ORIGINAL ARTICLE

Urinary proteomics to diagnose chronic active antibody-mediated rejection in pediatric kidney transplantation – a pilot study

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SUMMARY

Chronic antibody-mediated rejection (cABMR) is the main cause of long-term renal graft loss. Late-stage diagnosis is made by detecting donor-specific antibodies (DSA) in blood combined with typical histomorphological lesions in renal allografts. There is a need for noninvasive biomarkers for cABMR that might permit screening and earlier diagnosis. In a case control study of 24 pediatric renal transplant recipients, urine samples were analyzed using capillary electrophoresis and mass spectrometry. Patients were matched with 36 pediatric renal transplant patients without cABMR. Statistical analysis used the nonparametric Wilcoxon test to identify 79 significant biomarkers, which were combined to a support vector machine-based classifier. After validation in an independent test cohort of eight pediatric patients with and 12 without cABMR, the area under the receiver operating characteristic (ROC) curve (AUC) for detection of cABMR was 0.92 (95% CI 0.71–0.99) with a sensitivity of 100% (95% CI 63–100%) and a specificity of 75% (95% CI 43–95%). Combining this classifier with the urinary proteomic marker CKD273 improved the detection of patients with cABMR with misclassification in only 2/20 of the patients. These data indicate that a biomarker pattern derived from urinary proteomics allows the detection of cABMR in pediatric renal transplant recipients with high sensitivity and moderate specificity.

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Introduction

After pediatric kidney transplantation, it is important to maintain the right balance between over- and under-immunosuppression to avoid severe infections and immunosuppressant side effects as well as graft rejection. Over the last decades, prevention and treatment of acute cellular rejection have been very successful. However, chronic active antibody-mediated rejection (cABMR) is the leading cause of long-term graft failure. Up to 63% of late kidney transplant losses can be attributed to cABMR [1]. The presence of donor-specific anti-HLA antibodies (DSA) significantly reduces graft survival to a half-life of 5.1 years compared to 13.2 years if no DSA are present [2]. Children may be at higher risk because of the greater exposure to nonadherence in teenage years. The diagnosis of cABMR depends on the presence of impaired renal function, detectable DSA and a conclusive renal biopsy [3]. In fact, studies showed a high prevalence (approximately 50%) of DSA in pediatric renal transplant recipients at median of 0.25 years post-transplant, but in many cases, the presence of DSA did not predict deterioration of graft function [2]. It has been shown that urinary proteome analysis by capillary electrophoresis-mass spectrometry (CE-MS) can identify kidney injuries with a high level of sensitivity and specificity [4]. Using CE-MS and protocol biopsies as reference, we previously established a set of urinary naturally occurring peptides that could reliably detect acute cellular rejection of renal allografts (aTCMR) at an early stage during the first-year post-transplant in adult patients [5]. This test showed an area under the curve (AUC) of 0.89 ($P < 0.0001$) in receiver operating characteristic (ROC) analysis, with 93% sensitivity and 78% specificity. CE-MS has also been used for another proteome-based classifier named "CKD273" that is able to predict progression of renal pathologies in advance to standard clinical parameters, like albuminuria [4]. The use of urine for proteome analysis has been shown to be superior to blood serum or plasma in several respects [6]: (i) it can be obtained noninvasively in large quantities, (ii) its low-molecular weight proteins are soluble and can be analyzed in a mass spectrometer without additional manipulation, and (iii) its peptide composition is highly stable, since degradation by proteases has been completed in the bladder before voiding.

We hypothesize that it is also possible to establish a urinary proteome pattern for early diagnosis of cABMR in pediatric patients.

Methods

Patient characteristics

As part of the IMMRES (Immune Response of Pediatric Renal Transplant Recipients challenged by Sensitization, Vaccination or Non-Adherence; Cross-sectional and Prospective Analyses of the International CERTAIN Registry Cohort) study, urine samples from 24 patients with a diagnosis of cABMR as defined by the Banff classification criteria [8] and 36 control patients (DSA-test negative, normal kidney biopsy or no biopsy) were analyzed in order to resolve the urinary proteome of each patient. Exclusion criteria were acute infection of the urinary tract and lack of informed parental consent. Patient matching was performed within the Cooperative European Paediatric Renal TransplAnt INitiative (CERTAIN) registry [9]. Matching criteria were source of transplant (living donor versus deceased donor), age group (2–5.9, 6–11.9, and 12–18 years, sex and time post-transplant (6–11 months, 1–5, >5 years). Each patient with cABMR was matched to at least one patient without cABMR. Demographic data are listed in Table 1. Following the guidelines of the clinical proteome analysis [7], 2/3 of the total cohort were randomly allocated to the training set (16 cases/24 controls) and 1/3 to the test set (6 cases/12 controls). Furthermore, the matching criteria were applied to the training set including estimated glomerular filtration (eGFR).

