

Detection of Acute Rejection by Proteome Analysis of Urinary Samples in Renal Transplant Recipients

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Diagnosis of acute rejection after renal transplantation is still confined to allograft biopsy upon functional graft impairment. This study evaluates proteomic analysis of urinary samples as a non-invasive method to detect acute rejection in patients.

A novel high throughput method, capillary electrophoresis on-line coupled to mass spectrometry (CE-MS) was used for analysis. Analyses included 29 patients without acute rejection and 19 patients with different grades of subclinical or clinical acute rejection (BANFF Ia to IIb). In addition, 10 patients with urinary tract infection were examined.

Using support vector machines, a specific polypeptide pattern was identified by CE-MS that segregated patients with acute rejection from patients without. This pattern was present in acute tubulointerstitial rejection, but not in two samples with vascular rejection. Among the samples with tubulointerstitial rejection (n=17), one sample could not be classified correctly. With regard to urinary tract infection, several polypeptides were found enabling correct diagnosis in all samples with infection as well as differentiation between infection and rejection. Potentially confounding variables like acute tubular lesions, tubular atrophy, tubulointerstitial fibrosis, and calcineurin inhibitor toxicity did not affect correct diagnosis. Likewise, degree of proteinuria, hematuria, allograft function, and different immunosuppressive regimens did not interfere.

Blinded analysis of 26 samples (Table 3) with and without rejection showed correct diagnosis by CE-MS in 14 out of 17 cases for patients without rejection. Six out of nine samples from patients with acute renal rejection were classified correctly as acute rejection. One sample is misclassified. Further two samples are classified as non-acute rejection and the blinded re-evaluation of the corresponding biopsies showed a borderline classification. The proteome pattern in borderline rejection may be different, which would explain why urinary samples in these patients were misclassified.

Detection of acute rejection by CE-MS is a promising non-invasive tool for the post-transplantation surveillance of renal allograft recipients. Further efforts are necessary to establish separate patterns for vascular rejection and to explore whether acute rejection can be diagnosed when urinary tract infection is simultaneously present.

Technology:

