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A urinary metabolite constellation to detect acute rejection in kidney allografts

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ABSTRACT

Background: To validate a novel method for post-transplant surveillance to detect kidney allograft rejection via a characteristic constellation of the urine metabolites alanine, citrate, lactate, and urea investigated by nuclear magnetic resonance (NMR) spectroscopy a first prospective, observational study was performed.

Methods: Within the UMBRELLA study 986 urine specimens were collected from 109 consecutively enrolled renal transplant recipients, and metabolite constellations were analyzed. A metabolite rejection score was calculated and compared to histopathological results of corresponding indication and protocol allograft biopsies ($n = 206$).

Findings: The metabolite constellation was found to be a useful biomarker to non-invasively detect acute allograft rejection (AUC = 0.75; 95% confidence interval (CI) 0.68–0.83; based on 46 cases and 520 control samples). Combined analysis of the metabolite rejection score and the estimated glomerular filtration rate (eGFR) at the time of urine sampling further improved the overall test performance significantly (AUC = 0.84; 95% CI 0.76–0.91; based on 42 cases and 468 controls). Regarding the time course analysis in patients without rejection episodes the test results remained well below a diagnostic threshold associated with high risk of acute rejection. In other cases, a marked increase above this threshold indicated acute allograft rejection already six to ten days before diagnostic renal biopsies were performed.

Interpretation: A combination of an NMR-based urine metabolite analysis and eGFR is promising as a non-invasive test for post-transplant surveillance and to support decision making whether renal allografts need histopathological evaluation.

Research in context

Evidence before this study

Kidney allograft rejection is still one main predictor of long-term allograft loss. Regular clinical follow-up examinations, often in combination with renal allograft biopsy, are standard of care. However, even with serial biopsies, it would be unlikely that all rejection episodes could be detected upon onset. Thus, attempts have been made to develop novel biomarkers to detect acute rejection. We searched PubMed for relevant diagnostic studies published before July 2019 using the search terms “urine”, “biomarker”, and “renal allograft rejection”. We also manually searched original articles. None of the identified biomarkers in the literature has been established in the clinical routine so far. We have previously introduced a urinary metabolite constellation comprising the metabolites alanine, citrate, lactate, and urea normalized to urine creatinine as indicator of renal allograft rejection.

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Added value of this study

The results from the UMBRELLA study indicate a significant progress in the development of a non-invasive diagnostic test to reliably detect acute renal allograft rejection using the recently introduced urinary metabolite constellation alone or in combination with eGFR.

Implications of all the available evidence

This test gives a valuable support in biopsy decision making. In case of a high metabolic rejection score a renal biopsy might be recommended to clarify this suspicious finding. A test result pointing to an intermediate risk might trigger closer patient follow-up and renal biopsy might be considered in case of appearance of further signs and symptoms. Kidney biopsy might be waived in favor of regular follow-up visits in case of a test result associated with low rejection risk.

1. Introduction

Despite major improvements in treatment regimens for kidney transplant patients, allograft rejection is still a substantial threat and one of the main predictors of long-term allograft loss [1]. Regular clinical follow-up examinations in combination with laboratory measurements of serum creatinine levels are standard procedures in post-transplant surveillance for acute rejection. Increasing serum creatinine levels above a patient-specific baseline value with or without the appearance of clinical symptoms typically trigger a renal allograft biopsy for histopathological evaluation. This often implies diagnosis in more advanced stages of dysfunction, whereas earlier stages, where functional impairment is not yet clinically detectable, often remain unrecognized. Therefore, protocol biopsies have been introduced to potentially detect acute rejection in a subclinical state [2]. However, even with serial biopsies it is unlikely that all rejection episodes are detected upon onset, not to speak of risks and complications [3] associated with such a costly approach.

Thus, attempts have been made to develop novel tests based on biomarkers to detect acute rejection non-invasively [4–11]. Probably due to high costs of analysis and lack of platform standardization, none of the tests has been established in the clinical routine so far. Recently, we established a urinary metabolite constellation indicating renal allograft rejection using inexpensive and standardized nuclear magnetic resonance (NMR) spectroscopy that represents the basis for an in-vitro diagnostic test [12]. We were able to detect acute renal rejection with an area under the curve (AUC) value between 0.72 and 0.74 for an initial test set. Untargeted biomarker discovery followed by exhaustive statistical modelling yielded a final urine metabolite constellation comprising the metabolites alanine, citrate, lactate, and urea normalized to urine creatinine that are mathematically combined in a multiple logistic regression model. Interestingly, these metabolites had already been reported individually to be associated with renal disturbances such as tubulitis due to rejection [7], acidosis [13], respiratory chain dysfunction in mitochondria [14], and decreased glomerular filtration [15]. Hence, the novel metabolite constellation may reflect a set of metabolic processes that determine pathophysiological and biochemical properties at least in part on the mitochondrial level due to hypoperfusion, organ swelling, and respiratory chain dysfunction.

Here we further investigate our newly established metabolite constellation [12] and report the results from an independent clinical validation study reviewing the clinical utility of the metabolite constellation as a novel biomarker for renal allograft rejection. The study is based on a reasonable number of consecutively enrolled patients with their respective urine samples and renal allograft biopsies, including both protocol and for-cause biopsies.

