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Pretransplant systemic metabolic profiles in allogeneic hematopoietic stem cell transplant recipients - identification of patient subsets with increased transplant-related mortality

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A B S T R A C T

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is used in the treatment of high-risk acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS); however, the treatment has high risk of severe transplantation-related mortality (TRM). In this study, we examined pretransplantation serum samples derived from 92 consecutive allotransplant recipients with AML or MDS. Using nontargeted metabolomics, we identified 1274 metabolites including 968 of known identity (named biochemicals). We further investigated metabolites that differed significantly when comparing patients with and without early extensive fluid retention, pretransplantation inflammation (both being associated with increased risk of acute graft-versus-host disease [GVHD]/nonrelapse mortality) and development of systemic steroid-requiring acute GVHD (aGVHD). All three factors are associated with TRM and were also associated with significantly altered amino acid metabolism, although there was only a minor overlap between these three factors with regard to significantly altered individual metabolites. Furthermore, steroid-requiring aGVHD was especially associated with altered taurine/hypotaurine, tryptophan, biotin, and phenylacetate metabolism together with altered malate-aspartate shuttle and urea cycle regulation. In contrast, pretransplantation inflammation was associated with a weaker modulation of many different metabolic pathways, whereas extensive fluid retention was associated with a weaker modulation of taurine/hypotaurine metabolism. An unsupervised hierarchical cluster analysis based on the 13 most significantly identified metabolites associated with aGVHD identified a patient subset with high metabolite levels and increased frequencies of MDS/MDS-AML, steroid-requiring aGVHD and early TRM. On the other hand, a clustering analysis based on metabolites that were significantly altered for aGVHD, inflammation, and fluid retention comparison groups identified a patient subset with a highly significant association with TRM. Our study suggests that the systemic pretransplantation metabolic profiles can be used to identify patient subsets with an increased frequency of TRM.

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is increasingly used in the treatment of aggressive myeloid malignancies, especially acute myeloid leukemia (AML) and high-risk myelodysplastic syndromes (MDS) [1,2], even though it is still associated with a relatively high risk of transplantation-related mortality and morbidity [3]. The allo-HSCT process is initiated with either myeloablative or less toxic nonmyeloablative reduced-intensity conditioning, and the antileukemic effect of allo-HSCT is thus mediated by both the chemotherapy effect and immune-mediated anti-leukemic effect caused by graft-versus-host or specific graft-versus-leukemia reactivity [4]. The immune-mediated antileukemic effects are especially important in reduced-intensity conditioning patients [4].

The risk of transplantation-related mortality (TRM) after allotransplantation varies between patients, and important causes are infections, alloimmune/inflammatory reactions, and vascular/endothelial dysfunctions. The immunoregulatory status of the recipients will be important for the risk of infections [5,6], but pretransplantation signs of inflammation (i.e., increased C-reactive protein [CRP] levels [7]) are also associated with TRM and the risk of developing acute and chronic graft-versus-host disease (GVHD) [8,9]. Furthermore,

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endothelial dysfunction is involved in the development of both immune-mediated acute GVHD (aGVHD) and chronic GVHD (cGVHD) [10–12] and is also involved in the pathogenesis of several other post-transplantation complications (e.g., veno-occlusive disease) [10–12]. Thus GVHD is an important cause of TRM, but pretransplantation excessive fluid retention (probably a clinical sign of endothelial dysfunction) [13] and pretransplantation signs of inflammation [7] are also associated with the risk of TRM.

Cellular metabolism and metabolic mediators are important for the functional regulation of both immunocompetent/inflammatory cells and endothelial cells [14–17]. The systemic metabolic profile may therefore also be important in the functional regulation of immunocompetent or endothelial cells, or the profiles may reflect the functional status of these cells. In this context, we have investigated how the pretransplantation systemic metabolic profile of allotransplant recipients is (i) modulated by signs of inflammation (increased CRP levels), (ii) reflects pretransplantation/peritransplantation endothelial cell dysfunction (i.e., increased permeability/excessive fluid retention), or (iii) correlates with the later development of aGVHD. We have also compared the effects of these factors on the overall pretransplant metabolic profile.

Metabolomics, which is the profiling of metabolites, has emerged as a powerful tool to identify potential prognostic biomarkers in allotransplant recipients, and a link between metabolomic profiles and post-transplantation complications (including GVHD and fluid retention) has previously been described [18–21]. Metabolic targeting has therefore been suggested as a possible therapeutic strategy in the prophylaxis or treatment of GVHD [22–24]. The aims of the present study were to further analyze and map the serum levels of key metabolites and metabolic profiles and their significance in the allo-HSCT process. We investigated a consecutive group of patients with AML and high-risk MDS undergoing allo-HSCT. Our results indicate that GVHD is associated with altered pretransplantation levels of several immunoregulatory metabolites. In contrast, metabolites associated with increased pretransplantation levels of CRP and early fluid overload/retention showed minimal overlap with aGVHD-associated metabolites; an observation suggesting that their impact on posttransplant morbidity/mortality is not caused by a further modulation/exacerbation of aGVHD-associated metabolic alterations. The recipients could be subclassified into distinct subsets based on their pretransplantation metabolomics profiles. We hypothesized that with further exploration, metabolomic profiling may be a plausible method for individualized prophylactic therapy by identifying patients at particular risk for development of severe complications in the allo-HSCT course.

MATERIAL AND METHODS

Patients and rationale for patient classification

The study included 92 consecutive allotransplant recipients diagnosed with either AML (de novo or secondary AML) or high-risk MDS (54 men and 38 women; median age 54.5 years, range 17-73 years). All patients underwent transplantation with granulocyte colony stimulating factor–mobilized peripheral blood stem cell grafts derived from HLA-matched family donors. Our department is responsible for allo-HSCT with family donors in a defined geographical area of Norway, and the present patients represent a consecutive group. Our study should therefore be regarded as population based. The median follow-up of patients was 104 months, and all patients had a follow-up time of at least 25 months.

The characteristics of the patients are summarized in Table 1. None of the patients received parenteral nutrition or supplementary nutrition by an enteral tube (i.e., they all had their natural feeding at the time of sampling). All patients received cyclosporine-based GVHD prophylaxis; 88 patients

Table 1
Clinical and Biological Characteristics of the 92 Patients Included in the Study*

Demographic data at transplantation	
Gender	
Male	54
Female	38
Age (yr), median (range)	54.5 (17-73)
Height (cm), median (range)	175 (149-197)
Weight (kg), median (range)	71 (42-133)
BMI (kg/m^2), median (range)	23.3 (16.6-39.7)
Diagnosis	
AML de novo	64
Secondary AML	10
MDS high-risk	18
Pretransplantation status for the AML patients	
CR1	58
CR2	12
≥CR3	2
No complete remission	2
Pretransplantation hematological status	
WBC ($\times 10^9$/L)	3.6 (0.5-13.7)
Hb (g/dL)	10.4 (7.8-14.1)
Platelets ($\times 10^9$/L)	166 (6-779)
CRP (mg/L)	6 (1-120)
LDH (IU/dL)	181 (92-498)
GVHD prophylaxis	
Methotrexate + cyclosporine	88
Other cyclosporine-based regimen	4
Conditioning therapy	
Busulfan + Cyclophosphamide	52
Fludarabine + Busulfan	21
Fludarabine + Treosulfan	16
Antithymocyte Globulin + Cyclophosphamide	1
Fludarabine + Busulfan + Thiothepa	1
FLAMSA [26]	1
Days until 3 consecutive days above indicated level	
Neutrophils $>0.2 \times 10^9$/L	15 (10-29)
Platelets $>20 \times 10^9$/L	14 (9-35)
Prognostic parameters	
Excessive fluid retention	
Yes	37
No	51
NA	4
Inflammation	
Yes	36
No	56
aGVHD	
Yes	32
No	50
Death before day 100 without aGVHD	10
Death during 2 years of follow-up	
Overall mortality	53
Death within day 120 because of relapse	5
Nonrelapse death within day 100	12

BMI indicates body mass index; CR, complete hematological remission; FLAMSA, fludarabine, Ara-C and amsacrine; Hb, hemoglobin; LDH, lactate dehydrogenase; NA, not applicable; WBC, white blood cell count.

