Supplementary material

Table of Contents

- **Table S3**: Impact of the chosen treshold on the diagnostic performance of the model including all 10 proteins (model10)
- **Table S4**: Proteins included in the different statistical models. The models including 6 proteins were chosen based on their abundance in urine samples.
- **Table S5:** Subclassification of the number of biopsies that were classified using the model compared to the biopsy result for the training cohort
- **Table S6:** Subclassification of the number of biopsies that were classified using the model compared to the biopsy result for the validation cohort
- **Table S7:** Overview of the sensitivity analyses of the diagnostic accuracy of the urinary marker in presence or absence of hematuria, leucocyturia and bacteriuria

Supplementary Methods

Urinary sample collection and preparation

Urine samples were centrifuged at 2,000g at 4°C for 20 minutes to remove cell debris and casts within 2 hours after collection. The supernatans was then stored at -20°C until shipment to the analytical centre. Upon arrival, the samples were stored at -80°C. After a first concentration determination using the Pierce™ BCA Protein Assay Kit (Thermo Scientific), 2mg of protein was processed on a Amicon Ultra-0.5 Centrifugal Filter Unit with a 10kDa molecular weight cut-off membrane (Merck Millipore). The protein concentration of the concentrated samples was again determined using the same BCA Protein assay. Subsequently, 100µg of protein was loaded on the Pierce™ Albumin Depletion Kit (Thermo Scientific) spin columns to deplete the samples from human albumin. After albumin depletion, the protein concentration was determined a last time. 20µg of protein was denatured in 0.1% Rapigest (RapiGest ™ SF, Waters). After denaturation, proteins were reduced by adding 2µl of 200mM TCEP (Tris(2-carboxyethyl)phosphine; Thermo Scientific) and incubating the sample for 1h at 55°C. Afterwards, samples were alkylated by adding 2µl of 375mM IAA (Iodoacetamide; Thermo Scientific) for 30 min. at room temperature protected from light. To precipitate the proteins, 1ml of pre-chilled acetone was added and incubated overnight at -20°C. After a centrifugation step (10,000g, 15min., 4°C), the protein pellet was resuspended in 20µl 200mM TEAB (Triethylammonium bicarbonate; Sigma-Aldrich). 1µg of trypsin (Trypsin Gold, Mass Spectrometry Grade; Promega) was added to digest the proteins while incubating overnight at 37°C. The digestion was stopped and Rapigest was hydrolyzed by adding HCl to a final concentration of 200mM (30 min. at room temperature). After a centrifugation step (10,000g, 15 min., 4°C), the pellet was removed and the samples were diluted in 2% acetonitrile, 0.1% formic acid to a final concentration of 0.2µg/µl. All samples were spiked with 4fmol/µl GFP ([Glu1]-Fibrinopeptide B human; Sigma-Aldrich).

LC-MS/MS proteomics

In total, 1µg of the peptide mixture, spiked with 20fmol GFP, was loaded on the LC column. The tryptic peptide mixture was analysed on a Nano Acquity Ultra Performance LC system (Waters) using a nanoACQUITY UPLC Symmetry C18 Trap Column (180 µm x 20 mm; Waters) coupled to a ACQUITY UPLC Peptide BEH C18 nanoACQUITY column (100 µm X 100 mm; Waters). A linear gradient of mobile phase B (98 % acetonitril, 0.1 % formic acid, pH = 2) from 5 to 45% in 68 min. was followed by a steep incease to 90% mobile phase B in

3 minutes. The flow was set at 400nl/min. The nano-LC was coupled online to the LTQ Velos Orbitrap mass spectrometer (Thermo Scientific) via the nanospray ion source (Thermo Scientific). The LTQ Velos orbitrap was set up in MS/MS shotgun mode, where a full MS1 precusor scan (300 – 2000m/z, resolution 60,000) was followed by a maximum of 10 collision induced dissociation (CID) MS2 spectra of the 10 most intense precursor peaks. CID spectra were obtained in the linear ion trap of the mass spectrometer. The normalized collision energy used in CID was set at 35%. We applied a dynamic exclusion of 30s for data dependent acquisition.

