Assessment of urinary 3-indoxyl sulfate as a marker for gut microbiota diversity and abundance of Clostridiales

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ABSTRACT

Objectives: After allogeneic hematopoietic stem cell transplantation (allo-HCT), urinary levels of 3-indoxyl sulfate (3-IS) correlate with the relative abundance of bacteria from the class Clostridia (RAC), and antibiotic treatment is considered the major determinant of this outcome. A high RAC has been associated with favorable outcome after allo-HCT and protection from Clostridium difficile infection (CDI). We assessed correlations between alpha diversity, RAC and urinary 3-IS.

Methods: Fecal and urinary specimens were analyzed from 40 non-allo-HCT hospitalized patients before and 9 ± 2 days after initiation of intravenous antibiotic treatment. Fecal microbiota were analyzed by 16s RNA sequencing and urinary 3-IS was analyzed by liquid chromatography-tandem mass spectrometry. Receiver operating characteristic (ROC) analysis was performed to assess the predictive value of 3-IS.

Results: At a RAC cutoff of <30%, the binary logarithm of 3-IS (medium 3-IS: ≤2.5; high 3-IS: >2.5) was predictive with an accuracy of 82% (negative predictive value: 87%, positive predictive value 67%). Accuracy was improved by combing antibiotic history with 3-IS levels (accuracy 89%, npv 88%, ppv 92%).

Conclusion: In conjunction with patient antibiotic history, 3-IS is a candidate marker to predict RAC.

Introduction

Over the last decade, evidence supporting the regulatory impact of the human gut microbiota on central body functions has been gathered. Due to these developments, the existence of different axes connecting the gut microbiota with other organ systems, i.e. the gut-brain, gut-liver, gut-lung and gut-immune axis, can now be backed up by sound evidence. Particularly the loss of bacteria from the class Clostridia seems to play a crucial role in this context and has been shown to be associated with low urinary levels of 3-indoxyl sulfate (3-IS), a gut microbiota-derived metabolite of tryptophan. Within the class Clostridia, members of the order Clostridiales, such as Clostridium scindens, have also been shown to play a significant role in the regulation of CDI. In spite of intense research efforts, prediction of CDI in clinical practice remains a major challenge. Large cohorts have failed to deliver more specific risk factors besides...
age >65 years, previous antibiotic exposure and/or hospitalization. Therefore, a biomarker-based approach is warranted, preferably associated with low turn-around times and low costs. The occurrence of CDI is, however, a rare endpoint and requires large study population. Therefore, we aimed to assess the correlation between alpha diversity and abundance of Clostridiales with urinary 3-IS levels after antibiotic treatment in a non-allo-HCT inpatient population further explore the predictive capacity of 3-IS as a biomarker of reduced diversity. As a secondary endpoint, the urinary metabolites p-cresyl sulfate and phenyl sulfate were explored.

Results

After excluding patients who did not receive any antibiotic treatment for at least 7 days (n = 1) or patients without any urine or stool specimens (n = 2), a total of 40 patients (age 56.9 ± 17.5 years, 17/40 (42.5%) female) was eligible for further analysis. Antibiotics that led to inclusion into the study were mainly penicillin derivatives (12/40; 30.0%), followed by carbapenems (12/40; 30.0%), cephalosporins (9/40; 22.5%), fluoroquinolones (5/40; 12.5%), trimethoprim/sulfamethoxazole (1/40; 2.5%) and macrolides (1/40; 2.5%). Twenty of 40 (50.0%) patients received at least one additional antibiotic prior to collection of the second fecal specimen. However, administration of more than one antibiotic did not have any significant impact on our results.