The study protocol was approved by the Institutional Review Board or Independent Ethics Committee of each participating center and was conducted according to the Declaration of Helsinki. All parents/guardians provided written informed consent, with assent from patients when appropriate for their age.

Definition of cABMR

Human leukocyte antigen antibodies were measured prior to engraftment and at least annually post-transplant by the LABScreen single-antigen beads Luminex kit (One Lambda, Canoga Park, CA, USA) which uses single HLA-coated beads and enables identification of IgG alloantibody specificities against HLA-A, -B, -C, -DRB1/3/4/5, -DQA1, -DQB1, -DPA1, and -DPB1 antigens locally in all centers taking part in the IMMRES-trial. Because no clinically validated cut-off for the Luminex assay is recommended by the provider company, a mean fluorescence intensity of ≥1000 was used to define the cut-off for antibody positivity. For

Table 1. Demographic data (mean ± SD). (a) Comparison cABMR/controls. (b) Comparison trainings- and test-set. (c) Underlying diagnoses.

(a)	Patients with cABMR	Controls	*P*-value
Number	24	36	
Age (years)	14 ± 4	14 ± 4	0.73
Gender (male/female)	15/9	20/16	0.60
Time post-transplant (years)	6 ± 4	7 ± 4	0.53
eGFR (ml/min/1.73 m^2)	42 ± 15	61 ± 22	0.001
Donor source (LD/DD)	4/20	7/29	0.88
Detection of DSA (%)	100	0	<0.001
cABMR in graft biopsy (%)	100	0	<0.001
Urine albumin/creatinine ratio (mg/μmol)	47 ± 83	29 ± 38	0.20

(b)	Trainings set	Test set	*P*-value
Number	40	20	
Age (years)	14 ± 3	13 ± 3	0.75
Gender (male/female)	23/17	11/9	0.62
Time post-transplant (years)	7 ± 4	6 ± 3	0.43
eGFR (ml/min/1.73 m^2)	51 ± 11	62 ± 31	0.32
Donor source (LD/DD)	8/32	3/17	0.64
Detection of DSA (%)	100	100	1.0
cABMR in graft biopsy (%)	100	100	1.0
Urine albumin/creatinine ratio (mg/μmol)	19 ± 19	71 ± 113	0.20

(c)	cABMR trainings set	cABMR test set	Controls trainings set	Controls test set
CAKUT	7	3	8	5
Glomerulopathy	3	2	6	3
Cystic kidney	3	1	3	1
Systemic	1	0	2	1
Other	0	0	1	1
Unknown	2	2	2	3

cABMR, chronic antibody-mediated rejection; DSA, donor-specific antibodies; eGFR, estimated glomerular filtration; LD/DD, living donation/deceased donation]; SD, standard deviation.

high-resolution typing CTS-Sequence kits (Heidelberg, Germany) and Olerup-SSP kits (Saltsjöbaden, Sweden) were used. In case of positivity, it was determined whether a detected antibody was donor-specific (DSA). In most centers also MFI values of DSAs were available, some centers did not provide these values.

Patients with DSA underwent a renal graft biopsy, which was analyzed by the local pathologist, using the current update of the BANFF 2007 classification [8]. Here, cABMR was diagnosed by the local pathologist if the local analysis was in accordance with the cABMR criteria of the BANFF classification. No central pathological analysis was performed.

CE-MS analysis

Spot urine samples of 5–10 ml collected midstream in sterile collection bags were immediately frozen at −20 °C until shipment. The detailed method for urinary proteome analysis has been published previously [11]. Briefly, urine was prepared for proteome analysis, CE-MS was performed using a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA, USA) on-line coupled to a Micro-TOF MS (Bruker Daltonic, Bremen, Germany), and data were processed (calibration, normalization) with proprietary software packages. All detected peptides were deposited, matched and annotated in a Microsoft SQL database, allowing further analysis and comparison of multiple samples. MOSACLUSTER Software 1.7.0 (Biomosaiques Software GmbH, Hannover, Germany) using support vector machines (SVMs) was applied. Candidate biomarkers were sequenced using tandem mass spectrometry.