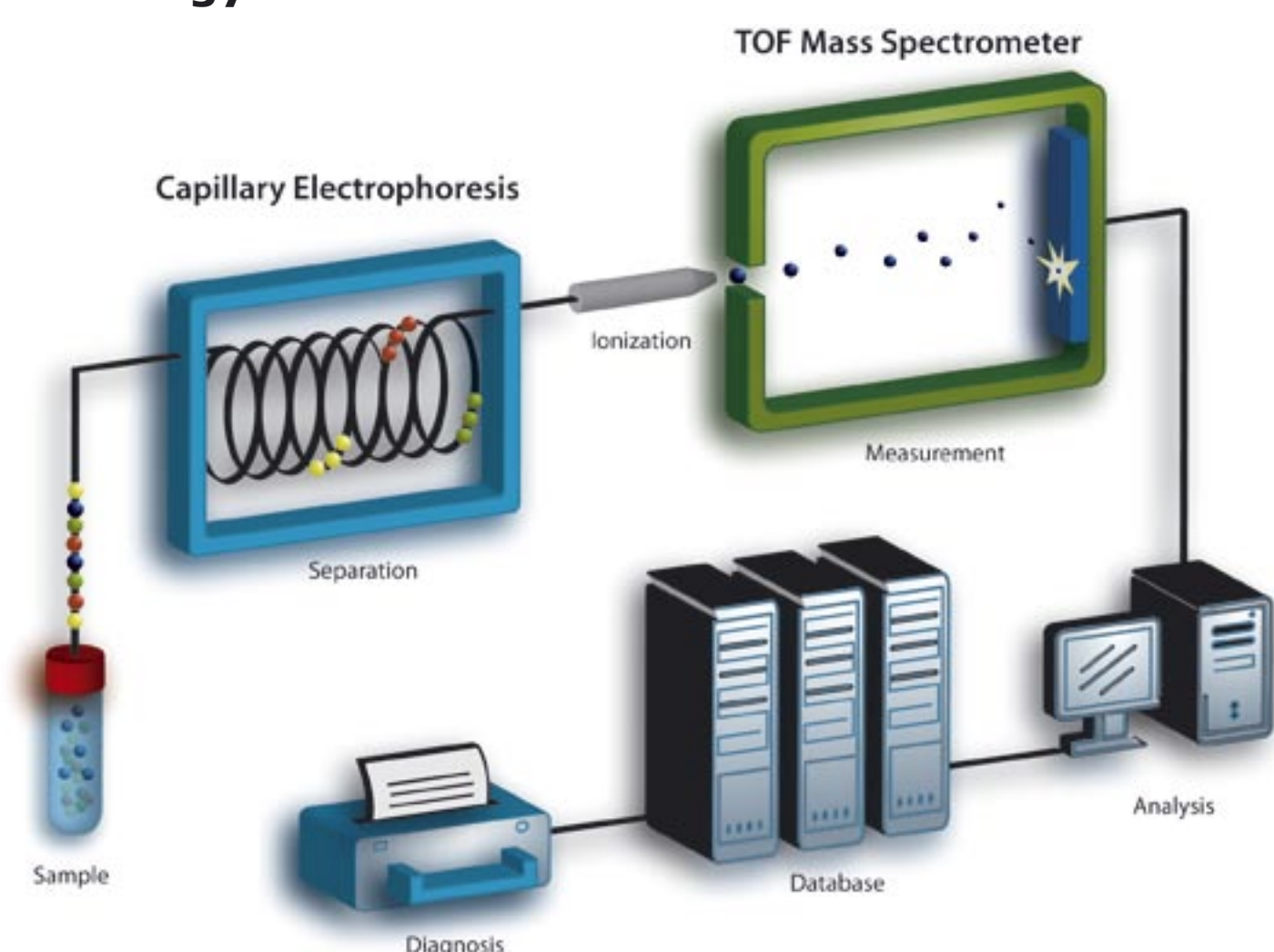


Figure 1. Schematic drawing of the on-line coupling of capillary electrophoresis to the mass spectrometer used to separate and identify proteins and polypeptides in body fluids by their charge and size. After electrophoretic separation, the polypeptides are ionised by the application of high voltage and analysed in the mass spectrometer (ESI-TOF). The combination of the two instruments initially yields a mass spectrogram of mass per charge plotted against migration time. These data are subsequently electronically deconvoluted and normalized.