* Unless otherwise stated, the data are presented either as the number of patients or median (variation range) for the indicated parameter. One of the AML patients transplanted with detectable leukemia was treated according to the FLAMSA sequential treatment. aGVHD is defined as GVHD requiring high-dose steroid treatment before day +100 after allo-HSCT.

received cyclosporine combined with methotrexate, whereas 4 exceptional patients received other cyclosporine-based regimen; only 1 of the 4 exceptional patients received antithymocyte globulin. None of the patients had any clinical evidence of infectious or inflammatory disease at the time of sampling or at the start of conditioning therapy, and none of the patients received systemic steroid therapy or any other additional drugs before or during the conditioning therapy that could predispose to fluid retention. All patients received intravenous fluid supplementation according to the same highly standardized regimen, and intravenous furosemide therapy was also administered according to the same clinical guidelines to all patients. For patients with later fluid retention, the weight increase and the need for systemic furosemide started before the stem cell transplantation. None of the patients received prophylaxis or treatment for veno-occlusive disease. Finally, signs of preconditioning inflammation were defined as a CRP level ≥10 mg/L based on previous studies investigating the prognostic impact of preconditioning CRP levels in allotransplant recipients [7].

Patients could be stratified into groups for statistical analysis based on the following criteria: aGVHD was defined as GVHD requiring high-dose systemic steroid treatment before day 100 after allo-HSCT (i.e., grade B or higher according to the International Bone Marrow Transplant Registry grading system [25]), whereas pretransplantation inflammation was defined as CRP ≥10 mg/L (see above), and excessive fluid retention/overload was defined as at least 10% weight gain compared to the weight immediately before the start of conditioning therapy. Missing data were excluded from statistical analyses. The follow-up time for each patient was at least 26 months.

All samples were collected less than 14 days before start of conditioning therapy when the patients had a stable clinical situation without any clinical or laboratory signs of infectious or inflammatory diseases. For AML or MDS patients who had received previous chemotherapy, the samples were collected at least 4 weeks after the last treatment, and all AML patients were then in complete hematological remission at the time of sampling.

All patients were included after written informed consent and the study was conducted according to the guidelines of the Declaration of Helsinki. The regional ethics committee (REK) approved the inclusion of patient samples into a registered biobank (REK Vest 1759/2015) and the use of samples in the present study (REK Vest 305/2017).

Sample collection and analysis of metabolic profiles

All samples were collected from patients before the start of their pretransplant conditioning therapy. Blood was collected into sterile plastic tubes (BD Vacutainer SST Serum Separation Tubes; Becton-Dickenson; Franklin Lakes, NJ) and allowed to coagulate for 120 minutes at room temperature before centrifugation (300 *g* for 10 minutes). Serum was prepared within 2 hours, immediately aliquoted, and later stored at −80°C.

All samples were analyzed by Metabolon (Morrisville, NC) as described previously [27], using the Metabolon's nontargeted Global HD4 platform. The company is ISO 9001: 2015 certified for analytical and diagnostic testing of biological specimens. Raw data were extracted, peak-identified, and quality control processed using Metabolon's hardware and software. Metabolon maintains a library based on authenticated standards that contains the retention time/index, mass to charge ratio (m/z), and chromatographic data (including mass spectrum (MS)/MS spectral data) on all molecules present in the library. Biochemical identifications are based on three criteria: retention index within a narrow retention time/index window of the proposed identification, accurate mass match to the library ± 10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards.

Statistical and bioinformatical evaluation

After log transformation and imputation of any missing values, Welch's 2-sample *t*-test was used to test whether two means were different for two independent samples, and *P* values are given (statistical significance set as $P < .05$). The false discovery rate (FDR) for a given set of compounds is indicated by the *q*-value [28]. The overall data were analyzed using principal component analysis and the random forest technique [29]. The software tool MetaboAnalyst version 5.0 was used for statistical enrichment analysis. Hierarchical cluster analyses were performed using the J-Express software (MolMine AS, Bergen, Norway); all values were then median variance standardized and log(2) transformed. The Benjamini-Hochberg procedure was calculated using a false discovery rate of 20%. Additional statistical analyses, such as Fisher's exact test, were performed using the Statistical Package for the Social Sciences (SPSS Inc. Chicago, IL).

RESULTS

Nontargeted metabolomic profiling of patients before allogeneic HSCT

Using ultrahigh performance liquid chromatography-tandem mass spectrometry, we detected a total of 1274 biochemicals in serum of the 92 analyzed allotransplant patient samples. Of these, 940 biochemicals were fully characterized with known identity (named biochemicals constituted 73.8%), whereas 28 were named and partially characterized, and 306 were of unknown structural identity (nonidentified). The distribution of the 940 biochemicals into main metabolic pathways was as follows: lipids (24.7%), xenobiotics (18.8%), amino acids (17.4%), peptides (3.9%), nucleotides (3.4%), cofactors and vitamins (2.8%), carbohydrates (1.9%), and energy (0.8%) pathways, as well as 26.2% that were only partially characterized or nonidentified (Supplementary Figure S1).

Minimal overlap between individual metabolites associated with acute GVHD, inflammation and fluid retention

All patient samples were stratified into 3 comparison groups for statistical analysis after global untargeted metabolomics: (i) patients with and without aGVHD (32 versus 50 patients, respectively), (ii) patients with and without pretransplant inflammation (36 versus 56 patients, respectively), or (iii) patients with and without excessive fluid retention (37 versus 51 patients, respectively; missing data for 4 patients) (Figure 1). Ten patients were unclassified for aGVHD because of early mortality before day 100 (i.e., death before the traditional time limit for development of aGVHD) without signs of aGVHD; these patients died from sepsis with multiorgan failure (7 patients), multiorgan failure because of toxicity without documented infection (2 patients) and early AML relapse (1 patient). Four patients were not evaluated for fluid retention because of missing data.

The 1274 significantly altered biochemicals, including both identified and unidentified metabolites, that increased or decreased for patients with versus without fluid retention, inflammation or aGVHD is presented in Supplementary Table S1. With a significant *P* value corresponding to <.05 we would expect to find 47 differentially expressed metabolites by coincidence alone (5% of 940 metabolites). As can be seen from Supplementary Table S1, the number of identified metabolites was significantly higher than this when comparing groups with and without fluid inflammation (131 identified

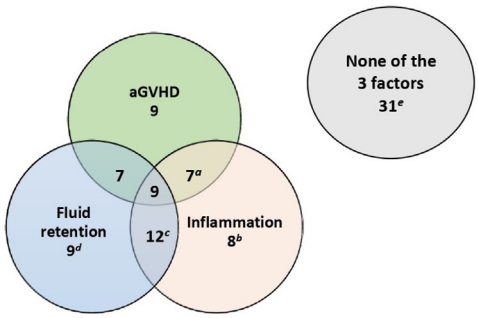

Figure 1. Stratification of the 92 allotransplant patients into transplantation-related risk factor groups. The Venn diagram presents the stratification of patients into the 3 comparison groups with or without the following factors: (i) aGVHD, (ii) excessive fluid retention, and (iii) pretransplantation inflammation, which were used for statistical analysis after samples were analyzed with the nontargeted HD4 metabolomics platform. A total of 31 patients did not have any of the 3 factors. There were 10 patients who were unclassified for aGVHD because of early death before day 100 and were therefore not included in this statistical comparison; these can be found among the following patient groups (superscript *b* includes 1 patient, superscript c includes 5 patients, superscript *d* includes 1 patient, and superscript *e* includes 3 patients); data were also missing for evaluation of fluid retention for 4 patients (superscript *a* includes 1 patient, and superscript *e* includes 3 patients).

metabolites), whereas the number of differentially expressed and identified biochemicals was lower for patients with/without aGVHD (72 identified metabolites) and with/without fluid retention (73 identified metabolites). Finally, there is an unbalance between metabolites that were increased versus decreased; if identification of significant metabolites was mainly based on coincidence, one would expect close to equal number of increased versus decreased levels among the significant metabolites.

The metabolic profiles associated with the 2 adverse prognostic parameters preconditioning inflammation and excessive fluid retention show only a minimal overlap

Nine patients had the 3 transplant-related risk factors aGVHD, inflammation, and excessive fluid retention (Figure 1). Even though previous clinical studies have demonstrated associations both for pretransplant signs of inflammation [7] and for early excessive fluid retention with nonrelapse mortality/aGVHD [30], these associations did not reach statistical significance in our present study that included relatively few patients (Fisher's exact test). Similarly, the individual metabolites that differed significantly when comparing patients with or without aGVHD, showed only a minor overlap with metabolites that significantly differed for patients with or without pretransplantation inflammation (Supplementary Table S2; 10 overlapping metabolites) and also with or without fluid retention (Supplementary Table S2; 3 overlapping metabolites). In contrast, there was a significant association between patients with pretransplantation inflammation and extensive fluid retention (Fisher's exact test, $P = .0078$); however, we identified only 18 overlapping metabolites that were significantly altered ($P < .05$) when comparing patients with versus without pretransplantation inflammation or excessive fluid retention (Supplementary Table S3; including 9 identified metabolites and 9 of unknown structural identity). The 9 identified metabolites were diverse, whereas 4 metabolites belong to amino acid metabolism. Among these 18 metabolites (Supplementary Table S3), the fold change ratio was similar for 13 of these metabolites with mostly increased levels. These 18 metabolites represent 17% and 10% of the metabolites associated with excessive fluid retention and inflammation, respectively.