2. Methods

2.1. Study design

The prospective, observational UMBRELLA study consecutively enrolled 109 patients after kidney or combined pancreas-kidney transplantation at the transplant center of the University Hospital Regensburg (Germany). Standard immunosuppressive treatment included an induction therapy, tacrolimus, mycophenolate mofetil/mycophenolic acid, and steroids. From all patients informed consent was obtained to collect urine samples and clinical data (Fig. 1). Patients were followed for one year starting at the day of transplantation (day 0). Urine samples were taken daily during the early post-transplantation phase as part of the normal routine (further referred as Phase I). After discharge from the hospital (routinely around day 15, in this paper described as Phase II), each patient was scheduled for five regular control visits at days 56 (±7), 84 (±14), 182 (±28), 273 (±28) and 364 (±28) after transplantation. Additional unscheduled visits were documented the same way. The study was approved by the Ethics Committee of the Medical Faculty of the University of Regensburg (Vote 03–082, dated Jan 27th 2011). Written informed consent was received from participants prior to study inclusion.

2.2. Kidney biopsies and histopathological analysis

According to our center standard, two protocol kidney biopsies were performed on day 14 ± 2 and on day 84 ± 14. Additional for-cause biopsies were taken when a rejection was suspected. Histopathological analysis was done using the actual at that time used BANFF classification [16].

2.3. Urine sampling and preparation

Spontaneous mid-stream urine was collected in standard plastic urine cups and analyzed for protein concentration, hematuria and leukocyturia by dipstick and microscopic inspection. Aliquots of 1.8 ml were frozen at −20 °C within five hours after collection and stored at −20 °C until analysis. For NMR spectroscopy, aliquots were allowed to thaw at room temperature. A volume of 600 µl urine was mixed with 150 µl of Axinon® urine additive solution in a centrifuge tube. The samples were centrifuged at 20.000 *g* for ten minutes at 20 °C, and 600 µl of the supernatant were transferred to 5 mm NMR tubes and kept at 2–6 °C until measurement.

2.4. NMR analysis

All measurements were carried out on a Bruker Avance II + 600 MHz NMR-spectrometer and a PATXI 1H/D-13C/15 N Z-GRD probe. All samples were warmed to 37 °C target temperature in the integrated preheating block prior to the measurement. Samples were measured in batches of up to 93 samples per run. Each run included one Axinon® urine calibrator sample and two Axinon® urine control samples in order to assure consistent measurement and reproducibility conditions throughout the whole run. NMR spectra underwent automatic data processing and quality control as part of the Axinon® system based on spectral properties, such as offset and slope of the baseline in selected spectral regions as well as properties of selected signals, e.g. signal position, shape and width. Urinary metabolite quantification and test results were generated fully automated by the Axinon® renalTX-SCORE® system as described in detail previously [12].