In order to identify the optimal cut-off value of the classifier, ROC curve analysis was used to ensure highest sensitivity and specificity of the classifier in the training cohort. This cut-off value was used in the test cohort to grade patients with and without cABMR.

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This study was conducted according to the European Kidney and Urine Proteomics Program quality guidelines and it therefore complies with all recommendations for biomarker identification and qualification in clinical proteomics regarding study design, statistical methods, definition of study and control groups, and usage of an independent test set for confirmation [6].

Statistical analysis

For statistical analysis, the nonparametric Wilcoxon test (R-based statistical software, version 2.15.3) was used for 5616 peptides [10]. Only proteins/peptides that were detected at a frequency of 90% in at least one of the diagnostic groups of the training set were considered for statistical analysis (n = 335). A P-value of <0.05 was set as significance level. Potential biomarkers of statistical significance (n = 79) were combined to SVM-based classifiers with the use of MOSACLUSTER software 1.7.0 (Biomosaiques Software GmbH) [11]. An independent test cohort was used to validate the generated cABMR-classifier for the detection of cABMR in children. The combination of the cABMR- and the CKD273-classifiers was performed by addition of both scores. The cut-off for this combination was defined based on the combination of both classifiers in the training set.

Demographic data were compared by the Fisher's exact or chi-square tests, $P < 0.05$ was considered significant. Analysis was carried out using MEDCALC 12.7.5.0 (MedCalc software, Mariakerke, Belgium), in addition to the ROC curve analysis.

Sequence analysis

In order to obtain sequence information CE- and LC-MS/MS were used as complementary approaches, using an Orbitrap Velos FTMS (Thermo Finnigan, Bremen, Germany) as the connected mass spectrometer [12]. CE-MS/MS has the advantage of direct matching (mass and CE-time) to the peptides quantified by CE-MS. On the other hand, LC-MS/MS exhibits higher sensitivity due to the improved loading capacity of the LC-column, consequently a better coverage of sequence information. A nonpossible direct matching of the sequence to the CE-MS data is the main disadvantage of LC-MS/MS. However, a property of CE is that the migration time is dependent on the net positive charge of the peptide. At pH 2 (pH of the running buffer) this (charge) is a function of the number of basic amino acids present [13]. Therefore, the sequence can be matched indirectly by the number of basic amino acids.

Data files were searched against the UniProt human nonredundant database using Proteome Discoverer 1.2 (Thermo Fischer Scientific, Waltham, MA, USA) and the SEQUEST search engine. Relevant settings were as follows: no fixed modifications, oxidation of methionine and proline as variable modifications. The minimum precursor mass was set to 790 Da, maximum precursor mass to 6000 Da with a minimum peak count of 10. The high confidence peptides were defined by cross-correlation (Xcorr) ≥1.9 and rank = 1. Precursor mass tolerance was 5 ppm and fragment mass tolerance was 0.05 Da. False discovery rate settings cannot guarantee that the correct sequence is assigned to a fragmentation spectrum generated in an MS/MS experiment [14].

Results

Demographic and clinical data of all patients are listed in Table 1a and b. There were no differences between patients with cABMR and controls in mean age, time post-transplant, sex, or donor source as well as in urine albumin-creatinine ratio. The distribution of the different primary renal diseases is depicted in Table 1c. Most patients had CAKUT as the primary renal disease. For some control patients, the primary renal disease was unknown because patients initially presented with terminal renal failure. Table 2 describes the type of DSA and the respective MFI values (if available) in the individual patients as well as the main graft biopsy findings according to the BANFF classification. Most patients had active and chronic lesions of antibody mediated rejection. As not all pathologists performed complete BANFF scoring, information is not complete in all patients. Patients of the control group were negative in routine DSA monitoring and biopsies performed in the 6 months before DSA-testing did not show the BANFF criteria of cABMR (n = 31). In five control patients, no graft biopsies were performed prior to enrollment.