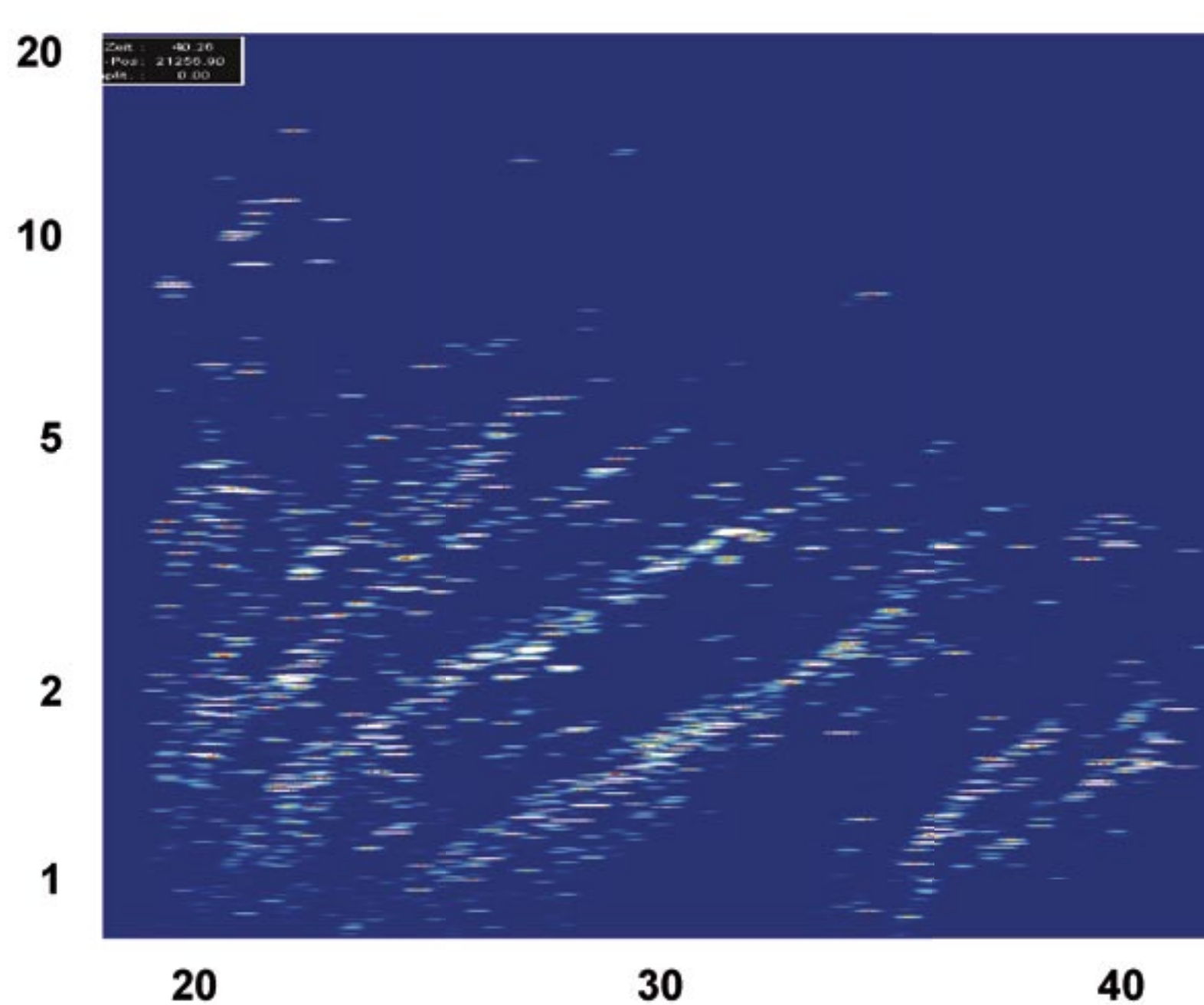


Figure 2: Graphic depiction of CE-MS data. All polypeptides detected in the sample are defined by the two coordinates mass (shown here in kDa on a logarithmic scale) and normalized migration time (in min). Signal intensity, which is color coded, is utilized as measure for the relative abundance of each polypeptide. To establish disease-specific polypeptide patterns and to define biomarkers for disease, individual data from a groups of patients (e.g. rejection) are compiled and compared with others (e.g. control).