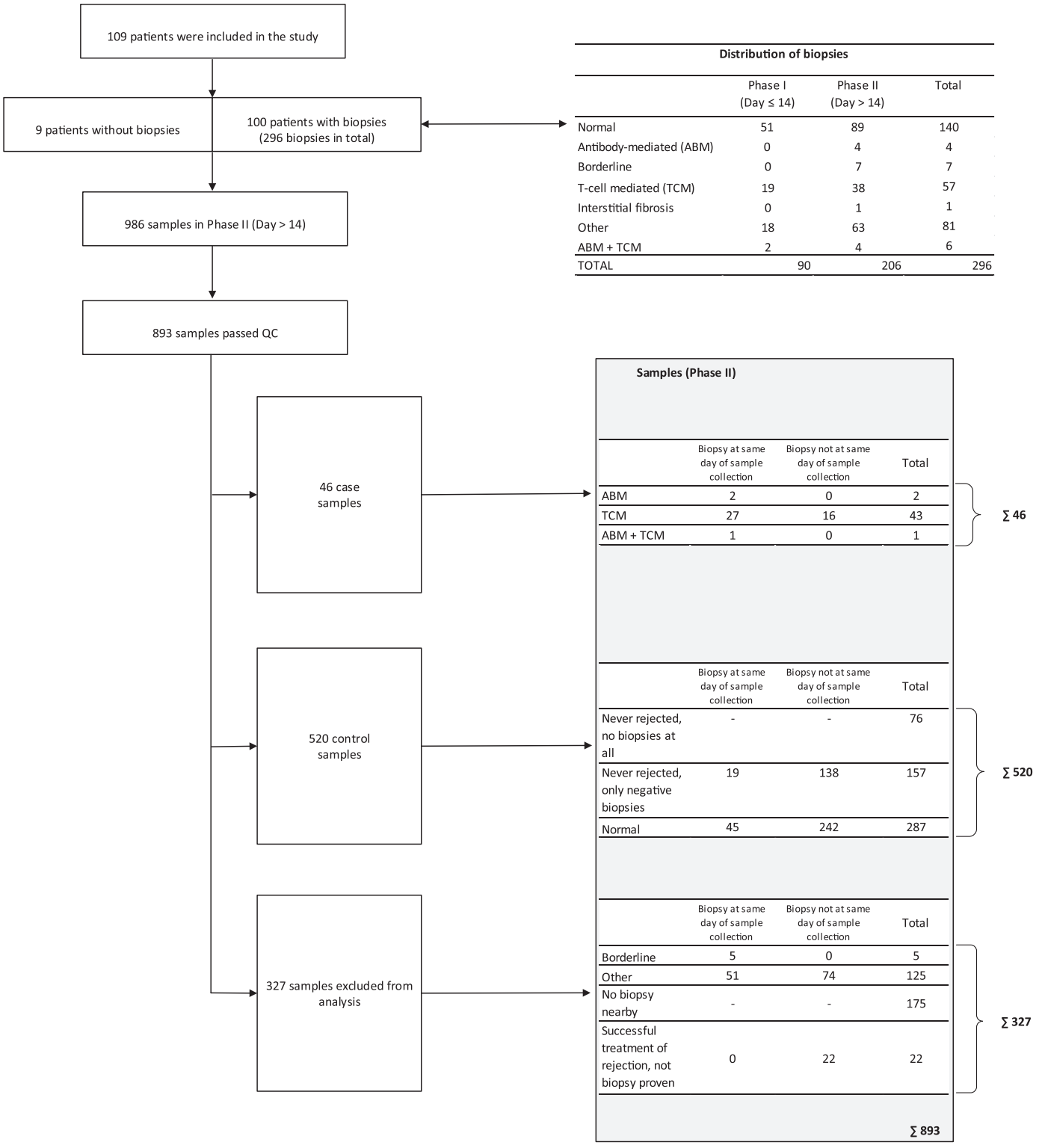

Fig. 1. Urine sampling and classification for evaluation of the NMR test

Urine samples were collected from 109 patients for urinary metabolite profiling after transplantation from day 15 (Phase II) to month 12. 296 allograft biopsies were performed in 100 patients. A metabolite rejection score was calculated for 893 urine samples passing all quality controls. These samples were further classified as either case or control dependent on respective biopsy findings. Therefore, urine samples taken within a 7-day time window before a biopsy showing acute rejection were considered as cases. Samples taken after biopsies with normal findings, as well as all samples from patients never experiencing rejection ('never rejected, only negative biopsies'), and patients without biopsies due to an uneventful post-transplant course ('never rejected, no biopsies at all'), were considered as controls. Samples associated with biopsies revealing borderline rejection, chronic allograft damage (interstitial fibrosis) or non-immunological damages (other) were excluded from analysis. Samples without a corresponding biopsy within a 7-day time window or after a biopsy-proven rejection, but without histological proof of successful rejection treatment could not be used for validation of the metabolite rejection score. The remaining set of samples included 46 cases and 520 controls. .

Table 1
Patient characteristics.

Number of patients	109	
Gender		
Male	65	60%
Female	44	40%
Age [years]		
Range	18–74	
Mean ± SD	52.7 ± 12.8	
Ethnic origin		
Caucasian	105	96%
African	2	2%
Other	1	1%
Not available	1	1%
CMV serology (IgG)		
Positive	49	45%
Negative	60	55%
HLA mismatch		
0 mismatch (HLA–A/HLA–B/HLA–DR)	37/19/30	34/17/28%
1 mismatch (HLA–A/HLA–B/HLA–DR)	48/38/46	44/35/42%
2 mismatch (HLA–A/HLA–B/HLA–DR)	23/51/32	21/47/29%
Not available (HLA–A/HLA–B/HLA–DR)	1/1/1	1/1/1%
Donor		
Living/Deceased	22/87	20/80%
Donor age [years]		
Range	7–86	
Mean ± SD	52.4 ± 17.3	

2.5. Statistical analysis

All statistical analyses were carried out with the R statistical software v3.0.2 [17]. The scoring model evaluated in this work is a multiple logistic regression model that had been established previously [12]. This model was applied to the data from the UMBRELLA validation cohort (predict.glm from the stats package), in this work. We used area under the receiver operating characteristics curve (AUC), sensitivity, and specificity to evaluate the ability of the previously developed metabolite rejection score to discriminate urine samples from patients with acute rejection and those showing no rejection. AUC computations and pairwise comparisons were carried out using the package pROC v1.5.4 and cvAUC 1.1.0 for pooled repeated measures data. Specifically we use the DeLong test (roc.test in pROC) for testing AUC differences.

An analysis of confounding effects was performed on the subset of control samples in the UMBRELLA study cohort and for each possible confounder individually. As possible confounders we analyzed the clinical parameters urinary tract infection (UTI), gender, donor type, recipient and donor age, ischemia time and post-transplant time with regard to the metabolite constellation score. The numerical parameters (age and times) were investigated using Pearson correlation analysis and the non-numerical parameters (e.g. gender and donor type) using Wilcoxon rank-sum test (two-sided; from the stats package).

Correlation coefficients were computed using Pearson correlation (cor from the stats package).

All results are reported according to STARD guidelines [18].