An overall analysis of metabolic differences between patients with versus without excessive fluid retention/inflammation/GVHD; results from principal 2-component and random forest analyses

We compared the groups with versus without excessive fluid retention/inflammation/acute GVHD by using principal 2-component analyses, but this statistical method revealed very little separation between any of the groups. The results from one representative principal 2-component analyses are presented in Supplementary Figure S2 (for excessive fluid retention).

We also performed random forest analyses for all three comparisons and the overall results are shown in Supplementary Table S1. The predictive accuracy was relatively low for all the comparison groups (60-67%) and should be interpreted with caution. However, an overview of the 30 top-ranked metabolites for each comparison group (aGVHD/inflammation/fluid retention) showed that especially amino acid metabolites/metabolism differed between the groups for all 3 comparisons (Supplementary Figures S3, S4, and S5).

Comparison of metabolite profiles for patients with and without later aGVHD; significantly increased levels of several amino acid metabolites in aGVHD pretransplant patient samples

A total of 89 metabolites differed significantly ($P < .05$, Welch's two-sample *t*-test) between patients with and without later development of aGVHD; 72 of these metabolites were identified whereas 17 were of unknown structural identity. The overall results are summarized in Table 2.

First, the 27 amino acid metabolites constituted a major subset, and most of these metabolites showed increased levels for patients later developing aGVHD. These amino acid metabolites reflected differences especially in tryptophan metabolism (5 metabolites), which is in accordance with the random forest analysis (see below). Also, additional differences were found for lysine metabolism (6 metabolites) and leucine, isoleucine, and valine metabolism (6 metabolites). Although we cannot exclude the possibility that some of these metabolites reached statistical significance because of coincidence, several observations strongly suggest that there is only a minor impact of coincidence when looking at the overall results for the amino acid metabolites.

- Using a P value = .05, we would only expect to identify a maximum of 11 metabolites by coincidence (of the 222 amino acid metabolites detected in our analysis). The possibility of identifying 27 significantly altered amino acid metabolites by coincidence alone is very low (Binomial test, $P = .00002$).
- The frequency of metabolites reaching statistical significance is significantly higher for amino acid metabolites (27 out of 222) compared with non-amino acid metabolites (45 out of 718; Fisher's exact test, $P = .0058$).
- If metabolites were identified by coincidence, we would expect equal numbers of metabolites with increased and decreased levels in aGVHD patients. However, all except 4 amino acid metabolites showed increased levels, and this is a highly significant difference from an equal distribution (Binomial test, $P = .00026$).

We identified a very heterogeneous group of 25 lipid metabolites; the largest single subset was four metabolites reflecting altered androgenic steroid metabolism. Third, a smaller and heterogeneous subset of xenobiotics was also identified. Fourth, only a small number of carbohydrate/energy and nucleotide metabolites reached statistical significance (data not shown). In contrast to the amino acid metabolites, the metabolites belonging to lipid and xenobiotic classes did not differ significantly with regard to the number of metabolites expected by coincidence alone, differing metabolites in each of the groups compared with the other metabolic subsets, or the increased versus decreased levels for identified metabolites.

We also did a pathway enrichment analysis of the significantly altered metabolites ($P < .05$) identified for patients with versus without later development of aGVHD, and the most enriched pathways reflected differences in amino acid metabolism, including taurine, tryptophan, biotin, malate-aspartate shuttle and arginine- and ornithine metabolism (Figure 2).

The twenty individual metabolites showing the most significant differences ($P < .01$) when comparing preconditioning metabolic profiles for patients with and without later aGVHD are listed in Supplementary Table S5. These metabolites included eight amino acid metabolites (three associated with tryptophan metabolism), 2 peptides, 2 lipids, 1 xenobiotic, and

Table 2
Significantly Altered Metabolites in Pretransplantation Patients With a Later Development of aGVHD Compared to Patients Who Did Not Develop aGVHD

Main Class and Subclass and Number of Metabolites	No.	Metabolite Name
Amino acid	27	
Alanine and aspartate metabolism	1	*Aspartate*
Histidine metabolism	2	Hydantoin-5-propionate, *1-methylhistamine*
Lysine metabolism	6	Lysine, N2-acetyllysine, fructosyllysine, 2-aminoadipate, pipecolate, N-acetyl-2-aminoadipate,
Tyrosine metabolism	1	Tyramine O-sulfate
Tryptophan metabolism	5	N-acetylkynurenine (2), kynurenate, N-formylanthranilic acid, xanthurenate, *serotonin*
Leucine, isoleucine and valine metabolism	6	Isovalerylglycine, isovalerylcarnitine (C5), β-hydroxyisovaleroylcarnitine, N-carbamoylvaline, isobutyrylcarnitine (C4), 3-hydroxyisobutyrate
Methionine, cysteine, SAM and taurine metabolism	2	*Hypotaurine, taurine*
Urea cycle; arginine and proline metabolism	2	Urea, argininate
Creatine metabolism	1	Creatinine
Guanidino and acetamido metabolism	1	Guanidinosuccinate
Peptide	8	
Dipeptide	3	*Glycylvaline, threonylphenylalanine, valylglycine*
Polypeptide	1	*glu-gly-asn-val*
Fibrinogen cleavage peptide	2	Fibrinopeptide A, Fibrinopeptide B (1-9)
Acetylated peptides	2	Phenylacetylcarnitine, phenylacetylglutamine
Lipid	25	
Long-chain saturated fatty acid	2	*Nonadecanoate (19:0), arachidate (20:0)*
Long chain monounsaturated fatty acid	1	*Erucate (22:1n9)*
Long-chain polyunsaturated fatty acid (n3 and n6)	3	*Stearidonate (18:4n3), nisinate (24:6n3), docosadienoate (22:2n6)*
Fatty acid metabolism (also BCAA metabolism)	2	Propionylcarnitine (C3), 2-methylmalonylcarnitine (C4-DC)
Fatty acid metabolism (acyl glycine)	1	Picolinoylglycine
Docosanoid	1	*14-HDoHE/17-HDoHE*
Endocannabinoid	2	*N-stearoyltaurine, N-stearoylserine*
Inositol metabolism	1	Myo-inositol
Phosphatidylserine (PS)	1	*1-stearoyl-2-oleoyl-GPS (18:0/18:1)*
Lysophospholipid	1	*1-stearoyl-GPS (18:0)*
Sphingosines	2	*Sphingosine, sphingosine 1-phosphate*
Sterol	1	*Beta-sitosterol*
Corticosteroids	1	Cortolone glucuronide (1)
Androgenic steroids	4	11β-hydroxyetiocholanolone glucuronide, 5α-androstan-3α,17α-diol monosulfate, 5α-androstan-3α,17α-diol disulfate, 11β-hydroxyandrosterone glucuronide
Primary bile acid metabolism	1	*Glycochenodeoxycholate 3-sulfate*
Secondary bile acid metabolism	1	Glycoursodeoxycholic acid sulfate (2)
Xenobiotic	9	
Benzoate metabolism	4	4-methylguaiacol sulfate, 4-methylcatechol sulfate, p-cresol sulfate, 3-phenylpropionate (hydrocinnamate)
Food component/plant	2	4-vinylguaiacol sulfate, vanillic acid glycine
Bacterial/fungal	1	1H-indole-7-acetic acid
Chemical	2	3,5-dichloro-2,6-dihydroxybenzoic acid, 3-bromo-5-chloro-2,6-dihydroxybenzoic acid

Systemic levels of metabolites that were significantly decreased in patients with aGVHD are shown in italics, whereas the other listed metabolites were significantly increased. The metabolites were classified into main metabolic (sub)classes, with the number of metabolites in each main class/subclass shown in parenthesis. Only the 72 named metabolites are shown in the table (of the 89 identified metabolites), and only main classes were at least 5 significantly altered metabolites were found are presented (i.e., 69 of the 72 named metabolites).