We then applied a label-free quantification algorithm, taking the union of all peptide identifications that were at least 3 times highly confidently identified in the training cohort as reference. We modeled retention time curves between all samples, which allowed us to recalibrate retention time correctly from one sample to another. Mass recalibration was checked and reperformed only when necessary. We allowed retention time differences for the top of the peaks of 30 sec and mass differences of 5 ppm. For each peptide, consensus values were calculated. For each reference feature in each individual run, the precursor ion was searched in MS1 using this consensus time and mass information. Within a matching window of RT +/- 1 min and m/z +/-5ppm, the ion with maximum intensity was selected. The whole peak, which is roughly a Gaussian-shaped curve, was then drawn and scored by the number of MS1 scans which made up the peak, and by the quality of the bell shape of the peak. This resulted in one intensity and score for each peptide in each sample. Together with the peptide information on retention time (T) and mass (M), this information was compared to the reference values and ΔT and ΔM values were calculated. Next, we inspected the peptides individually by comparing the ΔT and ΔM for each run. Deviating intensities for ΔT and ΔM were annotated as missing values. We then repeated this process using the same set of identifications as reference set, but changing the retention times to random retention times outside the reference retention time window. We finally compared the number of peptides that were retained and the percentage of missing values of the original process with the decoy approach, in order to calculate the false discovery rate.

We used this in-house developed software described above because it allowed us to have a clear overview of the data enrichment process and moreover, none of the referred methods were scalable for the amount of data we have.

Supplementary Figures

Figure S1: Number of proteins that are needed in a model based on cross validation in training cohort to get a good classification of phenotypes

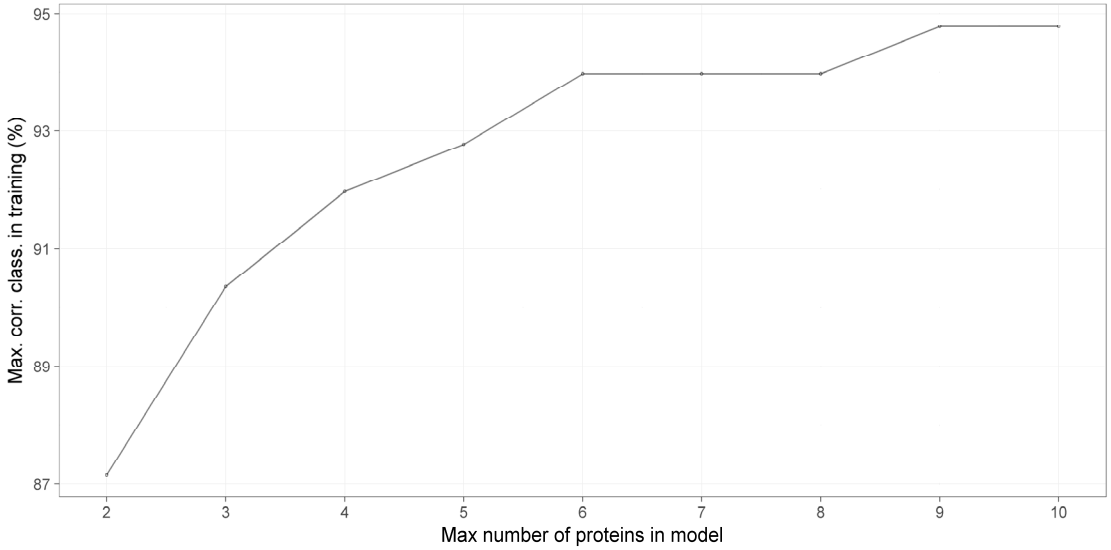


Figure S2: The unmodeled proteinuria data (in g/g creatinine) reached an area under the curve of 0,75 (95% CI 0,67 to 0,83) for the training cohort (A) and 0,76 (95% CI 0,66 to 0,85) for the validation cohort (B).