3. Results

From January 2011–October 2012 a total of 109 patients undergoing renal transplantation were enrolled into the UMBRELLA study for urinary metabolite analysis. Patient characteristics are given in Table 1. 296 allograft biopsies were conducted in 100 patients. 167 out of 296 biopsies were performed because of clinical signs of graft dysfunction or unclear renal dysfunction, whereas 129 were protocol biopsies. For nine patients, no biopsies were done during study (e.g. due to antithrombotic therapy or perirenal liquid). Histopathological evaluation resulted in 140 normal findings and 156 abnormal findings (antibody-mediated, borderline, *T-cell* mediated, interstitial fibrosis, other either alone or in combination, all details are given in Fig. 1). Within the subgroup of biopsies with abnormal findings a significant proportion of biopsies revealed acute cellular rejection (57/156 ≈ 36.5%).

986 urine samples were collected in Phase II of the study (day ≥ 15 after transplantation) and analyzed by NMR profiling. 893 samples passed quality control and subsequently a metabolite rejection score was calculated (Fig. 1). 33 out of the 986 samples could not be measured due to insufficient sample volume. 60 samples did not pass spectral quality criteria (shim and phasing issues: $n = 34$, interfering background signals: $n = 18$, extremely low biomarker concentrations: $n = 6$, disinfectant contamination: $n = 2$). As allograft rejection is a process over several days rather than a sudden event [4], we defined all urine samples within a seven day time window before a biopsy showing rejection as cases ($n = 46$). For the majority of these case samples, the biopsy was carried out exactly at the day of sample collection (30/46 ≈ 65.2%). All urine samples taken on or after the day of a renal biopsy without histopathological signs of rejection were defined as controls. The same was done for all samples of patients without need for any biopsy due to uneventful post-transplant courses, yielding a total of 520 control samples. The remaining samples taken after a biopsy with confirmed rejection were excluded to avoid bias from therapeutic intervention as the rejection status of the respective patient was unclear unless a further biopsy was taken proving successful rejection treatment. In addition, samples associated with biopsies of categories borderline, interstitial fibrosis and other or without any biopsy nearby were excluded as well (Fig. 1).

3.1. Clinical test validation

In order to validate the diagnostic performance of the already established metabolite constellation [12], we applied the case/control definition described above and evaluated the test results for the independent UMBRELLA study against the corresponding histopathological findings. The area under the receiver operating characteristic (ROC) curve of the metabolite constellation for discriminating case ($n = 46$) and control samples ($n = 520$) collected ≥day 15 days post-transplant showed an AUC of 0.75 (95% CI: 0.68–0.83; Fig. 2a). The performance dropped slightly, if only those control samples were considered for the ROC analysis that were collected at the day of biopsy (AUC: 0.72, 95% CI: 0.62–0.82; based on 46 cases and 64 controls).

If also urine samples associated with borderline rejection, interstitial fibrosis or other changes were included into the test validation (by handling borderline associated samples as cases and interstitial fibrosis and other samples as controls), an AUC of 0.71 was calculated (95% CI: 0.64–0.79; based on 57 cases and 720 controls; Fig. 2b).

The metabolite constellation failed to discriminate urine samples from patients with and without acute rejection immediately after transplantation (Phase I; AUC: 0.48, 95% CI: 0.43–0.53; based on 134 cases and 548 controls).

In order to investigate the effect of repeated measures (i.e. multiple samples from the same patient) on the performance, we calculated the AUC value in three alternative ways. First, a single sample was randomly chosen per patient and for this reduced data set the performance was computed. This was done 1000 times yielding a mean AUC of 0.71 ± 0.05 SD (with mean 95% CI: 0.56 ± 0.06 SD–0.86 ± 0.04 SD based on 16 cases and 73 controls). Second, due to the low number of cases in the first approach, we accepted some repeated measures by taking all case samples into consideration. For the control samples, again only one sample per patient was chosen and the AUC was calculated in 1000 iterations yielding a mean AUC of 0.77 ± 0.02 SD (with mean 95% CI: 0.68 ± 0.02 SD–0.86 ± 0.01 SD based on 46 cases and 73 con-