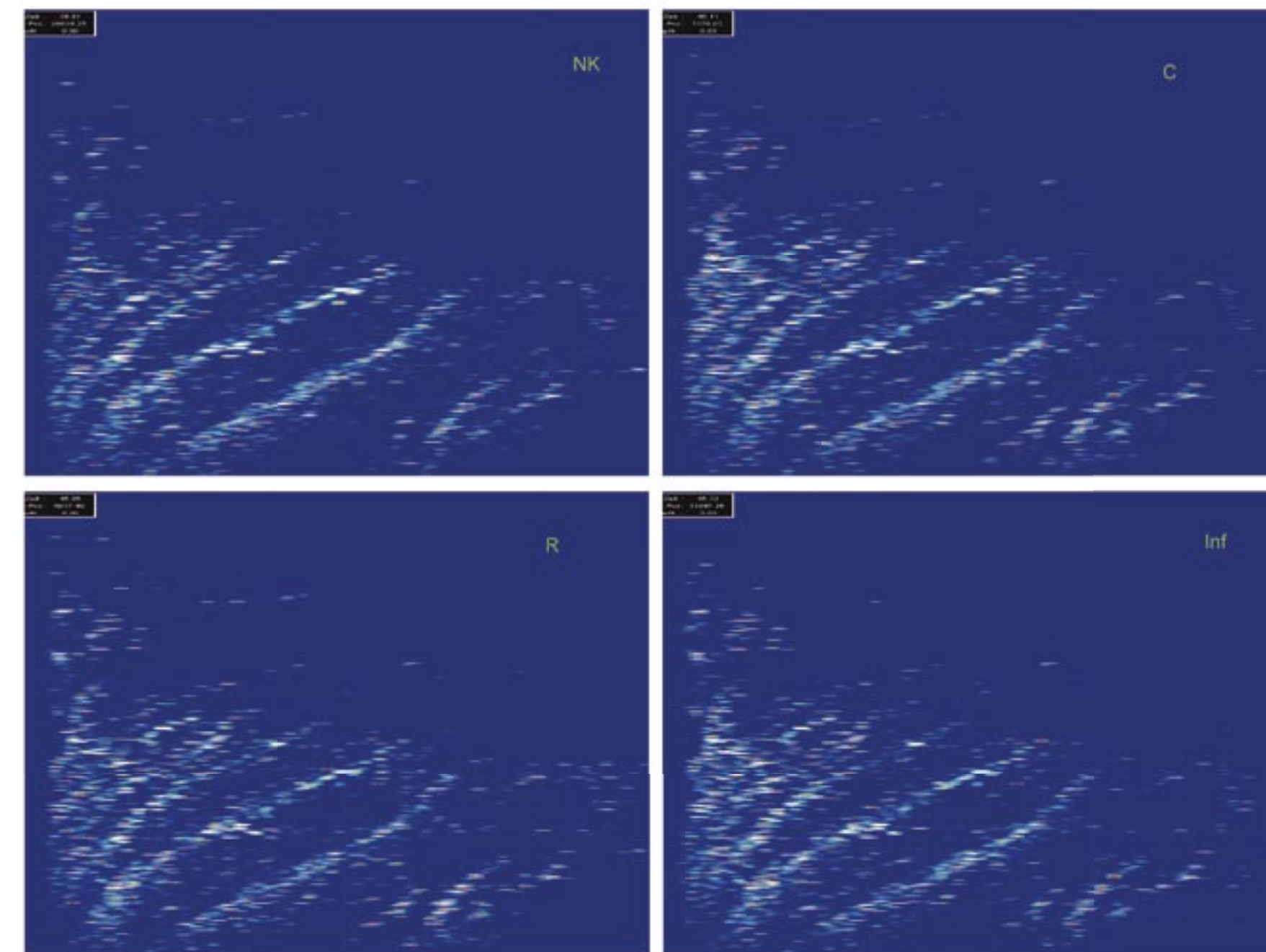


Figure 3: Compiled polypeptide pattern from normal controls (NK), patients with renal transplants without pathological findings (C), patients with rejection (R) or patients with urinary tract infection. While the data appear very similar at first sight, closer examination reveals an array of indicative differences (see below).

Table 1: 62 urinary polypeptides that allow discrimination between renal transplant patients and healthy non-transplanted subjects. Shown are the internal ID tag in the Mosaiques Database, mass, normalized migration time, and frequency of occurrence in transplant patients as well as normal controls.