Following the guidelines of the clinical proteome analysis [7], we randomly selected 2/3 of the total cohort as training set (16 cases/24 controls) and 1/3 as test set (6 cases/12 controls). Comparative urinary proteome analysis with the use of the CE-MS approach of patients with cABMR and controls from the training set revealed 79 peptides associated with cABMR. The peptide patterns of both groups are shown in Fig. 1. Some of the potential biomarker peptides were shown to be increased while others were decreased compared to control. Peptide sequence information was available for 46 of the 79 biomarker candidates (see Table S1). Most of the sequenced peptides were fragments of collagen

Table 2. Donor-specific antibodies (DSAs) and graft biopsy findings in the case group.

Patient	Glomerulopathy (cg > 0)	Glomerulitis (g > 0)	Peritubular capillary basement membrane multilayering	Peritubular capillaritis (ptc > 0)	Fibrous intimal thickening in arteries	IF/TA (*i* > 0)	c4d positivity (cd4 > 0)	Donor specific antibodies
1	yes	yes	yes	yes	no	yes	no	DR16 (MFI 6364)
2	no	no	yes	yes	no	no	yes	DQA1 (MFI 11491), DQB1 (MFI 12961)
3	yes	yes	yes	yes	yes	yes	yes	DQ2 (MFI 5188)
4	yes	yes	yes	yes	no	yes	yes	DQ7 (12089), DQ8 (7827), DQ9 (8799), Cw 7 (7111)
5	no	yes	yes	yes	no	no	no	DRB 4 (1662)
6	yes	yes	yes	yes	no	yes	yes	A1 (5306)
7	no	no	yes	no	no	yes	yes	B44 (MFI 3074), DQ2 (MFI 8807), DR53 (MFI 5512)
8	yes	yes	yes	yes	no	no	no	B45 (MFI 1639), DQ3 (MFI 15750)
9	yes	yes	yes	yes	yes	yes	no	DQ6 (MFI 11306)
10	yes	unknown	yes	unknown	yes	yes	no	Cw5 (MFI 5155), DQ6 (3685)
11	yes	yes	yes	yes	no	yes	yes	DR53 (MFI 4417)
12	yes	yes	yes	yes	yes	yes	no	A2 (MFI 11774), A24 (MFI 9229), DQ (MFI 3532)
13	yes	no	no	yes	yes	yes	no	B7 (24300), A3 (20500), A26 (17200), A38 (8700)
14	no	unknown	no	unknown	yes	yes	no	A26
15	no	yes	yes	yes	no	yes	no	DQB1, DQA1
16	yes	yes	yes	yes	yes	yes	no	A11 (MFI 2677), DR 52 (MFI 4965)
17	no	unknown	no	unknown	no	yes	yes	DRB1, DRB3, DRB1
18	yes	unknown	yes	unknown	yes	yes	yes	HLA DR3 (MFI 6200), DR52 (MFI 6200)
19	yes	unknown	yes	unknown	yes	yes	no	DQB1, DQA1, C07
20	yes	no	yes	no	no	yes	no	DQ3 (MFI 2806)
21	yes	yes	yes	yes	yes	yes	no	DQB1
22	yes	yes	no	yes	yes	yes	yes	DQB1, DQA1
23	yes	yes	yes	yes	yes	yes	no	DQ6 (MFI 16388)
24	yes	yes	yes	yes	no	yes	no	A2 (MFI 2740), DQ8 (MFI 21757)

If available, values for mean fluorescence intensity of DSAs are given. Patients of the training set are written black on white, patients of the test set.

alpha-1(I) chain (51%) and collagen alpha-1(III) chain (17%). We were also able to find differences in fragments from other collagen fragments (COL1A2, COL2A1, COL18A1, COL24A1). Further identified peptides were fragments of alpha-1-antitrypsin, retinol-binding protein 4, fibrinogen alpha chain, neurosecretory protein VGF, Ig kappa chain C region, beta-2-microglobulin, and annexin A1. In Fig. 2a,b, the fold changes of the 47 sequenced candidate biomarkers are shown. Within the COL1A1 fragments the fold change was heterogeneous (Fig. 2a). The other collagen fragments mostly had a negative-fold change, which demonstrates a decrease in the collagen peptides in cABMR. Furthermore, some of the collagen peptides belong to the same region of the collagen protein. In Fig. 2b, three peptides (alpha-1-antitrypsin, annexin A1, neurosecretory protein VGF) showed a positive-fold change, whereas four peptides (beta-2-microglobulin, fibrinogen alpha, Ig kappa chain C region, retinol-binding protein 4) had a negative fold change. In Fig. 2a,b, the peptides marked with # are also included in the CKD273-classifier (56% of the collagens, fibrinogen alpha chain, and neurosecretory protein VGF). Furthermore, some of the peptides included in the cAMBR and CKD273 classifier are derived from the same ancestor protein, e.g., collagen alpha-1 type I, II, and III, alpha-1-antitrypsin, and beta-2-microglobulin.