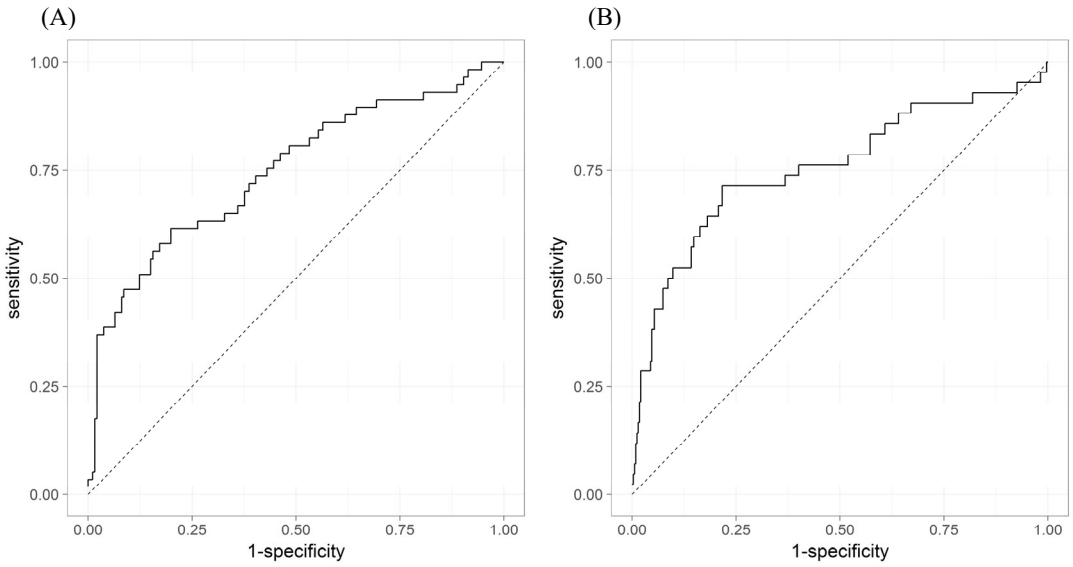


Figure S3: Receiver Operating Characteristic (ROC) curves are shown for the full model with 10 proteins for DSA positive (left panel) and DSA negative patients (right panel) using the validation data set. For the DSA positive ABMRs, the AUC value is 92,4% (95% CI 84% - 100%). For the DSA negative ABMRs, the AUC is 88% (95% CI 83% - 93%).

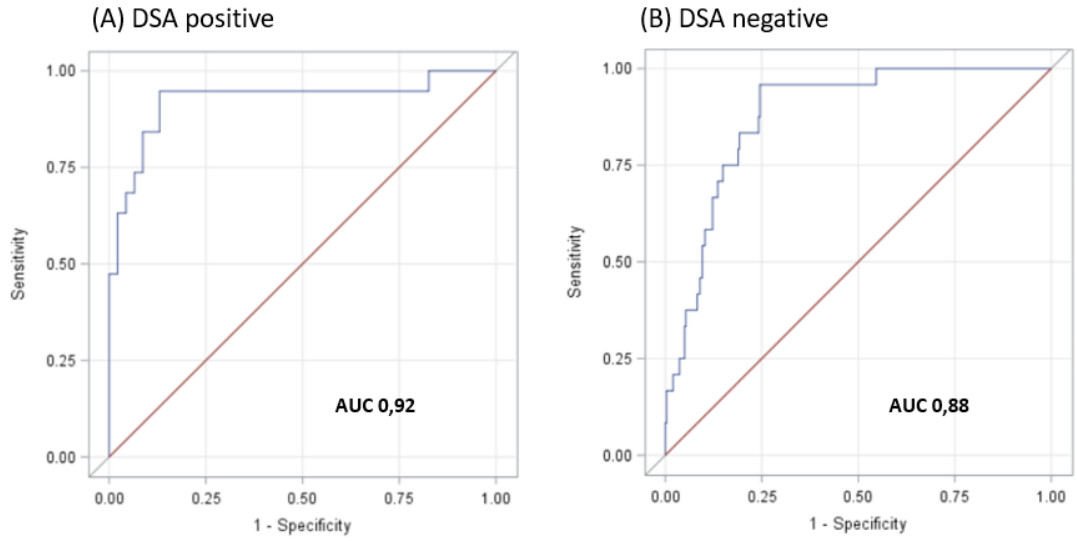


Figure S4: Probability scores using the 10-protein model for ABMR, TCMR, no rejection (NR) and mixed ABMR/TCMR cases.

