the ADMA distribution, was chosen to stratify patients in high (≥0.97 µM) and low (<0.97 µM) ADMA groups in the context of the INOS polymorphisms.

Supplemental Figure 3



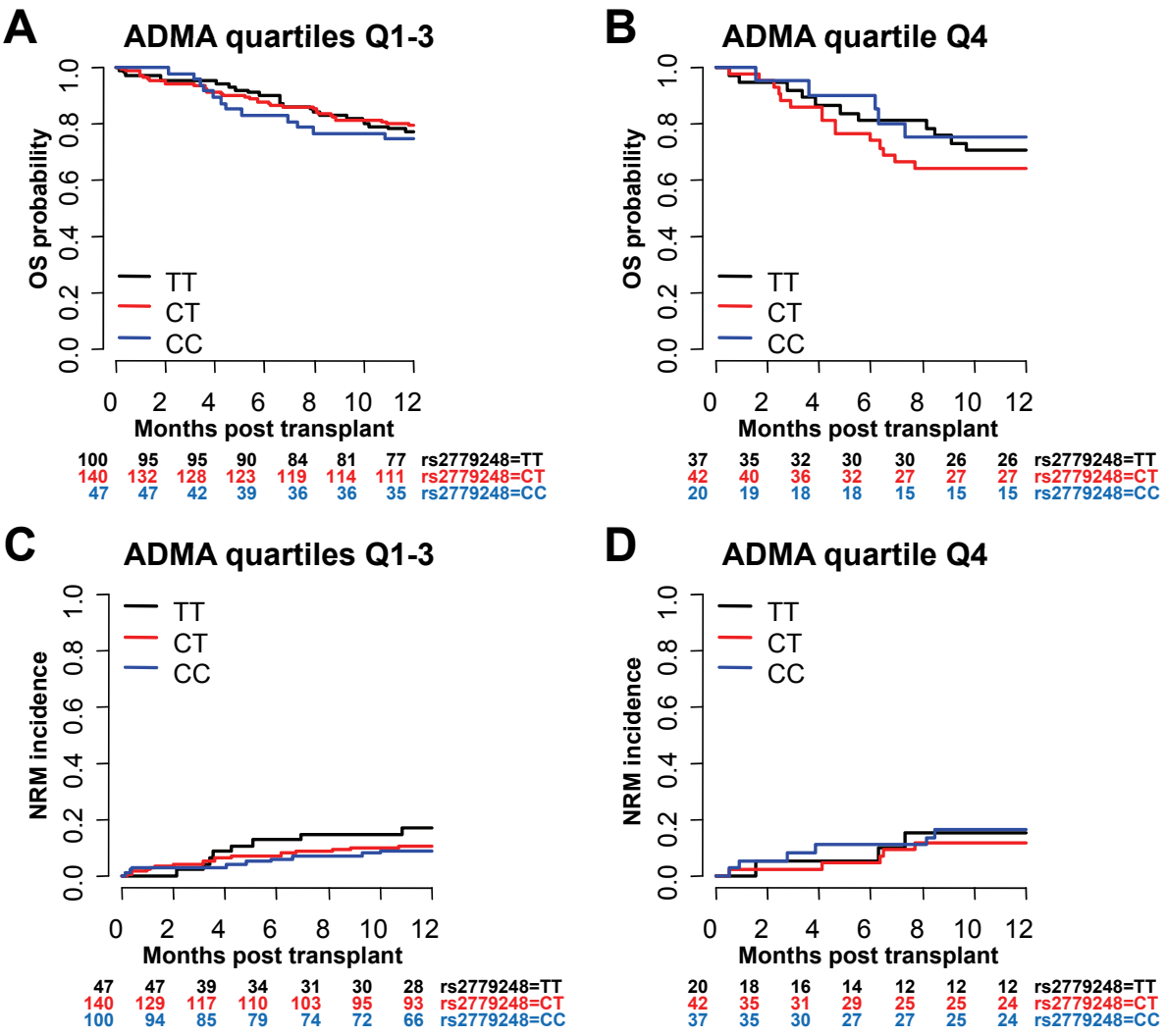
Supplemental Figure 3. Impact of the of inducible nitric oxide synthase (INOS) gene SNP rs1137933 on overall survival (OS) and non-relapse mortality (NRM) in the first post-transplant year in patients with low (<0.97 µM, Q1-3) and high (≥0.97 µM, Q4) pre-transplant asymmetric dimethylarginine (ADMA) serum levels.
(A, B) There was no significant influence of the INOS gene SNP rs1137933 on the effect of ADMA on OS within the first year after allogeneic stem cell transplantation (alloSCT) in patients with low (Q1-3) and high (Q4) pre-transplant ADMA serum levels, respectively.
(C, D) Similarly, the INOS gene SNP rs1137933 had no significant impact on the incidence of NRM in the first post-transplant year alloSCT in patients with low (Q1-3) and high (Q4) pre-transplant ADMA serum levels, respectively.

Supplemental Figure 4



Supplemental Figure 4. Impact of the nitric oxide synthase (INOS) gene SNP rs2297518 on overall survival (OS) and non-relapse mortality (NRM) in the first post-transplant year in patients with low (<0.97 µM, Q1-3) and high (≥0.97 µM, Q4) pre-transplant asymmetric dimethylarginine (ADMA) serum levels.
(A, B) The INOS gene SNP rs2297518 did not significantly influence the effect of ADMA on OS within the first year after allogeneic stem cell transplantation (alloSCT) in patients with low (Q1-3) and high (Q4) pre-transplant ADMA serum levels, respectively.
(C, D) There was no significant influence of the INOS gene SNP rs2297518 on the incidence of NRM in the first post-transplant year alloSCT in patients with low (Q1-3) and high (Q4) pre-transplant ADMA serum levels, respectively.

Supplemental Figure 5



Supplemental Figure 5. Impact of the nitric oxide synthase (INOS) gene SNP rs2779248 on overall survival (OS) and non-relapse mortality (NRM) in the first post-transplant year in patients with low (<0.97 µM, Q1-3) and high (≥0.97 µM, Q4) pre-transplant asymmetric dimethylarginine (ADMA) serum levels.
(A, B) The INOS gene SNP rs2779248 did not affect the effect of ADMA on OS within the first year after allogeneic stem cell transplantation (alloSCT) in patients with low (Q1-3) and high (Q4) pre-transplant ADMA serum levels, respectively.
(C, D) Accordingly, the INOS gene SNP rs2779248 had also no significant impact on the incidence of NRM in the first post-transplant year alloSCT in patients with low (Q1-3) and high (Q4) pre-transplant ADMA serum levels, respectively.