Alpha diversity in fecal specimens decreased significantly under antibiotic exposure (Shannon Index: pre 4.23 ± 0.39 (95% CI 4.10–4.36) vs post 3.75 ± 0.65 (95% CI 3.53–3.97); p < 0.001; Figure 1A; inverse Simpson’s Index: pre 56.92 ± 17.51 (95% CI 51.09–62.76) vs post 41.29 ± 25.03 (95% CI 32.82–49.76); p < 0.01; Faith’s Phylogenetic Diversity: pre 9.54 ± 3.39 (95% CI 8.41–10.67) vs post 6.09 ± 3.59 (95% CI 4.88–7.31); p < 0.0001). However, further sub-analyses revealed that this was mainly due to carbapenems and/or penicillin derivatives, as opposed to other antibiotics (Figure 1B and C). The correlation between urinary 3-IS levels and alpha diversity was low (Shannon Index: spearman r² = 0.20; inverse Simpson’s Index: r² = 0.20; Faith’s Phylogenetic Diversity: r² = 0.18). However, after categorization of 3-IS levels (medium concentration: 18 samples, high concentration: 55 samples), we found that

Figure 1. Alpha diversity (Shannon Indices) (A) before and after antibiotic treatment (n = 40), (B) treatment with carbapenems and/or penicillin derivatives (n = 26), (C) “other” antibiotics (i.e. cephalosporins: n = 7, fluoroquinolones: n = 5, trimethoprim/sulfamethoxazole: n = 1, macrolides: n = 1) and (D) grouped by the urinary 3-indoxyl sulfate concentration (medium: −8 <log2(x) >2.5; high: log2(x) >2.5). Values were compared between different groups using Student’s t-test. ns, p > 0.05; ***, p < 0.001; ****, p < 0.0001.
alpha diversity of samples belonging to the medium 3-IS group was significantly decreased (Shannon Index: medium 3.39 ± 0.64 (95% CI 3.07–3.71) vs high 4.19 ± 0.41 (95% CI 4.08–4.30); p < 0.001; Figure 1D; inverse Simpson’s Index: medium 30.01 ± 20.46 (95% CI 19.84–40.18) vs high 55.49 ± 19.96 (95% CI 50.10–60.89); p < 0.001; Faith’s Phylogenetic Diversity: medium 4.29 ± 3.21 (95% CI 2.69–5.89) vs high 9.00 ± 3.35 (95% CI 8.09–9.91); p < 0.001) compared to the high 3-IS group. In our study population, no samples were categorized into the low 3-IS group. Assessment of associations between gender and age, weight, alpha diversity, as well as the concentration of urinary metabolites did not indicate an impact of gender on our results.

In concordance with alpha-diversity, the relative abundance of bacteria from the order Clostridiales within the medium 3-IS group was also significantly decreased (relative abundance of Clostridiales [%]: medium 23.0 ± 25.4 (95% CI 10.4–35.6) vs high 48.7 ± 15.9 (95% CI 53.0–44.4); p < 0.001; Figure 2A) compared to the high 3-IS group. The same holds true for samples from patients, who were treated with carbapenems and/or penicillin derivatives (Figure 2B). Figure 2C shows combined information on antibiotic treatment and urinary 3-IS concentration.

To further assess the power of 3-IS to predict decreased alpha diversities (Shannon Index; SI), as well as relative abundances of Clostridiales, a ROC analysis was performed. However, the required cutoff for a clinically relevant decrease in SI and relative abundance of Clostridiales had not been established in previous studies. Hence, we calculated the point of intersection of the kernel density estimates between the medium and high 3-IS group for the SI (3.7), as well as for the relative abundance of Clostridiales (29%). Furthermore, we computed ROC curves for different cutoff values (Supplementary Figures S1 and S2). The accuracy of prediction increased with lower cutoff values for SI and lower relative abundance of Clostridiales; however the number of patients in the respective groups also decreased. Only, 6/73 (8.2%) and 11/73 (15.1%) samples fell into the respective groups, if an SI below 3.0 and <10% Clostridiales were chosen as cutoffs. With
increasing cutoff values, the amount of patients in the groups increased, while the accuracy decreased. Based on these results, we choose a SI of 3.5, and a relative abundance of 30% Clostridiales as cutoff values for further ROC analyses (Figure 3 and Supplementary Figure S3). At a cutoff of 2.5, the binary logarithm of the 3-IS concentration (medium 3-IS: ≤2.5; high 3-IS: >2.5) predicted a SI <3.5 with an accuracy of 86% (Figure S3A). The respective negative and positive predictive values (npv and ppv) were 95% (npv) and 61% (ppv) (Figures S3B and S3C). A decreased relative abundance of <30% Clostridiales was predicted with an accuracy of 82%, (Figure S3D) using the same medium/high 3-IS cutoff. The npv and ppv were 87% and 67%, respectively (Figures S3E and S3F).