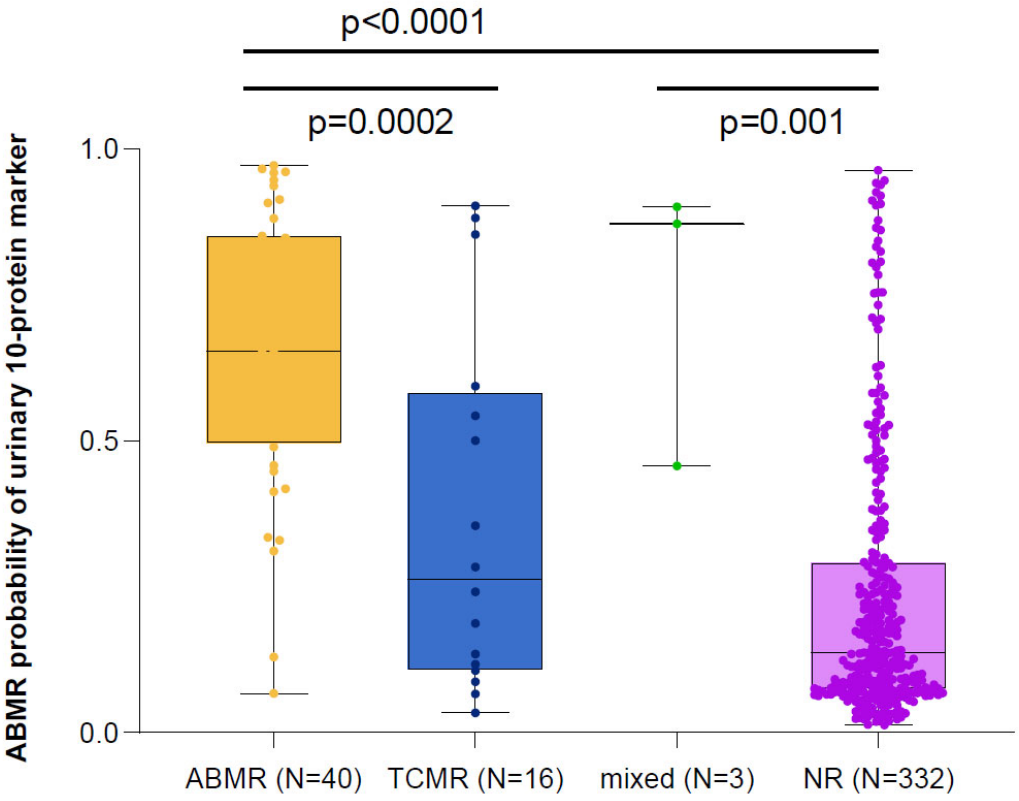
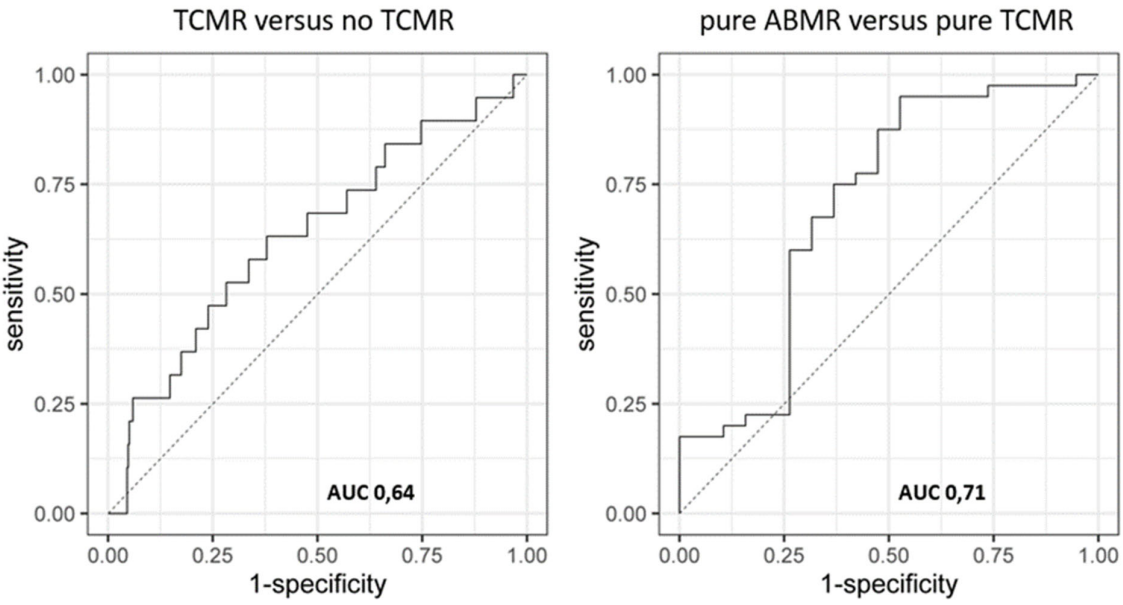