7 unidentified metabolites. Based on our overall analyses, we conclude that patients with and without later aGVHD differ in their pretransplantation metabolic profiles and the most important difference is altered levels of several amino acid metabolites.

Comparison of metabolic profiles for patients with and without preconditioning signs of inflammation; decreased levels of several amino acid metabolites is associated with inflammation

A total of 180 metabolites differed significantly ($P < .05$, Welch's 2-sample *t*-test) between patients with and without preconditioning signs of inflammation when serum samples were analyzed with the nontargeted HD4 Metabolon platform. Of these, 131 metabolites were identified and named, whereas 49 had unknown structural identity. These identified metabolites belong to different main metabolic pathways, and Table 3 lists only the main classes that included at least 5 metabolites in each class.

First, of the 117 metabolites, 38 metabolites were significantly altered for amino acid metabolism, mainly associated with histidine, leucine/isoleucine/valine, and methionine/cysteine/S-adenosylmethionine (SAM)/taurine metabolism. Decreased levels of 9 amino acids were seen (i.e., glycine, serine, threonine, alanine, asparagine, histidine, lysine, tryptophan, and methionine). Although we cannot exclude the

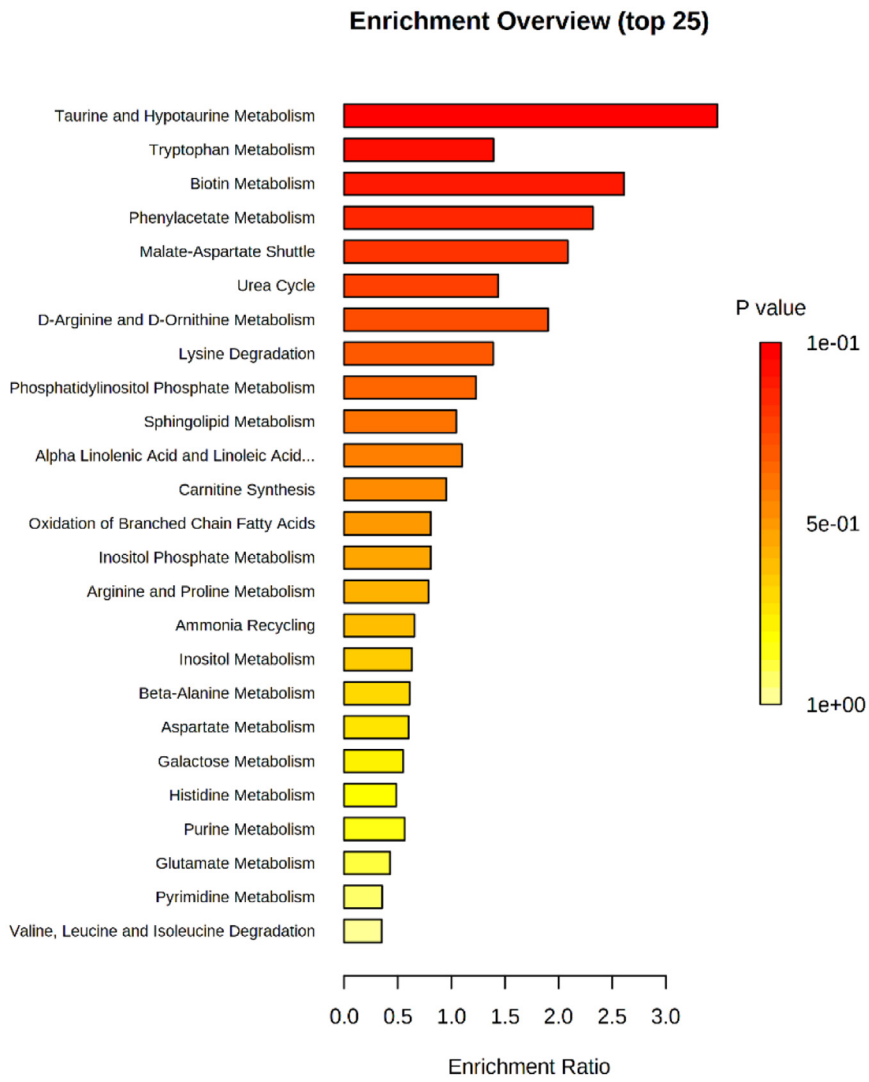

Figure 2. Metabolite pathway enrichment analysis to identify pathways enriched in the patient group with aGVHD compared to no aGVHD. The software tool MetaboAnalyst was used for analysis, based on the significantly altered identified metabolites with $P < .05$. The pathways with most significant P values are shown in *red*, whereas the least significant are in *yellow* (one-tailed P values).

possibility that some of these metabolites reached statistical significance because of coincidence, several observations strongly suggest that there is only a relatively small impact of coincidence for the whole group of amino acid metabolites.

- Using a P value of .05, we would only expect to identify a maximum of 11 metabolites by coincidence (of the 222 amino acid metabolites identified in our metabolomic analysis). The possibility of identifying 38 amino acid metabolites by coincidence alone is very low (Binomial test, $P < .00002$).
- If metabolites were identified by coincidence, we would expect equal numbers of metabolites with increased and decreased levels in patients with increased pretransplant CRP levels. However, all except one amino acid metabolite showed decreased levels, and this is a highly significant difference from an equal distribution (Binomial test, $P < .000002$).

Twelve peptide metabolites were also altered, and most of them were increased levels of metabolites derived from fibrinogen. Second, alteration of a large and heterogeneous group of 27 lipid metabolites was found. Third, xenobiotic metabolites included a large group of 29 metabolites; 12 of them reflected benzoate metabolism and 15 were food components. Metabolites reflecting carbohydrate/energy metabolites were less than five in each class and were therefore not listed. We performed a pathway enrichment analysis including all metabolites that differed significantly ($P < .05$) when comparing patients with and without pretransplant inflammation. Increased pretransplant CRP was associated with relatively weak modulation of a wide range of metabolic pathways, the only strong effect being altered amino acid metabolism and especially methylhistidine metabolism, but several other amino acid pathways were also included among the 25 top-ranked pathways (Supplementary Figure S6).

Of the metabolites that differed significantly between patients with and without pretransplant inflammation, 56 metabolites showed a P value <.01 (Supplementary Table S6). Of these, 33 were identified/named metabolites, 13 were classified as amino acids (plus 1 fibrinogen peptide), 8 lipid metabolites, and 6 xenobiotic metabolites (including 2 food components). Finally, we would emphasize that mannose and retinol (vitamin A) were the two most significant metabolites (q-values were 0.0004 and 0.0431, respectively).

Table 3
Significantly Altered Metabolites ($P < .05$) in Serum Samples From Patients With Pretransplantation Inflammation (CRP ≥10 mg/L) Compared to No Inflammation