Pr. ID	Mass	CE-T	NTX	NK	Pr. ID	Mass	CE-T	NTX	NK
6362	1059.7	32.6	0.72	0	71931	4336.8	26.3	0.94	0.47
5954	1075.5	32.9	0.72	0	72916	4449	20	0.78	0.09
6368	1158.7	33.4	0.72	0	73079	4465.2	20	0.88	0.02
8823	1174.7	33.7	0.72	0.01	73307	4491.7	26.2	0.97	0.47
12121	1281.7	32.5	0.72	0	73621	4520	19.9	0.75	0.05
12572	1297.7	32.8	0.72	0	73772	4550.8	23.2	0.81	0.05
14244	1356.9	34.6	0.75	0	73904	4567	23.3	0.91	0.04
15132	1338.9	34.8	0.76	0.01	74101	4591.8	23.3	0.91	0.01
23930	1716.3	20.6	0.88	0.85	75459	4771.1	20.2	0.88	0.64
26851	1829	21.2	0.97	0.67	76149	4861.2	20.5	0.75	0.01
46378	2629.6	19.8	0.88	0.07	76172	4864.7	23.8	0.88	0.18
46948	2658.3	19.3	0.94	0.58	76646	4934.1	22.9	0.88	0.07
48925	2743.1	19.7	0.89	0.11	76816	4960.3	20.6	0.88	0.47
49161	2752.5	19.9	0.81	0.4	77003	4991.2	30.4	0.78	0.02
52406	2907.3	35.8	0.38	0.76	77351	5059	24.4	0.88	0.15
53009	2938.9	33.8	0.13	0.87	77470	5090.3	20	0.88	0.18
62348	3473.9	19.2	0.72	0.04	77795	5172.1	24.9	0.91	0.04
64395	3630.5	21.8	0.91	0.65	78150	5276.4	20	0.91	0.2
65881	3769.9	19.2	0.84	0.14	78211	5292.4	20	0.94	0.13
66718	3832.7	21.6	0.88	0.29	78496	5394.4	20	0.84	0.14
67523	3906.6	24.1	0.91	0.21	78906	5508.4	25.2	0.81	0.05
68440	3996.8	21	0.91	0.62	79170	5627.4	20.3	0.72	0.02
68811	4032.4	22.3	0.75	0.05	79196	5638.5	20	0.75	0.02
68891	4041.1	20.5	0.75	0.29	79809	5910.4	20.6	0.75	0
68379	4082.8	21	0.81	0.01	80446	6236.9	21.1	0.94	0.68
69521	4088.8	21.1	0.88	0	81883	7511.2	28.3	0.75	0.04
69654	4114.9	21.1	0.78	0.02	82967	8559.2	19.4	0.91	0.24
69930	4139.9	20	0.84	0.07	84197	10199.3	21.1	0.94	0.69
71494	4305.9	25	0.91	0.11	85008	11474.1	21.6	0.81	0.01
71695	4322	25.1	1	0.33	85014	11490.7	21.6	0.78	0.01
71747	4324.8	21.3	0.88	0	86630	15816.1	19.2	0.84	0.09