Figure S5: Receiver Operating Characteristic (ROC) curves are shown for the full model with 10 proteins for TCMR versus no TCMR (left panel) and pure ABMR versus pure TCMR cases (right panel) using the validation data set. For TCMR versus no TCMR, the AUC value is 64,4% (95% CI 51,3% - 77,3%). For the comparison of pure cases of ABMR and TCMR, the AUC is 71% (95% CI 54,5% - 85,5%).



Supplemental Tables

Table S1: Histological characteristics of the biopsies included in the BIOMARGIN study in the validation phase (N=391) according to rejection subtypes
* All tubulitis >1 lesions in the ABMR group were t1i0 lesions

Table S1. Histological characteristics of the biopsies included in the BIOMARGIN study in the validation phase (N=391) according to rejection subtypes.				
Histological parameter	**ABMR (N=40)**	**Mixed rejection (N=3)**	**TCMR (N=16)**	**NR (N=332)**
Glomerulitis > 0	36 (90.0%)	3 (100%)	0 (0%)	9 (2.7%)
Peritubular capillaritis > 0	36 (90.0%)	3 (100%)	4 (25%)	21 (6.3%)
Microcirculation inflammation > 1	33 (82.5%)	3 (100%)	1 (6.3%)	8 (2.4%)
Transplant glomerulopathy > 0	22 (55.0%)	2 (66.7%)	0 (0%)	6 (1.8%)
C4d deposition	13 (32.5%)	1 (33.3%)	1 (6.3%)	63 (19.0%)
Interstitial inflammation > 0	1 (2.5%)	3 (100%)	14 (87.5%)	5 (1.5%)
Tubulitis > 0	4 (10.0%)*	3 (100%)	16 (100%)	24 (7.2%)
Total i score > 0	11 (27.5%)	3 (100%)	13 (81.3%)	40 (12.0%)
Intimal arteritis > 0	4 (10.0%)	1 (33.3%)	1 (6.3%)	1 (0.3%)
Tubular atrophy > 1	18 (45.0%)	1 (33.3%)	7 (43.8%)	88 (26.5%)
Interstitial fibrosis > 1	18 (45.0%)	1 (33.3%)	6 (37.5%)	90 (27.1%)
Arteriolar hyalinosis > 1	18 (45.0%)	1 (33.3%)	4 (25%)	62 (18.7%)
Vascular intimal thickening > 1	21 (52.5%)	0 (0%)	7 (43.8%)	102 (30.7%)
DSA positivity	16 (40.0%)	3 (100%)	1 (6.3%)	45 (13.6%)

Table S2: Selected list of unique peptides that were used for training the SVM model in the training cohort. Two unique peptides per protein were selected based on their ability to quantify the proteins using shotgun or targeted proteomics.