Main Class/Subclass of Metabolites (Number of Metabolites)	No.	Metabolite Name
Amino acid	38	
Glycine, serine, and threonine metabolism	5	Glycine, sarcosine, serine, threonine, O-acetylhomoserine
Alanine and aspartate metabolism	2	Alanine, asparagine
Histidine metabolism	6	Histidine, 1-methylhistidine, 3-methylhistidine, N-acetyl-3-methylhistidine, hydantoin-5-propionate, 1-methyl-5-imidazolelactate
Lysine metabolism	3	Lysine, N6,N6,N6-trimethyllysine, 6-oxopiperidine-2-carboxylate
Tyrosine metabolism	1	Catechol glucuronide
Tryptophan metabolism	2	Tryptophan, 6-bromotryptophan
Leucine, isoleucine, and valine metabolism	8	N-acetylleucine, 4-methyl-2-oxopentanoate, α-hydroxyisocaproate, 2-keto-caprylate, Isovalerylglycine, N-acetylisoleucine, 3-methyl-2-oxovalerate, 2,3-dihydroxy-2-methylbutyrate
Methionine, cysteine, SAM, and taurine metabolism	7	Methionine, N-acetylmethionine, S-methylmethionine, S-methylcysteine, S-methylcysteine sulfoxide, cysteine s-sulfate, cysteine sulfinic acid
Urea cycle, arginine, and proline metabolism	1	trans-4-hydroxyproline
Creatine metabolism	1	Guanidinoacetate
Polyamine metabolism	1	*5-methylthioadenosine (MTA)*
Glutathione metabolism	1	2-aminobutyrate
Peptide	12	
Gamma-glutamyl amino acid	3	Gamma-glutamylhistidine, gamma-glutamylmethionine, gamma-glutamylserine
Fibrinogen cleavage peptide	9	*Fibrinopeptide A, Fibrinopeptide A (2-15), Fibrinopeptide A (3-16), Fibrinopeptide A (5-16), Fibrinopeptide A (7-16), Fibrinopeptide A (8-16), Fibrinopeptide A, des-ala(1), Fibrinopeptide A, phos-phono-ser(3), Fibrinopeptide B (1-13)*
Lipid	27	
Fatty acid synthesis	1	Malonate
Long-chain polyunsaturated fatty acid (n3 and n6)	1	Nisinate (24:6n3)
Fatty acid, dicarboxylate	4	Undecanedioate (C11-DC), dodecanedioate (C12-DC), dodecenedioate (C12:1-DC), 3-carboxy-4-methyl-5-pentyl-2-furanpropionate (3-CMPFP)
Fatty acid, amino	2	2-aminooctanoate, N-acetyl-2-aminooctanoate
Fatty acid metabolism (also BCAA metabolism)	1	Propionylglycine
Fatty acid, monohydroxy	1	2-hydroxyoctanoate
Fatty acid, dihydroxy	1	*3,4-dihydroxybutyrate*
Endocannabinoid	3	N-stearoyltaurine, N-stearoylserine, N-palmitoylserine
Phospholipid metabolism	1	Glycerophosphorylcholine (GPC)
Lysophospholipid	2	1-oleoyl-GPA (18:1), 1-linoleoyl-GPA (18:2)
Glycerolipid metabolism	1	Glycerol 3-phosphate
Mevalonate metabolism	1	3-Hydroxy-3-methylglutarate
Sterol	1	3β-Hydroxy-5-cholestenoate
Pregnenolone steroids	1	*Pregnenediol disulfate (C21H34O8S2)*
Progestin steroids	1	5α-Pregnan-3β,20α-diol monosulfate (2)
Androgenic steroids	1	*Androstenediol (3β,17β) disulfate (1)*
Primary bile acid metabolism	1	Cholate
Secondary bile acid metabolism	3	Ursodeoxycholate, taurocholenate sulfate, 3b-hydroxy-5-cholenoic acid
Nucleotide	6	
Purine metabolism, (hypo)xanthine/inosine containing	2	N1-methylinosine Urate
Purine metabolism, adenine containing	1	*N1-methyladenosine*
Pyrimidine metabolism, uracil containing	2	5-methyluridine (ribothymidine), 3-ureidopropionate
Pyrimidine metabolism, cytidine containing	1	*Cytidine*
Cofactors/vitamins	5	
Nicotinate and nicotinamide metabolism	1	Trigonelline (N'-methylnicotinate)
Pantothenate/CoA metabolism	1	Pantoate
Tocopherol metabolism	1	Gamma-CEHC
Hemoglobin and porphyrin metabolism	1	Heme
Vitamin A metabolism	1	Retinol (Vitamin A)
Xenobiotics	29	
Benzoate metabolism	12	Catechol sulfate, 4-methylguaiacol sulfate, guaiacol sulfate, 3-methyl catechol sulfate, 4-ethylcatechol sulfate, 4-allylcatechol sulfate, methyl-4-hydroxyben-zoate, 4-acetylphenol sulfate, 3-methoxycatechol sulfate, 3-methoxycatechol sulfate, methyl-4-hydroxybenzoate sulfate, o-cresol sulfate

(*continued*)

Table 3 (*Continued*)

Main Class/Subclass of Metabolites (Number of Metabolites)	No.	Metabolite Name
Xanthine metabolism	2	3-methylxanthine, 7-methylxanthine
Food component/plant	15	Levulinate (4-oxovalerate), 2,3-dihydroxyisovalerate, dihydrocaffeate sulfate, dihydroferulate, dihydroferulic acid, sulfate, 3-ethylcatechol sulfate. Ferulylglycine, ferulylglycine, homostachydrine, menthol glucuronide, isoeugenol sulfate, syringol sulfate, (2,4 or 2,5)-dimethylphenol sulfate, vanillic acid glycine, 4-ethyl-2-methoxyphenol sulfate
Drug–topical agents	2	2,6-dihydroxybenzoic acid, hydroquinone sulfate
Chemical	6	Ethyl glucuronide, 3-acetylphenol sulfate, dimethyl sulfone, 1,2,3-benzenetriol sulfate, 2-methoxyresorcinol sulfate, 3-hydroxy-2-methylpyridine sulfate

Significantly increased systemic levels of metabolites in patients with inflammation are shown in italics, whereas the other metabolites listed were significantly decreased. The left part of the table lists the main class/subclass that the metabolites belong to and the number of metabolites in each metabolic class. Only main classes including at least 5 metabolites are listed (i.e., 117 of the 131 identified and named metabolites are shown).

Based on our overall analyses, we conclude that patients with and without preconditioning signs of inflammation differ in their pretransplant metabolic profiles, and similar to the aGVHD patient/comparison group this difference is mainly caused by altered levels of amino acid metabolites though there is a minimal overlap between individual metabolites and inflammation is mainly associated with decreased and not increased levels of amino acid metabolites.

Comparison of metabolic profiles for patients with and without excessive early fluid retention; association between fluid retention and altered amino acid metabolism is a main observation

A total of 107 metabolites differed significantly ($P < .05$, Welch's 2-sample *t*-test) between patients with and without excessive fluid retention when samples were analyzed by the nontargeted HD4 Metabolon platform; 74 of the metabolites could be classified whereas 33 were of unknown structural identity (Table 4).

The significantly altered metabolites were classified into three main metabolic pathways; amino acid metabolism, nucleotide metabolism and xenobiotics. These included 58 of the 71 known/named metabolites that differed significantly between patients with and without excessive fluid retention. Most metabolites differing between patients with and without excessive fluid retention are amino acids (38 metabolites) and unknown metabolites (33 metabolites), although also ten nucleotide metabolites and ten xenobiotics were detected. Altered amino acid metabolism included seven metabolites that reflected differences in branched-chain amino acid metabolism, seven metabolites that were associated with methionine, cysteine, SAM and taurine metabolism and six metabolites reflecting tryptophan metabolism. Taurine ($P = .0458$) is one of the major osmolytes in the renal medulla and possibly a regulator of renal blood flow.

We cannot exclude the possibility that some amino acid metabolites reached statistical significance by coincidence, but several observations suggest that the impact of coincidence is limited.

- Using a *P* value of .05, we would expect to identify a maximum of 11 metabolites by coincidence (of the 222 amino acid metabolites detected in our analysis). The possibility of identifying 38 amino acid metabolites by coincidence alone is low (Binomial test, $P = .00002$).
- The frequency of metabolites reaching statistical significance is higher for amino acid metabolites (38 out of 222) compared with non-amino acid metabolites (36 out of 718; Fisher's exact test, $P < .00001$).
- If metabolites were identified by coincidence, we would expect equal numbers of metabolites with increased and decreased levels for fluid retention patients. However, all metabolites except nine amino acid metabolites showed increased levels, and this is significantly different from an equal distribution (Binomial test, $P = .00103$).

The nucleotide metabolism group included four metabolites reflecting pyrimidine metabolism and six metabolites associated with purine metabolism. All these metabolites showed increased levels, and the fraction of differing nucleotide metabolites was significantly different from the fraction of non-nucleotide metabolites (Fisher's exact test, $P = .00213$).

Of the 74 significantly altered metabolites, ten were classified as xenobiotics (including five food components); however, only three such metabolites were among the 30 top-ranked metabolites in the random forest analysis (see results below). Finally, the large majority of significantly altered metabolites showed decreased levels for patients with excessive fluid retention (85 metabolites increased, 22 metabolites decreased).

We did a pathway enrichment analysis based on all metabolites that differed significantly when comparing patients with and without excessive early fluid retention. Increased fluid retention was associated especially with altered taurine and hypotaurine metabolism (Supplementary Figure S7).

Based on our overall analyses, we reached the same conclusion when comparing groups with and without pretransplantation fluid retention as for the GVHD/inflammation group comparisons; the major difference between patients is mainly caused by altered levels of amino acid metabolites whereas other metabolites show less variation; however, nucleotide metabolites may be a possible exception that show a relatively high fraction of differing metabolites.