For comparison, the predictive value of information from the patient chart was assessed. The administration of carbapenems or penicillin derivatives predicted decreased alpha diversities (accuracy 79%, npv 94%, ppv 48%, Figures S3A-C: red lines). The prediction of decreased relative Clostridiales abundances was more accurate using the antibiotic history than the biomarker 3-IS (accuracy: 86% vs 82%, npv: 94% vs 87%, ppv: 70% vs 67%, Figures S3D-F: red lines). The combining 3-IS concentration and antibiotic history resulted in an accuracy of 90% (Figure S3A: green line) for the prediction of a decreased alpha diversity (npv 93%, ppv 77%, Figures S3B-C: green lines) and an accuracy of 89% (Figure S3D: green line) for the prediction of decreased relative abundances of Clostridiales (npv 88%, ppv 92%, Figures S3E-F: green lines).

In order to identify other microbiota signatures that may correlate with urinary 3-IS levels, we calculated the generalized UniFrac distances between samples. As shown in Figure S4, antibiotic treatment (Figure S4A) as well as different 3-IS groups (Figure S4B) are associated with a significant shift (ANOSIM R = 0.511, p = 0.001) of gut microbiota composition. However, the 95% confidence levels (Figure S4; dotted lines) overlap markedly between the groups. Hence, a distinct clustering of samples based on their generalized

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**Figure 3.** Receiver operating characteristic curve for the binary logarithm of the 3-indoxyl sulfate concentrations [log$_2$(3-IS)] differentiating fecal samples with (A) decreased alpha diversity (Shannon Index <3.5) and (B) decreased relative abundance of *Clostridiales* (relative abundance <30%). Numbers on curve refer to a range of selected cut-off scores between “negative” and “positive” results. (x indicates the respective values for the treatment with carbapenems and/or penicillin derivatives; ♦ indicates the respective values for the treatment with carbapenems and/or penicillin derivatives and medium urinary 3-indoxyl sulfate concentration).
UniFrac distances was not possible. The generalized UniFrac distances between the samples are depicted in the Supplementary Figure S5.

All analyses were repeated for the urinary metabolites p-cresyl sulfate (low: binary logarithm < −0.31) and phenyl sulfate (low: binary logarithm < −0.90). However, these metabolites were less accurate than urinary 3-IS predicting decreased alpha diversities (p-cresyl sulfate: accuracy 24%; phenyl sulfate: accuracy 38%; 3-IS: accuracy 86%) and decreased relative Clostridiales abundances (p-cresyl sulfate: accuracy 24%; phenyl sulfate: accuracy 32%; 3-IS: accuracy 82%). Combining these urinary metabolites with the antibiotic history failed to improve prediction of our selected endpoints compared to the antibiotic history alone.