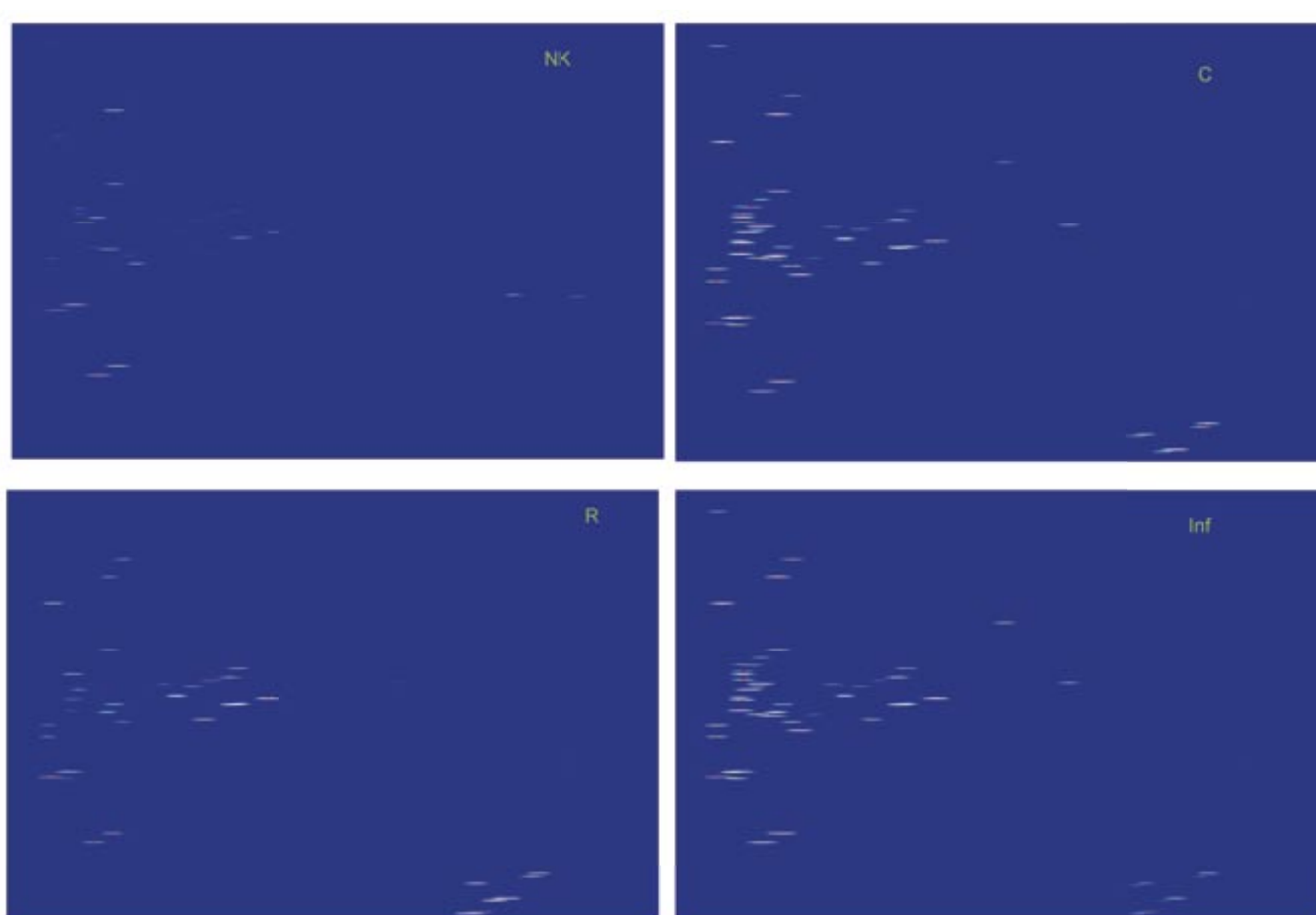


Figure 4: Graphic depiction of the 62 polypeptides that serve as discriminators between renal transplant patients and healthy controls. (see Table 1) within the compiled polypeptide pattern shown in Figure 3.

Table 2 A-C: Classification of patients based on urinary polypeptides. Shown are the internal ID tag in the Mosaiques Database, and frequency of occurrence in the respective patients

protein-ID	frequency by group [%]		protein-ID	frequency by group [%]		protein-ID	frequency [%]	
	infection	control		rejection	Control		rejection	infection
13422	70	8	527	59	12	2024	82	20
14094	70	12	1804	71	28	6455	71	20
2170	80	24	5342	59	16	1586	29	80
4995	60	4	8614	85	24	1972	29	80
14175	60	4	4103	47	8	5873	29	80
14620	60	4	4481	82	80	15005	18	70
6568	90	36	7150	12	52	19658	6	60
14233	60	8	9023	47	88	13666	12	70
			4062	41	84	6947	0	60
			7203	41	84			
			8835	41	84			
			18848	29	72			
			1079	24	68			
			1102	12	56			
			8223	24	68			
			4475	29	84			

2A: Distribution of potential biomarkers discriminating between patients with urinary tract infection and patients without infection and rejection (control) in renal allograft recipients. **2B:** Distribution of potential biomarkers discriminating between patients with acute renal rejection and patients without allograft rejection and patients with urinary tract infection. This patients. Using the combination of peptides also wed correct identification of all patients with acute rejection could be identified correctly. Two of the patient in the control group was misclassified samples belonged to misclassified as having infection. **2C:** Distribution of potential biomarkers discriminating between patients with acute renal rejection and patients without infection and rejection (control) in renal allograft recipients. Using the combination of peptides also wed correct identification of all patients with acute rejection could be identified correctly. Two of the patient in the control group was misclassified samples belonged to misclassified as having infection. and the other one with tubulointerstitial rejection appeared to be quite dilute (<800 polypeptides/sample) which might have led to unreliable classification. None of the control subjects were incorrectly identified as having rejection.