We used the 79 peptides to generate an SVM-based classifier for the diagnosis of cABMR. In an independent test set, the sensitivity of this classifier for the detection of cABMR was 100% (95% confidence interval [CI]: 63–100%), specificity was 75% (95% CI: 43–95%) with an area under the ROC curve (AUC) of 0.92 (see Table 3). Furthermore, there is no significant correlation between the eGFR and the cABMR scores of patients ($P = 0.64$).

Since other classifiers had previously been developed for the diagnosis of chronic kidney disease (CKD; CKD273-classifier), as well as for the diagnosis of acute cellular rejection of renal allografts in adult patients (aTCMR-classifier), we additionally classified the test set using these proteomic classifiers (Fig. 3). The diagnosis with the CKD273-classifier resulted in an AUC of 0.80 (95% CI: 0.57–0.94), which was not significantly worse than the cABMR-classifier. The AUC of the aTCMR-classifier was 0.64 (95% CI: 0.39–0.84). However, this was also not significantly ($P = 0.06$) lower than the AUC of the cABMR-classifier, although this could be an effect of the small test cohort.

Furthermore, we combined the classification scores of the cABMR-classifier with those of the CKD273-classifier in order to improve specificity. The combination of both

classifiers resulted in a not significantly better AUC of 0.93 (95% CI: 0.72–1.00) with a sensitivity of 88% (95% CI: 47–100%) and a specificity of 92% (95% CI: 62–100%) for the diagnosis of cABMR. With this combination the misclassification was only 10% of the patients, which was 5% less than with only the cABMR-classifier.

Discussion

This study shows for the first time that urinary proteome analysis by CE-MS can identify children with cABMR in a pediatric postkidney transplant population with an indication biopsy based on the serum creatinine increase in the case of positivity for DSA. These findings are independent of total urinary albumine excretion that is highly variable in both groups. No cut-off value for urinary albumine/creatinine ratio could be determined to distinguish between normal biopsy and cAMR in children with newly detected DSAs.

Because of these results, this noninvasive urinary proteome analysis may be used as a post-transplant-screening test in kidney transplanted children in whom *de novo* DSA are detected. Although the sensitivity of the cABMR-classifier is 100%, patients without cABMR will also be detected. Furthermore, combination with the CKD273-classifier, which can identify all patients without cABMR (with an established cut-off of 0.343), showed an increase in specificity (with a lower sensitivity as a price to be paid for) and therefore provides the opportunity to screen children with recently detected DSA. Nevertheless, this classifier might help to decide which patients might not need a renal graft biopsy, which is invasive and not without risks, since they are negative for this classifier and may have a low chance of experiencing cABMR. A follow-up trial in a sufficiently large prospective cohort that will allow calculating more precise positive and negative predictive values of the combined proteomics test is required before implementing this test in routine care. In longitudinal trials, it is important to test if this urinary proteomics classifier can identify cABMR in pediatric renal transplant recipients long before functional impairment as a result of cABMR to allow earlier diagnosis by renal graft biopsy and to initiate more intense immunosuppressive therapy before irreversible tissue damage occurs.

Although the biomarker profile contains the principal information for diagnosis, a sequence analysis of peptides is needed to gain insights into pathophysiological mechanisms. Using state-of-the-art tandem mass spectrometry, approximately 60% of the peptides that are included in this cABMR pattern could be identified. Some of the sequenced peptides, which showed a significant difference between the urine of children with or without cABMR, are well known with respect to kidney diseases, as for example, collagen chains and fibrinogen alpha fragments, which are differentially expressed in patients with cABMR compared to those without cABMR. Collagens are the main structural elements of the interstitial extracellular matrix (ECM), responsible for cell adhesion, tissue development and tensile strength [15]. The reduced abundance of urinary peptides derived from ECM proteins has previously been observed in other studies on kidney diseases [16]. In general, 30% of the marker peptides are also a component of the known CKD273-classifier (marked with # in Fig. 3) for the detection of CKD [15], which was recommended by the FDA in 2016 for