Discussion

To our knowledge, this is the first study assessing the utility of urinary 3-IS as a marker for reduced microbiota diversity in a non-allo-HCT population with antibiotic exposure. In allo-HCT patients, associations between gut microbiota alpha diversity and incidence of complications and mortality after allo-HCT have been reported from independent cohorts. Particularly the loss of bacteria from the class Clostridia was shown to play a crucial role in this context and to be associated with low urinary 3-IS levels. In our study, a decrease in urinary 3-IS levels correlated with reductions in alpha diversity and relative abundance of Clostridiales, yet the predictive value of urinary 3-IS at individual patient level was only moderate. This could, however, be improved by combination with the patients’ antibiotic history. While our study did therefore not confirm urinary 3-IS as an effective independent predictor for development of CDI, it showed that it may still be of value within the context of a risk assessment score. Such a score has already been validated for prediction of CDI outcome and recurrence, but not for prediction of disease onset. In a first step, the data from this pilot study could be used to design a large prospective cohort study in which established clinical risk factors for CDI (e.g. antibiotic exposure, age >65 years, immunosuppression, previous hospitalizations) and would be assessed along with urinary 3-IS levels. Since the current study suggests that the length and type of exposure to different antibiotic classes plays a crucial role in predicting RAC, specific care should be taken to establish multiple sampling time points and detailed documentation of antibiotic exposure. Furthermore, the results of the current study could be used to define RAC as an independent variable and categorize urinary 3-IS (high vs. low). Using a Cox regression approach, the different predictive factors for CDI could be weighted and integrated into a risk score. This score would then require further validation in a confirmatory cohort.

Limitations of our study included the potential effect of co-medications that were not part of the observation on the gut microbiota. None of the patients studied received antibiotics only. However, even if changes in the gut microbiota were partially induced by other drugs, this would not have altered the limited predictive value of 3-IS with respect to our endpoints. In contrast to previous studies, none of the patients studied was categorized into the low 3-IS group, as defined by Weber et al. Compared to other patients, allo-HCT patients experience massive antibiotic exposure prior to and after the infusion of stem cells. The result is a radically disturbed gut microbiota, often dominated by a single bacterial genus and a depletion of Clostridia. Discrimination of this population from patients with only moderately disturbed or intact gut microbiota requires less discriminatory power than identification of more subtle differences in gut microbiota signatures. We hypothesize that our study population of patients with little to moderate antibiotic exposure and thus less disturbed gut microbiota may explain why discriminatory power of 3-IS was less pronounced compared to an allo-HCT population.

In a large recently published study, Pallister et al. identified and validated five (hippurate, p-cresyl sulfate, phenylacetylglutamine, 3-phenylpropionate, and hyodeoxycholate) out of 292 screened metabolites from human serum that may serve as markers of gut microbiota alpha diversity. Only one of the identified metabolites, p-cresyl sulfate was part of our panel, but did not perform better than 3-IS. Opposed to the above mentioned study, our analysis was performed from a urine specimen and not from serum. Since the analysis by Pallister et al. focused on the
associations of these metabolites with specific nutritional habits in healthy volunteers, the data are not directly comparable with our results. Unfortunately, these data became available only after we had finished our analysis, such that we did not get a chance to include all of these metabolites into our analysis. However, it may be wise to include them into our future work.

Even though our study failed to confirm the potential of urinary 3-IS as an independent predictor of CDI, we identified carbapenems and penicillin derivatives as high-risk antibiotics with respect to reduction in microbiota diversity. In line with these findings, we recently confirmed exposure to these antibiotic classes as an independent risk factor for intestinal GvHD in allo-HCT patients. Concordantly, other studies have demonstrated these antibiotics as risk factors of CDI. Yet other studies have confirmed a link between development of CDI and intestinal GvHD. While no study has succeeded in integrating all of these aspects into a fully explanatory pathophysiological hypothesis, the close linkage of these clinical problems suggests that they may share a common underlying condition, e.g. a specific microbiota signature which may result in shifts in anti-inflammatory metabolites. With respect to the involvement of such metabolites, secondary bile salts and short chain fatty acids have been described as key regulators in CDI and GvHD, respectively.

These findings further underline the potential role of antimicrobial stewardship to reduce unneeded antibiotic exposure and promote short and focused treatment regimens and thus preserve a diverse and healthy gut microbiota. Besides the described findings in the areas of allo-HCT and CDI, current publications on the significant role of gut microbiota diversity in the pathophysiology of a diverse range of other medical conditions, e.g. hepatic encephalopathy, tumor growth and multiples sclerosis, suggest that protection of microbiota diversity may emerge as a new focus of antimicrobial stewardship besides protection from development of antibiotic resistance.