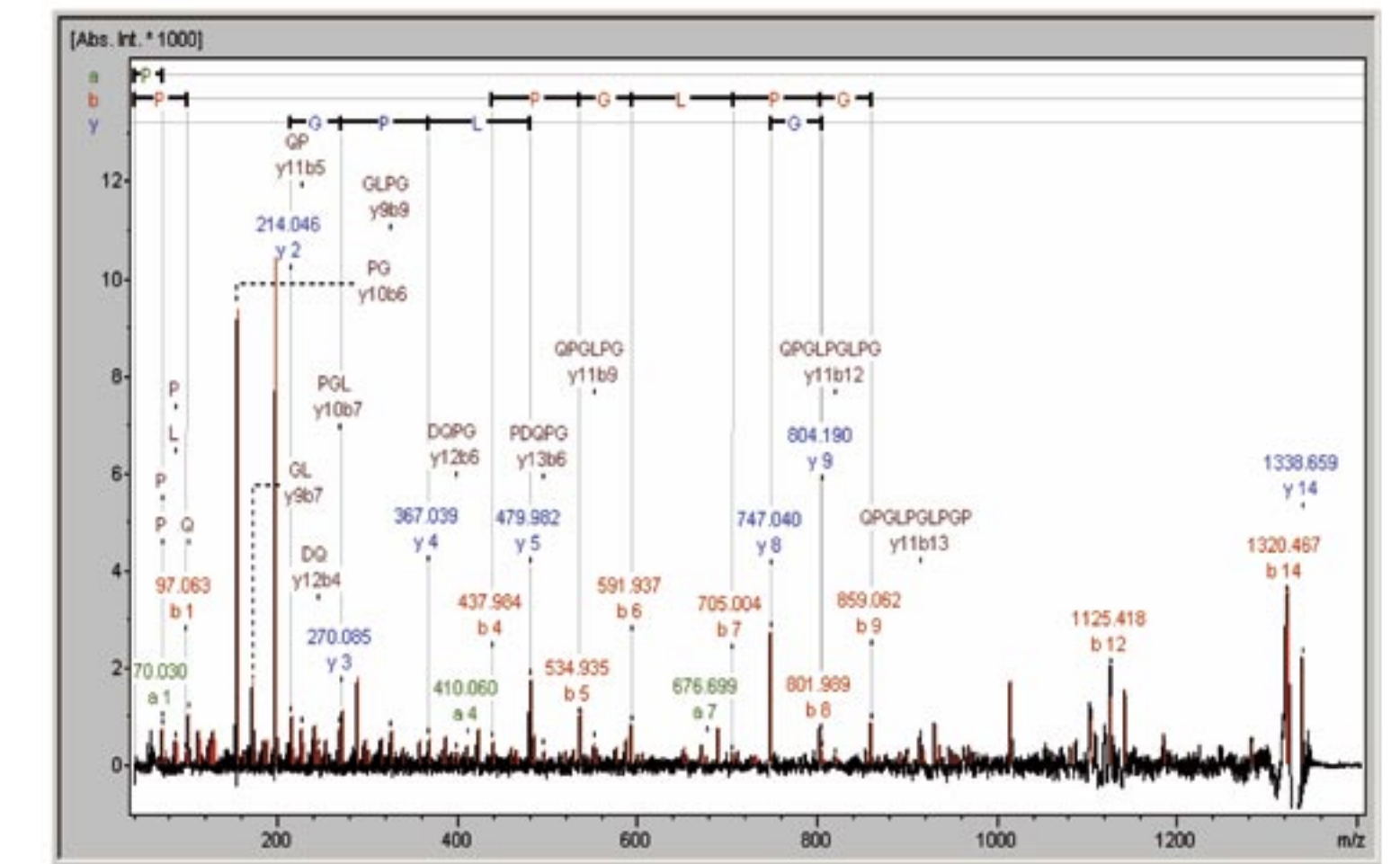


Figure 5: Identification of potential biomarker polypeptides with MS/MS. Fragment of collagen alpha 5(IV), (CA54_human); Mass: 1338.71; Sequence: PPDQPLGLPLGPP

Table 3: Validation experiments with blinded analysis of urine samples from patients with rejection, urinary tract infection, and without these conditions. The corresponding biopsies were re-evaluated blindly by the same pathologist who had initially examined the biopsies. (no AR: no acute rejection)

sample ID	aGrade initial diagnosis	aGrade re-evaluation	classification by proteome analysis
control (n=10)			
C1	no AR	no AR	rejection
C2	no AR	no AR	infection
C3	no AR	borderline rejection	control
C4	no AR	no AR	control
C5	no AR	no AR	control
C6	no AR	no AR	control
C7	no AR	no AR	control
C8	no AR	no AR	control
C9	no AR	no AR	control
C10	no AR	no AR	control
urinary tract infection (n=7)			
U1	no AR	no AR	rejection
U2	no AR	no AR	rejection
U3	no AR	no AR	infection
U4	no AR	no AR	infection
U5	no AR	no AR	control
U6	no AR	no AR	control
U7	no AR	no AR	control
rejection (n=9)			
R1	Ia	Ia	rejection
R2	Ib	Ia	rejection
R3	Ib	Ia	rejection
R4	Ib	Ib	rejection
R5	Ia	Ia	rejection
R6	Ib	Ia	control
R7	Ia	Ia	rejection
R8	Ia	borderline rejection	infection
R9	Ia	borderline rejection	control