Analysis of single metabolites versus metabolic profiles (i.e., metabolite classes): the results from Benjamini-Hochberg analyses

Our main results are based on the identification of metabolite classes/metabolic profiles and not on single metabolites and based on the statistical analyses described above, the contribution of coincidental metabolites among significant groups is low although it cannot be excluded. To further analyze differences between patients with regard to single metabolites, we performed Benjamini-Hochberg analyses to adjust for the number of comparisons when identifying significantly altered metabolites. When comparing patients with and without later aGVHD, none of the single metabolites remained significant after this analysis. For the fluid retention comparison group,

Table 4
Significantly Altered Metabolites ($P < .05$) in Pretransplantation Serum Samples When Comparing Patients With and Without Postconditioning Excessive Fluid Retention

Main Class/Subclass	No.	Metabolite Name
Amino acid	38	
Glycine, serine and threonine metabolism	3	Betaine, N-acetylserine, N-acetylthreonine
Alanine and aspartate metabolism	2	N-acetylalanine, hydroxyasparagine
Histidine metabolism	1	1-ribosyl-imidazoleacetate
Lysine metabolism	1	hydroxy-N6,N6,N6-trimethyllysine
Phenylalanine metabolism	1	2-hydroxyphenylacetate
Tyrosine metabolism	2	Vanillactate, 3-methoxytyrosine
Tryptophan metabolism	6	*Tryptophan betaine*, kynurenate, 8-methoxykynurenate, *serotonin*, indolelactate, indole-3-carboxylate
Leucine, isoleucine, and valine metabolism	8	*4-methyl-2-oxopentanoate*, 3-methylglutaconate, 3-methylglutarylcarnitine, *3-methyl-2-oxovalerate*, ethylmalonate, methylsuccinoylcarnitine, *3-methyl-2-oxobutyrate* Butyrylclycine (can be regarded both as a fatty acid and branched amino acid metabolite)
Methionine, cysteine, SAM and taurine metabolism	7	N-formylmethionine, N-acetylmethionine sulfoxide, 5-methylthioribose, 2,3-dihydroxy-5-methylthio-4-pentenoate (DMTPA), *cysteine*, lanthionine, *taurine*
Urea cycle, arginine and proline metabolism	2	Ornithine, 3-amino-2-piperidone, dimethylarginine (SDMA + ADMA)
Polyamine metabolism	2	5-methylthioadenosine (MTA), 4-acetamidobutanoate
Guanidino and acetamido metabolism	1	1-methylguanidine
Glutathione metabolism	2	*Cysteinylglycine, 2-aminobutyrate*
Nucleotide	10	
Purine metabolism, (hypo)xanthine/inosine containing	1	N1-methylinosine
Purine metabolism, adenine containing	4	N6-methyladenosine, N6-carbamoylthreonyladenosine, N6-succinyladenosine, N2,N2-dimethylguanosine
Purine metabolism, guanine containing	1	N2,N2-dimethylguanosine
Pyrimidine metabolism, uracil containing	3	Pseudouridine, 5,6-dihydrouridine, 3-(3-amino-3-carboxypropyl)uridine
Pyrimidine metabolism, cytidine containing	1	Cytidine
Xenobiotics	10	
Food component/plant	5	Erythritol, fucitol, mannonate, S-allylcysteine, ethyl α-glucopyranoside, vanillate glucuronide
Bacterial/fungal	1	N-methylpipecolate
Chemical	4	Diglycerol, triethanolamine, 2-methoxyresorcinol sulfate, thioproline

Metabolites that were decreased in patient samples with fluid retention are shown in italics, whereas the other metabolites showed increased levels. The left part of the table lists the main class/subclass that the metabolites belong to and the number of metabolites in each metabolic class. Only main classes including at least five metabolites in each class are listed.

4 unidentified/unnamed metabolites remained significant. Finally, for the inflammation comparison group, a heterogeneous group of amino acids (serine, asparagine, histidine, hydantoin-5-propionate, α-hydroxyisocaproate), lipids (malonate, nisinate (24:6n3), 1-oleoyl-GPA (18:1), 3β-hydroxy-5-cholenoic acid), carbohydrate (mannose), cofactors and vitamins (retinol/vitamin A), nucleotides (cytidine, 3-ureidopropionate), and exobiotic (menthol glucuronide) remained significant together with 8 unidentified metabolites. In our final hierarchical cluster analyses, we therefore focused on metabolite profiles formed by a group of metabolites (amino acids) and not on single metabolites. Focusing on metabolite profiles are also consistent with the function of amino acids and amino acid metabolites as a bioregulatory network [14-17,31,32].

Pretransplantation metabolomics profiles of allotransplant recipients: identification of patient subsets with increased frequency of TRM within 2 years after transplantation

As described below, we performed 2 unsupervised hierarchical cluster analyses based on metabolites identified in our 3 comparisons (i.e., transplantation-related risk factor groups) (Tables 2-4) and to limit the impact of coincidental changes in metabolite levels on the overall results, the analyses were either based on (i) metabolites reaching a P value $< .01$ or (ii) only the subclass of amino acids because statistical analysis suggested that this subclass includes only a limited number of metabolites identified by coincidence in our study.

As can be seen in Tables 2-4, there was minimal overlap between the individual metabolites that reached statistical significance for each of the three comparison/patient groups. None of the identified metabolites reached significance for all three comparisons, and some of the overlapping metabolites showed divergent effects (i.e., increased versus decreased) in two comparisons. We therefore investigated the metabolomic profiles for metabolites that were identified in the aGVHD comparison (i.e., excluding metabolites associated with the other 2 risk factors), and, to further reduce the impact of metabolites identified by coincidence, we only included metabolites with $P < .01$. We thus performed an unsupervised hierarchical cluster analysis based on the 13 identified/named metabolites that were significantly altered ($P < .01$, see Supplementary Table S5) when comparing the pretransplantation metabolomics profiles for patients with and without later aGVHD. These 13 metabolites included 10 amino acid metabolites/peptides, 2 lipid metabolites, and 1 xenobiotic; the 7 unidentified metabolites were left out from this clustering analysis (Supplementary Table S5). The clustering analysis is presented in Figure 3, and the patients could be classified into 2 main subsets. The right cluster included 26 patients with

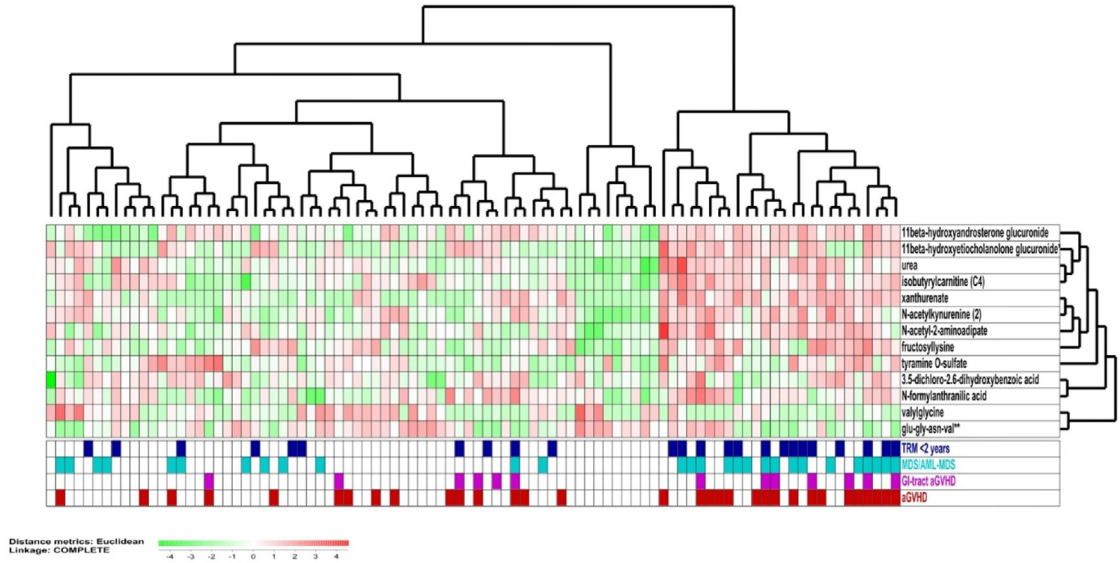

Figure 3. The pretransplantation metabolomics heterogeneity of allotransplant recipients. The figure presents an unsupervised hierarchical cluster analysis, including 92 patients receiving their first allogeneic stem cell transplant (shown at the top of the figure), and the analysis is based on the 13 identified metabolites that were significantly altered ($P < .01$) when comparing patients with and without steroid-requiring aGVHD. Altered metabolites, along with patient characteristics, are shown to the far right.

generally high pretransplantation metabolite levels whereas the left main cluster included 66 patients with generally lower metabolite levels.