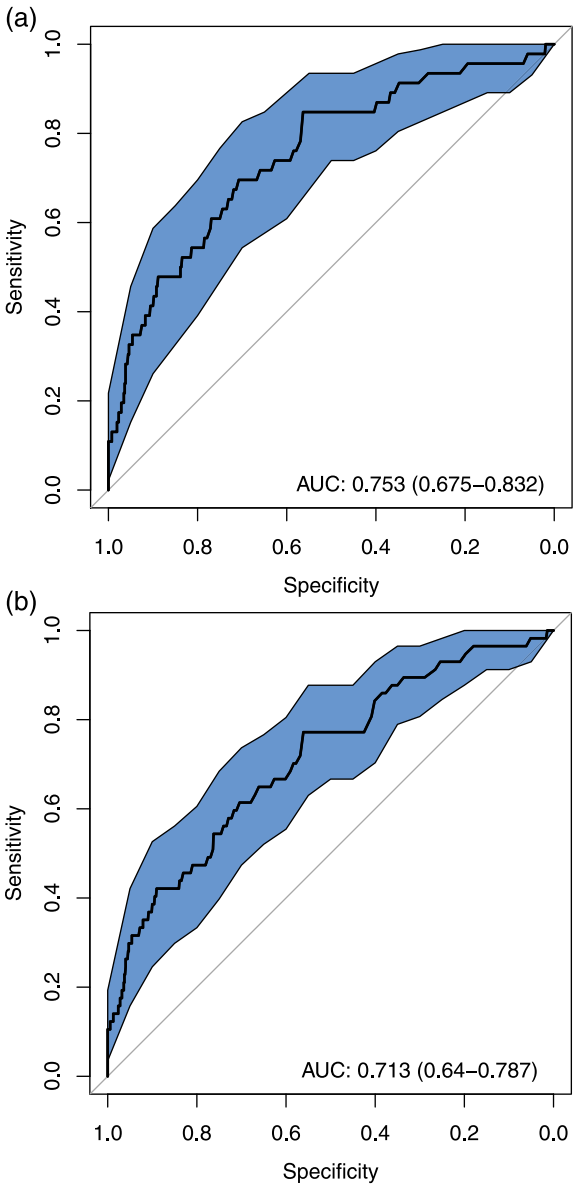

Fig. 2. Receiver-Operating-Characteristic Curve for the metabolite rejection score. The fraction of true positive results (sensitivity) and the fraction of false positive results (1–specificity) for the metabolite constellation are shown for patients in Phase II (≥day 15). The area under the curve (AUC) for discriminating patients with acute rejection (46 cases) and patients without rejection (520 controls) was 0.75 (95% CI: 0.68–0.83). Blue area represents upper and lower 95% confidence intervals of the ROC curve (Panel a). Panel b shows the corresponding analysis including samples associated with borderline rejection, interstitial fibrosis or other changes. By handling borderline associated samples as cases and interstitial fibrosis and other samples as controls, an AUC of 0.71 (95% CI: 0.64–0.79) was obtained based on 57 cases and 720 controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

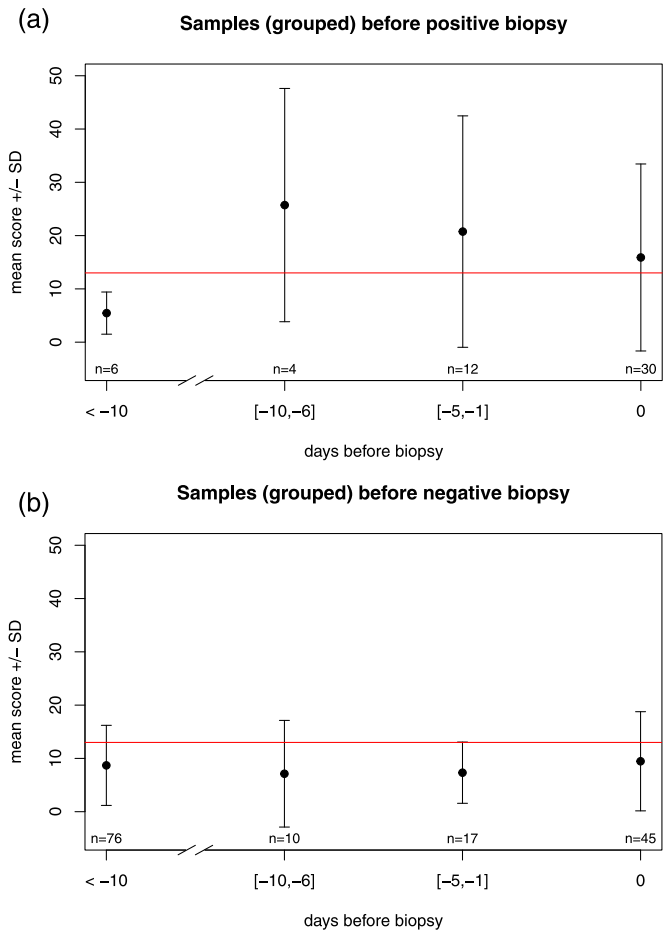

Fig. 3. Retrospective time courses of metabolite rejections scores. Time course for average values of the metabolite rejection score prior to a biopsy taken from patients who were diagnosed with acute cellular rejection (Fig. 3a) or who did not develop rejection (Fig. 3b), respectively. The number of corresponding samples is specified for each time interval (see *x*-axis).

trols). Third, we used the cvAUC package to account for repeated measurements in the AUC calculation that applies a 10-fold cross-validation (CV) with CV folds stratified by patient id. Using this method, the AUC accounts for 0.75 (95% CI: 0.67–0.84). Taken together, it can be seen that these values are comparable to our original calculation (AUC = 0.75, 95% CI: 0.68–0.83; see Fig. 2a).

In the previously described establishment of the metabolite constellation score [12] the cut-off values of 3.0 and 13.0 were associated with 90% sensitivity (i.e. to effectively rule out acute rejection) or 90% specificity (i.e. to rule in acute rejection), respectively. In the here described UMBRELLA cohort a metabolite rejection score cut-off set at 3.0 was associated with a sensitivity of 91% (95% CI: 79%–98%) and a specificity of 34% (95% CI: 30%–38%). A cut-off set at 13.0 was related to a specificity of 89% (95% CI: 86%–91%) and a sensitivity of 48% (95% CI: 33%–63%).