Summary and Conclusion:

This study demonstrates that acute tubulointerstitial rejection of renal allografts causes significant changes in the urine proteome. These changes occur even in cases with subclinical and histologically mild rejection. Specific polypeptide patterns were identified for patients with tubulointerstitial rejection, infection, and control transplant recipients without these conditions. In addition, urinary polypeptide patterns of transplanted patients are different from healthy subjects without transplant.

In summary, we have established urinary polypeptide patterns in transplant patients that allow to differentiate between patients with acute tubulointerstitial rejection, urinary tract infection, and control patients without rejection or infection. This method seems to provide an interesting non-invasive tool to monitor patients after renal transplantation and may aid in identifying patients with possible rejection who need further diagnostic workup with allograft biopsy. Additionally, this method may help to identify novel mediators of rejection and allograft injury.

References:

1. E. M. Weissinger, S. Wittke, T. Kaiser, H. Haller, S. Bartel, R. Krebs, I. Golovko, M. Haubitz, H. Hecker, H. Mischak and D. Fliser. Proteomic patterns established with capillary electrophoresis and mass spectrometry for diagnostic purposes. *Kidney Int.*, 65:2426-34 (2004).
2. T. Kaiser, S. Wittke, I. Just, R. Krebs, S. Bartel, D. Fliser, H. Mischak and E. M. Weissinger. Capillary Electrophoresis coupled to mass spectrometer for automated and robust polypeptide determination in body fluids for clinical use. *Electrophoresis*, 25:2044-55 (2004).
3. H. Mischak, T. Kaiser, M. Walden, M. Hillmann, S. Wittke, A. Herrmann, S. Knueppel, H. Haller and D. Fliser. Proteomic analysis for assessment of diabetic renal damage in humans. *Clinical Science*, 107:485-95 (2004).
4. E. M. Weissinger, T. Kaiser, N. Meert, R. De Smet, H. Mischak and R. C. Vanholder for the European Uremic Toxin Work Group. Proteomics: a novel tool to unravel the patho-physiology of uraemia. *Nephrology, Dialysis and Transplantation*, 19:3068-77 (2004).
5. W. Kolch, H. Mischak, M. J. Chalmers, A. Pitt, and A. G. Marshall. *Clinical Proteomics: a question of technology*. *Rapid Commun Mass Spectrom.*, 18:2635-2636 (2004).
6. M. Haubitz, S. Wittke, E. M. Weissinger, M. Walden, H. D. Rupprecht, J. Floege, H. Haller, H. Mischak. Urine Protein Patterns can serve as Diagnostic Tools in Patients with IgA Nephropathy. *Kidney Int.*, 67:2313-20 (2005).
7. S. Wittke, H. Mischak, W. Kolch, T. J. Ruedler, K. Wiedemann. Discovery of biomarkers in human urine and cerebrospinal fluid by capillary electrophoresis coupled mass spectrometry: towards new diagnostic and therapeutic approaches. *Electrophoresis*, 26(7-8):1476-1487 (2005).
8. W. Kolch, C. Neusüß, M. Pelzing and H. Mischak. Capillary electrophoresis - mass spectrometry as a powerful tool in clinical diagnosis and biomarker discovery. *Mass Spectrometry Reviews*, in press.
9. K. Rossing, H. Mischak, H.-H. Parving, P. K. Christensen, M. Hillmann, T. Kaiser. The impact of diabetic nephropathy and angiotensin II receptor blocker treatment on urinary polypeptide patterns in type 2 diabetic patients. *Kidney Int.*, in press.
10. D. Fliser, S. Wittke, H. Mischak. Capillary electrophoresis coupled to mass spectrometry (CE/MS) for clinical diagnostic purposes. *Electrophoresis*, in press.