Later aGVHD was associated with increased levels for most of the amino acid metabolites that differed significantly between patient groups (i.e., with versus without later aGVHD). This cluster analysis showed that increased levels of amino acid metabolites were seen especially for a minor subset of 26 patients (right main cluster), and this patient subset was also characterized by high frequencies of patients with steroid-requiring aGVHD, previous MDS and increased TRM within the first 2 years after transplantation (Table 5). The 2 main clusters did not differ significantly with regard to patient age, sex, body mass index, or sample storage time before metabolomics analysis (data not shown). Finally, the 2 main clusters did not differ with regard to the frequencies of patients with pretransplantation signs of inflammation or early excessive fluid retention (data not shown).

To investigate the metabolite profiles based on our overall results (i.e. metabolites associated with both aGVHD disease and the other two risk factors), we also did an unsupervised hierarchical clustering based on only the amino acid metabolites identified in all three comparisons (Figure 4). The analysis identified two main patient subsets. The upper main patient cluster included 62 patients and had a lower frequency of patients with TRM within two years posttransplant (8 out of 62 patients) than the lower main patient cluster (16 out of 30 patients, Fisher's exact test, $P = .0001$). The lower main cluster showed a slightly higher patient age than patients in the upper main cluster (mean age 55.3 versus 48.8 years, Mann-Whitney U-test, $P = .0271$), but the 2 clusters did not differ with regard to sex, previous MDS, absolute pretransplant CRP level (mg/L), absolute weight gain (kg) or frequencies of patients with later aGVHD, pretransplantation signs of inflammation, or early fluid retention.

The 2 patient subsets identified differed with regard to conditioning therapy (Figure 4); 29 of the 30 patients in the lower main cluster received BuCy myeloablative conditioning whereas the conditioning therapy for the 62 patients in the larger main upper cluster varied and only 23 patients received BuCy conditioning. We compared the TRM after 2 years only for the patients receiving BuCy conditioning, (i.e., for 2 patient subsets receiving the same conditioning but differing in their pretransplant amino acid profiles). Fifteen of the 29 BuCy

Table 5
A comparison of the two main patient clusters identified in the unsupervised hierarchical cluster analysis (Figure 3) for the 92 patients receiving their first allotransplantation

Patient Parameter	Left Patient Subset*	Right Patient Subset†	*P* Value
Steroid-requiring aGVHD‡	15 patients	17 patients	.0144
Gastrointestinal aGVHD‡	6 patients	7 patients	.433
MDS/AML-MDS	12 patients	16 patients	.0001
Overall nonrelapse death before day 100	9 patients	6 patients	ns
TRM within 2 years	10	14	.0004

The analysis was based on the 13 identified/named metabolites that were significantly altered ($P < .01$) when comparing patients with and without steroid-requiring aGVHD (Supplementary Table S5). The table presents the characteristics that differed significantly between the 2 main clusters; the numbers/values for each of the 2 clusters are presented together with the corresponding *P* value (Fisher's exact test for categorized variables). The *P* values are not corrected for the number of comparisons.

* The main cluster including 66 patients.
† The main cluster including 26 patients.
‡ Six patients in the right subset and four patients in the left subset died before day 100 without aGVHD and were therefore excluded from the statistical analysis.

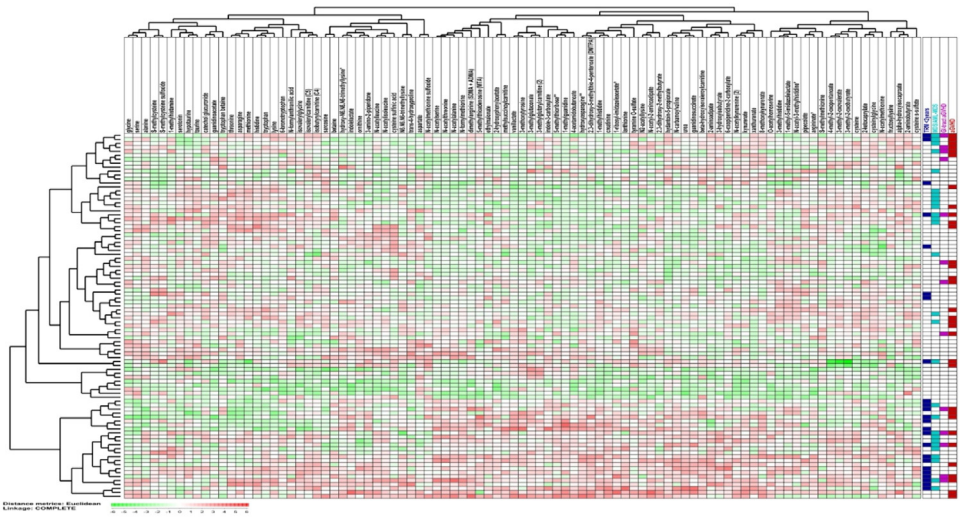

Figure 4. The pretransplantation metabolomic heterogeneity of patients treated with allogeneic stem cell transplantation. The figure presents an unsupervised hierarchical cluster analysis based on the 93 identified amino acid metabolites that differed significantly when comparing patients with versus without aGVHD/inflammation/fluid retention (Tables 2-4). The left part of the figure shows the clustering of the 92 patients receiving their first allogeneic stem cell transplant, and the top part of the figure shows the clustering of amino acids. The clinical characteristics of the patients are presented to the right in the figure.

patients among the patients within the lower main cluster died of TRM within 2 years after transplantation, whereas only 4 of the 23 BuCy patients within the other main cluster (i.e. among the 62 patients in the upper main cluster) showed TRM within the first 2 years after transplant (Fisher's exact test, $P = .0193$). These 2 groups of BuCy patients did not differ with regard to age. Thus the association between the amino acid metabolic profile and TRM is also seen when comparing patients receiving the same conditioning therapy (i.e., it does not depend on differences in conditioning intensity between the 2 main patient subsets identified in Figure 4). However, there was a significant difference in age between BuCy patients included in the upper cluster (with lower incidence of TRM) (mean age 44.5 years) and the lower cluster (with higher incidence of TRM) (mean age 54.1 years; Mann-Whitney U-test, $P = .0056$) suggesting that age-dependent differences may contribute to the amino acid profile of patients associated with higher TRM in the lower main cluster of our analysis.

DISCUSSION

Metabolites are important regulators of vascular, renal, endothelial and gastrointestinal functions, as well as the functions of various immunocompetent cells (Supplementary Tables S4, S7, S8). Furthermore, pretransplantation inflammation (altered immunocompetent cell activity), early postconditioning excessive fluid retention (modulated renal/vascular/endothelial function), and development of immune-mediated aGVHD are associated with adverse prognosis after allo-HSCT [33–35], and our present results show that all 3 factors are associated with specific modulations of the systemic pretransplantation metabolic profiles especially involving amino acid metabolism. However, there was only a minimal overlap between the metabolites associated with aGVHD and inflammation/fluid retention; these last 2 factors showed altered levels of other metabolites involved in regulation of inflammation or microvascular functions, respectively. Thus the prognostic impact of inflammation/fluid retention in allo-HSCT recipients is not caused by a further modulation/exacerbation of GVHD-associated metabolic alterations. Finally, severe morbidity/mortality after allo-HSCT increases with age [36], and additional aging-associated metabolic modulation may also contribute to the metabolic heterogeneity even though our patients were relatively young [37].

Modulation of systemic amino acid profiles are associated with prognosis in patients with colorectal [38], renal [39,40], lung [41], as well as head and neck cancer [42,43]; and, in patients with colorectal cancer, decreased levels of glutamine and histidine together with increased levels of phenylalanine are associated with systemic signs of inflammation [38]. The potential prognostic impact/association of systemic amino acid levels has not been investigated in human AML/MDS.

Transplantation-related death can be caused by infections, immune-mediated GVHD and vascular complications, and various immunocompetent cells, as well as endothelial cells, are important in the pathogenesis of these complications. Amino acids are important regulators of activation and function of these cells. First, various amino acids are important functional regulators of monocyte phenotypic polarization and macrophage activation (arginine, tryptophan, glutamine, branched chain amino acids, serine, glycine), dendritic cells (branched amino acids), T-cell activation and differentiation into various subsets (e.g., central memory/arginine, regulatory T cells/glutamine, T-cell suppression/phenylalanine, and serine/T cell activation/cysteine), regulatory T-cell development (glutamine, tryptophan), polymorphonuclear and monocytic myeloid-derived suppressor cells (arginine) [14,15,17,32,44,45]. Second, endothelial cells are important for immunoregulation and transvascular migration of immunocompetent cells (e.g., during infections or GVHD) [46,47], but endothelial dysfunction is also important in the pathogenesis of several other severe and potentially lethal post-transplantation complications [12]. A wide range of amino acids are involved in the regulation of endothelial functions, including both nonessential (glycine, proline, serine, cysteine, glutamine, aspargine, arginine) and essential amino acids (tryptophan, methionine, phenylalanine, branched-chain amino acids) [16,48]. Finally, amino acids are important in the regulation of gastrointestinal and skin inflammation [31,49], 2 organs that are commonly involved in aGVHD [25]. These observations illustrate that amino acids and amino acid metabolites function as a

biological network as will be further discussed below. Finally, several drugs for targeting of amino acid metabolism are now available for preclinical testing or in early clinical studies [23,50], although to the best of our knowledge they have not been evaluated in allotransplantation.