3.2. Retrospective time courses of metabolite rejection scores

Fig. 3 shows the time course for average values of the metabolite rejection score prior to a biopsy taken from patients who either developed an acute rejection (Fig. 3a) or who did not develop a rejection (Fig. 3b), respectively. On average, we observed a marked increase in metabolite rejection scores (above the 13.0 threshold associated with high risk of acute rejection) already six to ten days before the biopsies were performed that finally confirmed allograft rejection. In contrast, metabolite rejection scores remained below the 13.0 threshold in the group of patients not suffering from rejection.

3.3. Combination of metabolite rejection score and eGFR

Estimated glomerular filtration rate (eGFR; CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equation) is a standard marker to evaluate excretory renal function. Therefore, we investigated what performance can be achieved by combining our metabolite rejection score with eGFR. First, we examined the correlation between the eGFR and the metabolite score and its corre-

Table 2
Correlation of the estimated glomerular filtration rate (eGFR) with the metabolite rejection score and corresponding metabolites.

Correlation with eGFR	Pearson correlation (95% confidence interval)	*p*-value
Metabolite rejection score	−0.133 (−0.218 to −0.047)	0.0026
Alanine	0.242 (0.159–0.322)	<0.001
Citrate	0.273 (0.190–0.351)	<0.001
Lactate	0.018 (−0.069–0.105)	0.69
Urea	0.205 (0.120–0.286)	<0.001

sponding metabolites. As can be seen in Table 2, all correlations are below 0.50 suggesting that the parameters provide independent information and may improve performance when combined. A two dimensional interpretation template was established (Fig. 4a), using the metabolite rejection score thresholds (3.0 and 13.0) and an eGFR cut-off at 30 ml/min/1.73 m^2 which denotes the threshold between KDIGO (Kidney Disease Improving Global Outcomes) stages G3b and G4 [19].

In a first assessment, we only included urine samples taken on the same day as the corresponding kidney biopsies (Fig. 4a, 28 cases and 57 controls). The proportion of cases in samples associated with a high risk of rejection (metabolite rejection score ≥13 and eGFR <30 ml/min/1.73 m^2) was 61.5% (8/13 samples), in samples with intermediate risk (metabolite rejection score <13 and eGFR <30 ml/min/1.73 m^2 or metabolite rejections score ≥13 and eGFR ≥30 ml/min/1.73 m^2) 48% (12/25 samples), and in samples with low risk (metabolite rejection score <13 and eGFR ≥30 ml/min/1.73 m^2 OR metabolite rejections score <3 and eGFR <30 ml/min/1.73 m^2) 17% (8/47 samples), respectively.

In a second step, we assessed all cases and controls in the combined test, i.e. 42 cases and 468 controls (Fig. 4b). The number of cases and controls in this analysis is smaller than given in Fig. 1 (46 and 520, respectively) as some samples dropped out due to undocumented eGFR. 16 out of 37 (43.2%) of samples assigned to a high rejection risk were collected from patients with biopsy-confirmed allograft rejections. The relative risk (RR) of acute allograft rejection with NMR test result ≥13 and eGFR <30 ml/min/1.73 m^2 was RR 7.9 (95% CI: 4.6–13.3). In contrast, only eight out of 355 (2.3%) samples associated with a low risk of allograft rejection were taken from patients suffering from an acute rejection episode. This rate was significantly lower ($p = 8 \bullet 10^{-6}$) compared to the 17% observed for the biopsy-only analysis.

In order to numerically assess whether an improvement in performance can be achieved, we derived a single measure by taking the ratio of the metabolite rejection score and eGFR. The respective AUC for the combined measure to discriminate between rejection and no rejection was 0.84 (95% CI: 0.76–0.91, based on all urine samples, i.e. 42 cases and 468 controls; see Fig 4c). This AUC is significantly larger than the AUC obtained for the metabolite constellation alone (DeLong: *p*-value $= 2.4 \times 10^{-3}$) and the AUC for eGFR alone (0.75 (95% CI: 0.67–0.83); DeLong: *p*-value $= 2.7 \times 10^{-2}$).

3.4. Clinical confounder analyses

Urinary tract infection (UTI), cytomegalovirus (CMV) and BK virus infections are complications in kidney transplant recipients potentially changing the urine metabolome. A total of 48 UTI were documented for 566 urine samples (8.5%) without significant differences in the prevalence between case (6/46) and control (42/520) samples. UTI was associated with a significant increase of about four metabolite rejection score units (*p*-value $= 8 \times 10^{-8}$ using two-sided Wilcoxon rank-sum test) in the control urine samples. However, the average test results remained well below the 13.0 threshold in the group of patients without rejection. A systematic analysis on CMV and BK virus infections was not possible due to limited clinical events and sample size. However, for seven out of eight false-negative samples from patients with acute rejection, either BK virus infection ($n = 1$), CMV infection ($n = 5$), or acute UTI ($n = 1$) was documented at day of the biopsy or three to five days later. The remaining false-negative case had a history of CMV infection 37 days before renal biopsy.