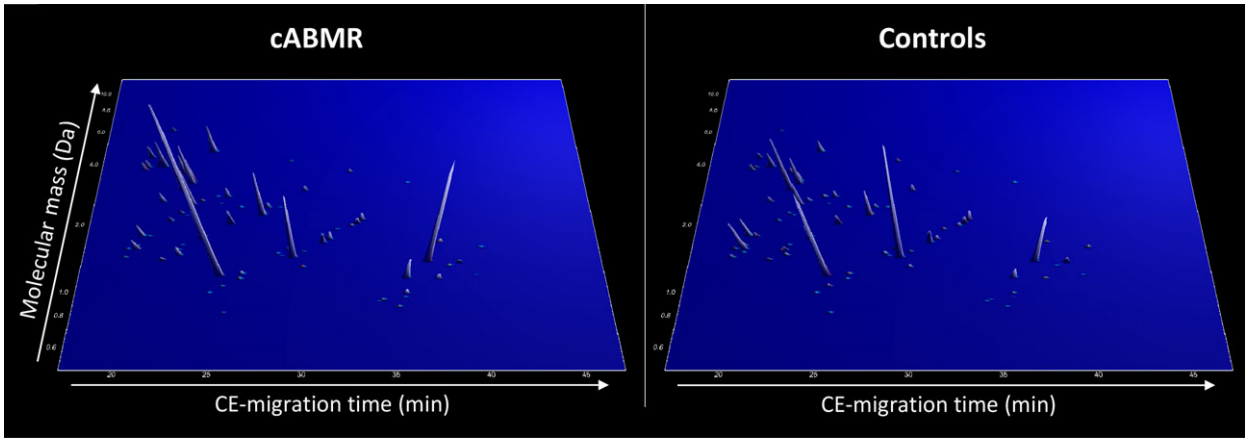

Figure 1 Proteome pattern. Urinary peptides obtained from children with chronic antibody-mediated rejection (cABMR) after kidney transplantation and controls from kidney transplanted children without cABMR. Seventy-nine indicative peptides with *P*-value <0.05 defined a cABMR-specific peptide pattern distinguishing patients from controls. Normalized molecular mass (Da) was plotted against normalized capillary electrophoresis-migration time (min). Mean signal intensity was given in three-dimensional depiction.