Acute GVHD was mainly associated with increased pretransplantation levels of amino acids metabolites. First, tryptophan is metabolized to kynurenines; immune cells are both sources and functional targets of kynurenines, possibly through negative feedback mechanisms [51] that inhibit T-cell proliferation and facilitate regulatory T-cell development [45]. Tryptophan metabolism is also important for gut immunoregulation [51] and the balance between immune tolerance and microbiota maintenance [45]. Second, several metabolites belonging to lysine metabolism were increased in patients who later had aGVHD; these levels are determined by the balance between intake, microbial production, and mitochondria-dependent catabolism mainly in the liver [52]. Third, later aGVHD was also associated with pretransplant alterations of branched amino acid metabolism; these amino acids are also involved in immunoregulation through effects on the phosphoinositide 3 kinase-Akt-mammalian target of rapamycin (PI3K-Akt-mTOR) pathway [44,53]. Finally, aGVHD was also associated with altered levels of several xenobiotics and a relatively large and heterogeneous group of lipid metabolites. Taken together these observations suggest that altered pretransplantation gastrointestinal or nutritional status contributes to the pretransplantation metabolomic modulation associated with later aGVHD.

Patients with signs of inflammation differed from the other patients regarding decreased levels of several amino acid metabolites, including the branched amino acids leucine/isoleucine/valine and methionine/SAM/cysteine/taurine, and decreased levels were observed for 8 amino acids including 4 essential amino acids. Such a broad decrease in amino acid levels together with the decreased levels of several food components/xenobiotics suggest that the gastrointestinal or nutritional status is altered in patients with signs of inflammation. Finally, the altered glutathione metabolites and gamma-glutamyl amino acid peptides suggests altered glutathione metabolism, whereas increased levels of fibrinogen peptides probably reflect the acute phase reaction [54].

Patients with signs of inflammation also showed alterations in glycine/serine/threonine and histidine metabolism (Table 3) which are relevant for endothelial/renal functions, immunoregulation or metabolic regulation. First, glycine levels were decreased; this amino acid affects various immunocompetent cells, including monocytes/macrophages (e.g., inhibits toll-like receptor 4–mediated cytokine release, neutrophils, and T cells) [55]. Glycine also has a cytoprotective role in kidney and endothelial cells, is involved in systemic circulatory regulation and inhibits endothelial cell proliferation and migration [55]. Second, histidine regulates amino acid metabolism and is involved in regulation of immunity and inflammation, including gut inflammation [56,57]. Thus several of the metabolites showing altered levels during pretransplantation inflammation can influence immunoregulation, including gut inflammation, as well as endothelial/renal function and systemic circulatory regulation.

The majority of significantly altered amino acid metabolites were increased for patients with extensive fluid retention, and in particular changes in metabolite levels belonged to metabolism of branched-chain amino acids (leucine/isoleucine/valine) and methionine/cysteine/SAM/taurine together with altered levels of nucleotide metabolites and certain food components (Table 4). The branched-chain amino acids are essential amino acids [53] that function as substrates for protein synthesis, signaling molecules and substrates for energy metabolism [44,53]. Leucine is important for cell signaling functions [53] that involve both protein synthesis, as well as glucose and lipid metabolism through inhibition of the energy sensor AMP-activated protein kinase [44]. Furthermore, they are also involved in immunoregulation through effects on the PI3K-Akt-mTOR pathway that functions as an amino acid sensor that maintains the amino acid balance [44,53]. These 3 amino acids maintain innate immune functions, stimulate immunoglobulin A (IgA) secretion and regulate the functions of dendritic cells and T cells [44]. They are additionally important for the function of the gastrointestinal tract through effects on both the gastrointestinal cells and the microbiota [44]. Furthermore, the tryptophan metabolites are also important for immunoregulation [58,59]. Methionine is an essential amino acid involved in metabolic regulation; it is an immunoregulator and a regulator of the cellular metabolism [60,61], it seems important in renal failure [62] and is a possible regulator of the small intestine function [63]. Taurine is a nonessential amino acid that influences cellular osmoregulation, antioxidation and energy/lipid metabolism [64–67]. The effects on the renal function and the circulation are probably mediated through interactions with the renin-angiotensin system [66] and through effects on endothelial cells [68].

Extensive fluid retention was also associated with increased levels of adenosine metabolites. Adenosine is a regulator of the circulation and microvessel permeability [69–72], but T cells also express adenosine receptors and adenosine is an important regulator of early steps of T cell activation [73–75]. The methionine metabolism SAM is a ligand for the adenosine receptor and can thereby inhibit monocytic cytokine release [76,77]. Finally, the observed altered levels of xenobiotics/food products suggest altered gastrointestinal function. Thus extensive fluid retention is associated with complex effects affecting the systemic circulation, microcirculation, fluid balance, renal function, immunoregulation, and possibly gastrointestinal regulation.

Fifty-five metabolites (including 22 amino acid metabolites) associated with chronic kidney disease were recently listed in a review article [78]. Altered tryptophan metabolism seems to be particularly important in early kidney disease [79]; we also observed altered levels of several tryptophan metabolites for patients with fluid retention, and increased levels of malate, ribonate and pseudouridine have also been described in chronic kidney disease [78]. Taken together, these observations suggest that the renal function is altered/modified in allotransplant recipients with fluid retention even though our patients with fluid retention had normal pretransplantation creatinine levels.

In our study, we used unsupervised hierarchical cluster analyses to subclassify allotransplant recipients based on their metabolomic profiles (Figures 3 and 4) and identified patient subsets with an increased risk of 2-year TRM. The TRM difference reached the highest statistical significance for the analysis that was based only on metabolites belonging to the subclass amino acid metabolism (Figure 4). We sought to reduce the impact of any coincidental metabolites by including only a single main metabolic pathway (i.e., amino acid metabolism), but in contrast to the other analysis (Figure 3) including individual metabolites with P values between 0.01-0.05. The increased statistical strength found when including less significant metabolites in our analysis, probably illustrates that metabolites with less significant P values ($.05 > P > .01$), with regard

to association with the risk factor better reflect the heterogeneity among risk factor–positive patients than the metabolites with the strongest associations to the whole group/patient subset ($P < .01$). The 2 patient subsets identified based on the overall amino acid differences differed only in frequency of TRM, although there was a minor age difference that reached borderline significance without correction for the number of comparisons, but neither the sex, frequencies of aGVHD, increased pretransplantation CRP, nor did early fluid retention differ significantly between the 2 identified subsets.

Our present study suggests that altered systemic amino acid metabolite profiles are associated with increased TRM, but we do not know whether the altered profile contributes to the development of fatal complications (e.g., alters immunoregulation or endothelial cell status) or simply reflects altered cellular functions during the development of these complications. Furthermore, our observations need to be validated in independent patient cohorts. Future studies also have to clarify whether our present observations are representative for allotransplant recipients in general (e.g., patients with other diagnoses and thereby different pretransplantation anticancer therapy) receiving different GVHD prophylaxis (especially with regard to the use of antithymocyte globulin) or conditional therapy, or receiving stem cells derived from different donors (e.g., unrelated or related haploidentical donors, umbilical cord blood stem cells). Finally, future studies should also be prospective and include more complete data (i.e., transplantation-related complications and mortality) for survival analyses using both Kaplan-Meier and multivariate Cox regression analysis.

To conclude, pretransplantation inflammation, early excessive fluid retention, and aGVHD are associated with increased morbidity and mortality after allo-HSCT. All 3 factors are also associated with different/nonoverlapping effects on the systemic metabolic regulation, which may affect regulation of circulation, microvascular permeability, renal, gastrointestinal, or immune functions. Analysis of differences in metabolomic profiles identify metabolites that are associated with aGVHD/inflammation/fluid retention and can be used to identify patient subsets with an increased risk of TRM.

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SUPPLEMENTARY MATERIALS

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