In addition, confounder analyses were performed for gender, donor type, recipient age, donor age, cold ischemia time, warm ischemia time, and post-transplant time in control urine samples. While the effect of gender was insignificant (*p*-value = 1), deceased donor transplantation was associated with a significant increase in the metabolite score (*p*-value $= 4.5 \times 10^{-2}$), but the average test results were below the 13.0 threshold. For recipient and donor age, cold and warm ischemia time as well as post-transplant time, observed correlation coefficients were below 0.20, suggesting no confounding effects.

4. Discussion

This independent clinical validation study in a representative cohort of the kidney transplant population indicates that acute renal allograft rejection can be diagnosed non-invasively using a novel test based on a characteristic urinary metabolite constellation. In combination with eGFR this test might represent a valuable support in biopsy decision making. In case of a high metabolite rejection score a renal biopsy might be recommended to clarify this suspicious finding. A test result pointing to an intermediate risk might trigger closer patient follow-up, and renal biopsy might be considered in case of appearance of further signs and symptoms. Kidney biopsy might be waived in favor of regular follow-up visits in case of a test result associated with low rejection risk. In the UMBRELLA cohort we applied this regime to all patients where a urine sample was available taken at the same day of kidney biopsy (28 cases and 57 controls as a subset of the complete test set of 46 cases and 520 controls). In this subset we would have missed eight cases of acute rejection, would have triggered biopsies which turned out negative in five cases, would have resulted in close follow-up in 25 cases, and would have correctly identified 47 patients, who did not benefit from biopsy.

Although acute rejection is nowadays treatable in the majority of cases, it is a known risk factor for chronic rejection and graft loss [1,20]. Preventive strategies include immunosuppression, which is initiated at the time of transplantation and often adjusted in response to clinical events [21]. Since the average metabolite rejection score was found to be already increased six to ten days before an acute rejection is documented with the diagnostic procedures actually available, this may give the opportunity to assess metabolite analysis for active patient surveillance in combination with preemptive anti-rejection therapy.

As a non-invasive test, the analysis of the urine metabolite constellation allows much closer follow-up than is currently possible with biopsies to detect even transient immunological injury. Future work is warranted to clinically interpret suspicious test results in asymptomatic patients in order to distinguish false positive test results from subclinical immunological injury.