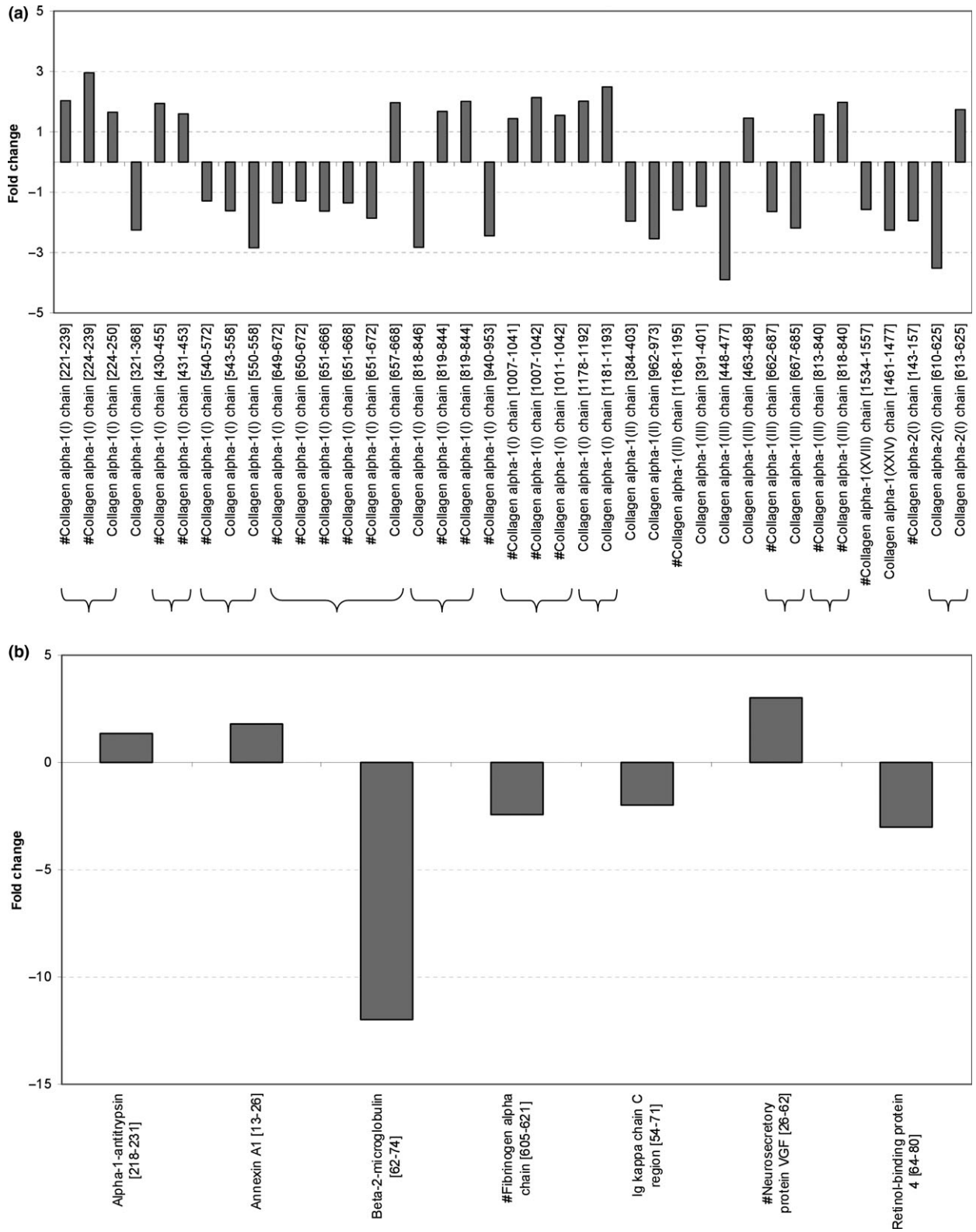

Figure 2 Important marker peptides of the proteomic pattern for chronic antibody-mediated rejection (cABMR). Fold change is shown of (a) collagen peptides and (b) other peptides. #Peptides also included in the CKD273-classifier. ⤷Peptides located in the same protein region.

Table 3. Performance of the different proteomics classifiers.

Classifier	AUC [95% CI]	Sensitivity (%) [95% CI]	Specificity (%) [95% CI]	Pos. predicted value (%) [95% CI]	Neg. predicted value [95% CI]
aTCMR	0.64 [0.39–0.84]	100 [63–100]	0 [0–27]	40 [19–64]	–
CKD273	0.80 [0.57–0.94]	50 [16–84]	100 [74–100]	100 [40–100]	75% [48–93]
cABMR	0.92 [0.71–0.99]	100 [63–100]	75 [43–95]	73 [39–94]	100% [66–100]
cAMBR + CKD273	0.93 [0.72–1.00]	88 [47–100]	92 [62–100]	88 [47–100]	92% [60–100]

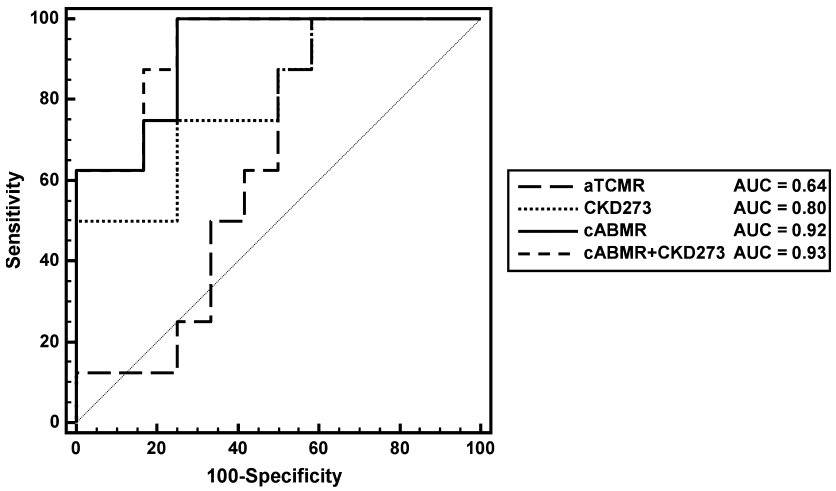

Figure 3 Receiver operating characteristic curve analysis of the test set. The black line represents the results of the chronic antibody-mediated rejection (cABMR)-classifier, the dotted line those of the CKD273-classifier, the line with the long dashes those of the acute T-cell-mediated rejection (aTCMR)-classifier, and the line with the short dashes those of the combination of cABMR- and CKD273-classifiers.