A1BG: Alpha-1 B glycoprotein; AFM: afamin ; APOA1: apolipoprotein A1; APOA4: apolipoprotein A4; IGHA1: Ig heavy constant α1; IGHA4: Ig heavy constant γ4; LRG1: leucine rich α2 glycoprotein 1; SERPINA1: alpha-1 antitrypsin; SERPINC1: antithrombin; TF: transferrin

GeneID	peptide
A1BG	ATWSGAVLAGR
A1BG	cEGPIPDVTFELLR
AFM	AESPEVcFNEESPK
AFM	FTDSENVcQER
APOA1	DLATVYVDVLK
APOA1	DYVSQFEGSALGK
APOA4	ISASAEELR
APOA4	SLAELGGHLDQQVEEFR
IGHA1	DASGVTFTWTPSSGK
IGHA1	TFTcTAAYPESK
IGHG4	TTPPVLDSDGSFFLYSR
IGHG4	YGPPcPScPAPEFLGGPSVFLFPPKPK
LRG1	ALGHLDLSGNR
LRG1	DLLLPQPDLR
SERPINA1	LSITGTYDLK
SERPINA1	SVLGQLGITK
SERPINC1	ADGEScSASMMYQEGK
SERPINC1	IEDGFSLK
TF	cSTSSLLEAcTFR
TF	DSGFQMNQLR

Table S3: Impact of the chosen treshold on the diagnostic performance of the model including all 10 proteins (model10).; TP: true positives; TN: true negatives; FP: false positives; FN: false negatives; PPV: positive predictive value; NPV: negative predictive value.

model10	TP	TN	FP	FN	Sensitivity	Specificity	PPV	NPV
Treshold training cohort								
0,1	59	165	24	1	0,98	0,87	0,71	0,99
0,3	57	182	7	3	0,95	0,96	0,89	0,98
0,81	33	188	1	27	0,55	0,99	0,97	0,87
Treshold Validation cohort								
0,1	42	128	220	1	0,98	0,37	0,16	0,99
0,3	41	263	85	2	0,95	0,76	0,33	0,99
0,81	16	330	18	27	0,37	0,95	0,47	0,92

Table S4: Proteins included in the different statistical models. The models including 6 proteins were chosen based on their abundance in urine samples.

model name	model description	proteins included in the model									
		A1BG	AFM	APOA1	APOA4	IGHA1	IGHG4	LRG1	SERPINA1	SERPINC1	TF
model10	all 10 proteins	x	x	x	x	x	x	x	x	x	x
model6A	6 proteins based on highest seq. coverages	x		x	x	x			x		x
model6B	6 proteins based on highest number of PSMs	x			x	x		x	x		x
model6C	6 proteins based on peptide intensities	x		x	x			x	x		x

Table S5: Subclassification of the number of biopsies that were classified using the model compared to the biopsy result for the training cohort. ABMR = antibody-mediated rejection; TCMR = T-cell mediated rejection; NL = normal.

	training cohort		
	total number of samples	classified as no ABMR with our model	classified as ABMR with our model
NR	146	139	7
TCMR	43	43	0
ABMR	35	2	33
ABMR+TCMR	25	1	24

Table S6: Subclassification of the number of biopsies that were classified using the model compared to the biopsy result for the validation cohort. ABMR = antibody-mediated rejection; TCMR = T-cell mediated rejection; NL= normal; GNF = glomerulonephritis; PVAN: polyomavirus-associated nephropathy.

	validation cohort		
	total number of samples	classified as no ABMR with our model	classified as ABMR with our model
NL	300	236	64
GNF	18	12	6
PVAN	13	6	7
PVAN + GNF	1	0	1
TCMR	15	9	6
TCMR + GNF	1	0	1
ABMR	35	2	33
ABMR + GNF	5	0	5
ABMR + TCMR	2	0	2
ABMR + TCMR + GNF	1	0	1

Table S7: Overview of the sensitivity analyses of the diagnostic accuracy of the urinary marker in presence or absence of hematuria, leucocyturia and bacteriuria

	total	TP	TN	FP	FN	Sensitivity	Specificity	PPV	NPV
Hematuria									
presence	157	26	85	45	1	96,3%	65,4%	36,6%	98,8%
absence	234	15	178	40	1	93,7%	81,6%	27,3%	99,4%
Leucocyturia									
presence	55	8	33	13	1	88,9%	71,7%	38,1%	97,1%
absence	336	33	230	72	1	97,1%	76,2%	31,43%	99,6%
Bacteriuria									
presence	29	3	17	9	0	100%	65,45%	25%	100%
absence	362	38	246	76	2	95%	76,4%	33,3%	99,2%