Patients and methods

Setting

At three sites, 43 patients with an expected systemic antibiotic treatment for at least seven days and no current antibiotic treatment were included. Patients undergoing allo-HCT were excluded from the study. Fecal and urine specimens were collected before and 9 ± 2 days after initiation of antibiotic treatment.

16s RNA analysis from fecal samples

DNA was extracted from fecal specimens using the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH, USA) following the manufacturer’s instructions. The extracted DNA was concentrated using a vacuum concentrator and the V3-V4 region of the bacterial 16S rRNA gene was amplified using the primers 341F and 802R published elsewhere. The 16S amplicon was purified using the Agencourt AMPure XP PCR Purification system (Beckman Coulter, Krefeld, Germany), processed (indexed, purified, normalized and pooled) and sequenced in a 300-bp paired-end run on the Illumina MiSeq as outlined in the Illumina 16S Sample Preparation Guide.

Urine specimens

Urinary 3-IS levels were analyzed as published. For categorization of 3-IS concentrations, the limits defined by Weber et al. for the binary logarithm of 3-IS were applied: low: < −8, medium: −8 − 2.5, high: ≥2.5. Correspondingly, binary logarithms of p-cresyl sulfate and phenyl sulfate were categorized (low and high concentration) using the cutoff value resulting in the highest accuracies for the prediction of a decreased alpha diversity (p-cresyl sulfate: −3.32, and phenyl sulfate: −2.79) and relative abundance of Clostidiales (p-cresyl sulfate: −0.3, and p-cresyl sulfate: −0.9).

Sequence data analysis and taxonomic profiling

The sequencing data was processed using the DADA2 pipeline and QIIME 2. Briefly, quality profiles of the reads were analyzed to determine positions at which read quality was greatly diminished. Reads were then trimmed at the identified positions (trunc_len_f = 280, trunc_len_r = 240) and processed by the QIIME DADA2 plugin with the denoise-paired option and standard parameters (trunc_q = 2, max_ee = 2, chimera_method = consensus). Rarefaction curves were determined based
on the feature table and analysis of the relative proportion of each bacterial taxon was made after the data were rarefied at a sequencing depth of 2,000 sequences per sample. Taxonomic classification was done by a Naïve Bayes classifier (sklearn)\textsuperscript{34}, which was trained on the SILVA database release 128 \textsuperscript{35}, where sequences were trimmed to only include the V3-V4 region of the 16S rRNA gen.

**Statistical analysis**

Statistical analyses were carried out using R for Statistical Computing (version 3.2.5, R Foundation for Statistical Computing, Vienna, Austria).\textsuperscript{36} The QIIME biom data was imported and the diversity scores were calculated using functions provided in the phyloseq R package.\textsuperscript{37} All continuous data was presented as mean (SD), presented as box- or violin-plots and tested with appropriate statistical analyses (two-sided t-tests). The optimal outcome-cutoff was determined by calculating the intersections of the Gaussian kernel density estimates (bandwidth = 0.2) between each category of the binary logarithm of the 3-IS concentration (medium: $-8-2.5$, high: $>2.5$). Receiver operating characteristic (ROC) analysis performed using the ROCR package.\textsuperscript{38} Community distances were compared using the Analysis of similarity (ANOSIM) test based upon generalized UniFrac (alpha = 0.5) distances measurements with 999 permutations using a combination of the vegan and GUniFrac package.\textsuperscript{39,40} All statistical tests were two-tailed, and a P value of <0.05 was considered to be statistically significant.

**Ethical statement**

This non-interventional study has been approved by the respective local institutional review boards and ethics committees.

**Conflicts of interest**

MJGTV is a consultant to: Berlin Chemie, MSD/Merck and Astellas Pharma; has served at the speakers’ bureau of: Astellas Pharma, Basilea, Gilead Sciences, Merck/MSD, Organobalance and Pfizer; received research funding from: 3M, Astellas Pharma, DaVolterra, Gilead Sciences, Merck/MSD, Morphochem, Organobalance, and Seres Therapeutics.

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