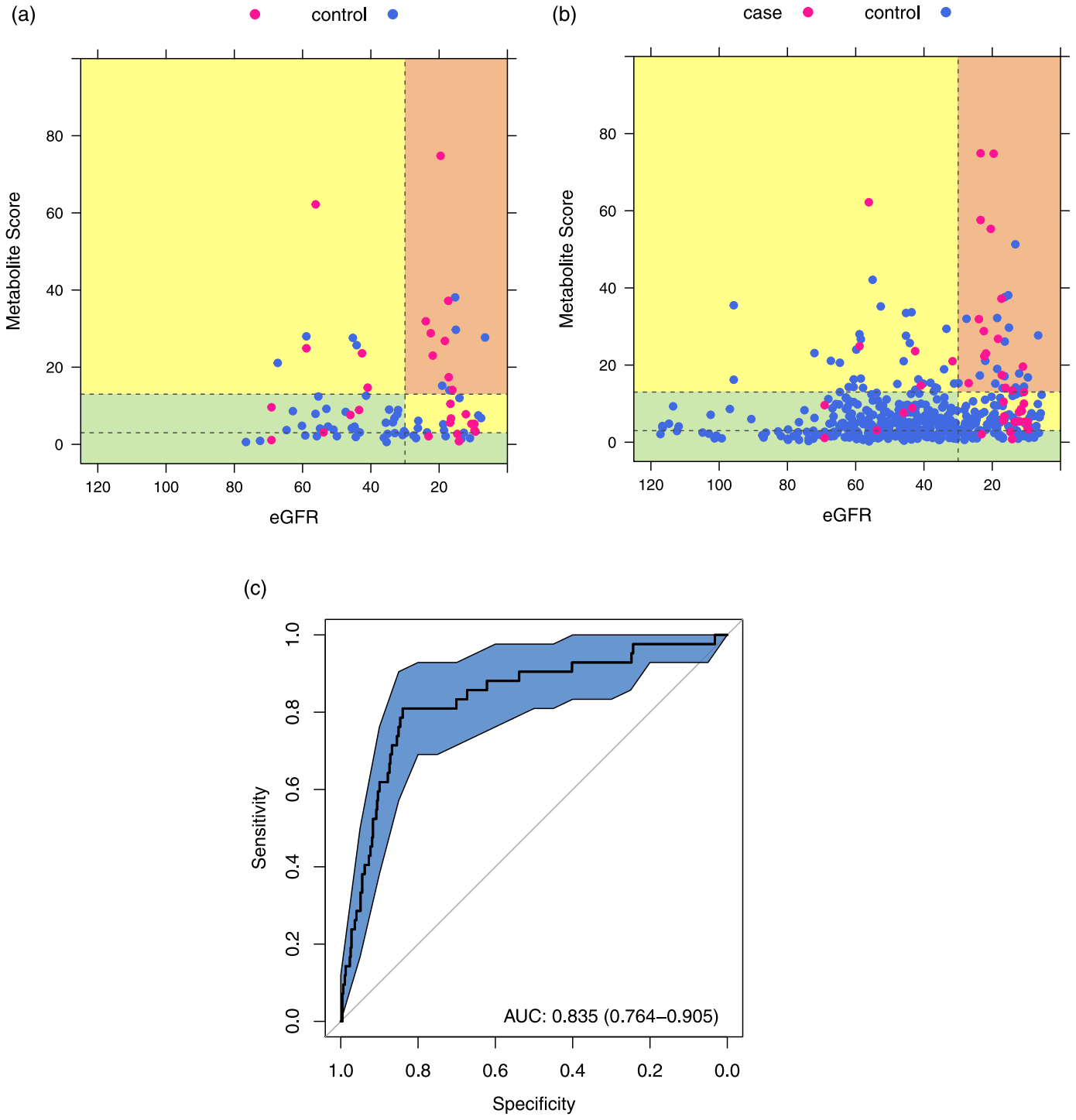

Fig. 4. Combination of metabolite rejection score and estimated glomerular filtration rate (eGFR).
To improve clinical usability of metabolite rejection score, a two-dimensional interpretation template was established. The thresholds used for the metabolite rejections were 3.0 and 13.0. eGFR values were discriminated in ≥30 and <30 ml/min/1.73 m^2. A high risk for rejection was noted with a metabolite score ≥13 and eGFR <30 ml/min/1.73 m^2 (red). Low risk is depicted in green (score <13 and eGFR ≥30 ml/min/1.73 m^2 or score < 3and eGFR <30 ml/min/1.73 m^2), with the remaining as intermediate risk (yellow). Panel a: Only urine samples taken at the same days as the corresponding kidney biopsies were analyzed (28 cases, red dots and 57 controls, blue dots). Panel b: All urine samples were analyzed, i.e. 42 cases and 468 controls. Panel c: In order to get an estimation for the performance of the combined measures, the ratio of the metabolite rejection score and the eGFR was assessed. The figure shows the ROC curve for this ratio based on the analysis of all urine samples (i.e. 42 cases and 468 controls) achieving an AUC of 0.84 (95% CI: 0.76–0.91). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

An important issue is to know factors that could interfere with test performance. A frequent complication in renal transplant recipients is urinary tract infection (UTI), which is associated with an inflammatory cytokine response and activation of the immune system [22,23]. Although in the present study UTI and deceased donor transplantation slightly influenced metabolite rejection scores, they remained below the diagnostic threshold for acute rejection in the group of patients without rejections. Nevertheless, simple dipstick testing for leukocyturia to rule out UTI might be advisable until further data are available. We further made anecdotal observa-

tions that systemic infections may mask expected score increases normally observed the days before an acute rejection episode. However, further substantiation is necessary in larger patient cohorts.

There are several limitations to our study. The metabolite analysis was not useful for detection of acute rejection within the first days after transplantation. From a physiological point of view, it is plausible that a metabolite-based test may be less reliable in the time period immediately after surgery, as the organ is recovering from ischemia/reperfusion damage. In addition, various degrees of hematuria are often detectable immediately after transplantation and this may also be an explanation for a disturbed urinary metabolome. Moreover, a catheter placed into ureter and/or urethra might influence test results in the same way. Nonetheless, once a stable metabolic homeostasis is reached approximately ten to 14 days post-transplant, the metabolite rejection score reliably indicates changes that are associated with the acute rejection process. The fact that the metabolite constellation was essentially developed with *T-cell* mediated rejection should be acknowledged. The small number of patients with antibody-mediated rejection in the UMBRELLA cohort prevented in-depth analysis of this subtype of allograft rejection and its specific influence on the metabolite constellation. To overcome this important shortcoming a further study program has already been initiated.

In conclusion, the metabolite constellation validated in the independent UMBRELLA study for the detection of acute renal allograft rejection provides a valuable non-invasive tool for close routine surveillance after renal transplantation.

Declaration of Competing Interest

SN, PP, JE and ES report personal fees from numares AG, outside the submitted work. In addition, SN, PP, and JE have a patent WO2018167157A1 pending that is directly related to this work. All other authors have declared that no conflict of interest exists.

CRediT authorship contribution statement

Miriam C. Banas: Conceptualization, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Sindy Neumann:** Formal analysis, Writing - original draft, Writing - review & editing. **Philipp Pagel:** Conceptualization, Formal analysis, Writing - review & editing. **Franz Josef Putz:** Data curation, Formal analysis, Writing - review & editing. **Bernhard K. Krämer:** Formal analysis, Validation, Writing - review & editing. **Georg A. Böhmig:** Formal analysis, Validation, Writing - original draft, Writing - review & editing. **Johannes Eiglsperger:** Formal analysis, Writing - review & editing. **Eric Schiffer:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing. **Petra Ruemmele:** Methodology, Writing - review & editing. **Bernhard Banas:** Conceptualization, Data curation, Formal analysis, Writing - original draft, Writing - review & editing.

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