use in clinical trials of adults with diabetes mellitus [17]. The similarity of both peptide patterns is not surprising since both diabetes mellitus and cABMR lead to a progression of fibrosis in the kidney and hence to progression of CKD [18,19]. However, other marker peptides are unique for cABMR: Annexin A1 has an important role in the epithelial cells defense against renal ischemia–reperfusion (I/R) injury [20]. The serum retinol-binding protein 4 concentration of patients with ESRD decreased significantly after kidney transplantation [21]. However, nothing is known with respect to kidney rejection [22]. The decreased concentration of the Ig kappa chain C region peptide can be a result of the increased deposition of immunoglobulins in the kidney tissue with respect of the immune response in the group of antibody-mediated rejection patients.

The CKD273-classifier has proven capable of very early detection of CKD which other methods have failed to identify. Consequently, it is feasible that this cABMR-pattern might also allow a diagnosis at a very early stage. Interestingly, the peptides in the cABMR-classifier differ completely from those in the urinary proteomic classifier published for acute cellular rejection [5], most likely because fibrosis does not play a significant role in acute rejection but represents graft deterioration caused by cABMR.

We used CE-MS, as it enables reproducible high-resolution analysis of thousands of urinary low-molecular weight peptides in less than 1 h [23]. Urinary analysis proved to be particularly suitable as samples can be stored at −20 °C [23]. It is easy to normalize differences in peptide concentration by using internal peptide standards generally present in urine [24]. To use this proteome test for clinical screening of cABMR, it has to be unaffected by other variable clinical parameters, such as age and gender. Therefore, statistical analysis of all cABMR-scores by patient's age, gender or CKD status has been performed. These parameters and the SVM scores demonstrated no significant influence, indicating that this classifier has a high degree of stability. No single biomarkers have been established in the last few years to support early diagnosis of acute or chronic kidney graft rejection. Therefore, we have chosen to evaluate a biomarker classifier rather than a single marker since a

distinct and well-defined pattern of biomarkers may better define the perturbations occurring in different disease stages than could a single biomarker [25]. However, as our pattern has not been compared in studies with other recently initiated and clinically used methods such as donor-derived cell-free DNA or other biomarkers, it remains to be evaluated whether the proteome pattern is superior or whether a combination with other biomarkers can increase positive and negative predictive value.

The limitation of this exploratory pilot study is the low number of patients. However, because of the fact that we were able to find relevant peptide biomarkers, which resulted in a good accuracy in their validation with an independent test cohort, we think that the sample size was efficient enough. This does not mean that the generated classifier does not need a further validation in a greater, multicenter cohort.

We conclude that the proteomic classifier established and validated in this study may be used as a first-line screening tool to identify children with cABMR after kidney transplantation in order to detect those who would profit from early graft biopsy, to avoid unnecessary biopsies and to implement intensification of immunosuppressive treatment if our findings could be confirmed by comparing DSA positive patients with and without development of future cAMR. The classifier should therefore be prospectively validated in a larger cohort of patients of all ages as well as in a longitudinal study evaluating whether the test can detect cABMR at earlier stages. If these trials are successful, the classifier could then be adopted as a routine diagnostic tool.

Authorship

Nele Kirsten Kanzelmeyer conducted the study and wrote the first version of the manuscript. Petra Zürbig, Harald Mischak and Jochen Metzger performed the proteome analyses. Alexander Fichtner, Kirstzina Rudzai, Tomas Seemann, Matthias Hansen and Simone Wyoda provided patients and clinical information for the study, Kai Krupka performed the CERTAIN data analyses. Burkhard Toenshoff, Anette Melk and Lars Pape designed the study and worked on the manuscript. All authors approved the last version of the manuscript.

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Conflict of interest

Harald Mischak is the co-founder and co-owner of Mosaiques Diagnostics GmbH, the company that developed CKD273. Petra Zürbig and Jochen Metzger are employees of Mosaiques Diagnostics GmbH. The other authors have no conflicts of interest to disclose.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Sequenced proteins of the cABMR pattern.

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