

Received: 2015.09.02
Accepted: 2015.11.04
Published: 2016.06.11

Effect of Immunosuppressive Therapy on Proteinogram in Rats

Authors' Contribution:

Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEF 1 **Karolina Kędzierska**
ABCDEF 2 **Krzysztof Sindrewicz**
ABCDEF 2 **Katarzyna Sporniak-Tutak**
BCDE 3 **Joanna Bober**
BCDF 3 **Małgorzata Stańczyk-Dunaj**
BCDEF 4 **Barbara Dołęgowska**
CDF 5 **Robert Kaliszczak**
CDF 6 **Jerzy Sieńko**
BCD 1 **Joanna Kabat-Koperska**
BCD 1 **Edyta Gołembiewska**
ABCDEF 1 **Kazimierz Ciechanowski**

1 Department of Nephrology, Transplantology and Internal Medicine, Pomeranian Medical University, Szczecin, Poland
2 Department of Maxillofacial Surgery, Pomeranian Medical University, Szczecin, Poland
3 Department of Medical Chemistry, Pomeranian Medical University, Szczecin, Poland
4 Department of Laboratory Diagnostics, Pomeranian Medical University, Szczecin, Poland
5 Department of Cardiology, Pomeranian Medical University, Szczecin, Poland
6 Department of General Surgery and Transplantology, Pomeranian Medical University, Szczecin, Poland

Corresponding Author: Karolina Kędzierska, e-mail: karolina.kedzierska@gmail.com
Source of support: Departmental sources

Background: It has been observed that the use of immunosuppressive drugs in patients after transplantation of vascularized organs may be associated with changes in the concentration of certain fractions of plasma proteins. The concentration of these proteins was correlated with an increased risk of occurrence of stage 3 chronic kidney disease (CKD). This article examines the effect of the most commonly used immunosuppressive drugs on the concentration of plasma proteins in Wistar rats.





Material/methods: The study involved 36 rats grouped according to the immunosuppressive regimen used (tacrolimus, mycophenolate mofetil, cyclosporine A, rapamycin, and prednisone). The rats in all study groups were treated with a 3-drug protocol for 6 months. The treatment dose was adjusted based on available data in the literature. No drugs were administered to the control group. The rats were sacrificed and blood samples collected to determine the concentration of plasma proteins using electrophoresis technique.

Results: Statistically significant differences were observed between protein concentrations within the studied groups. The differences related to the proteins with masses of 195 kDa, 170 kDa, 103 kDa, and 58 kDa.

Conclusions: (1) Immunosuppressive drugs caused changes in the proteinogram of plasma proteins. (2) The strongest effect on rat plasma proteins was exerted by a regimen based on rapamycin. Intermediate, weak, and weakest effects were observed in regimens based on cyclosporine A, tacrolimus, and mycophenolate mofetil, respectively.

MeSH Keywords: **Immunosuppression • Kidney • Kidney Transplantation**

Full-text PDF: <http://www.medscimonit.com/abstract/index/idArt/895856>

 3685  7  4  27



Background

Use of immunosuppressive drugs in patients after transplantation of vascularized organs may be associated with changes in the concentration of certain fractions of plasma proteins [1–3], although data in the literature are scarce.

In patients receiving immunosuppressive drugs (tacrolimus or cyclosporine A in monotherapy) following liver transplantation, elevated concentrations of specific serum proteins (e.g., apolipoprotein H, α_1 microglobulin, β_2 microglobulin of factor VII, and chromogranin A) can be observed [1]. The concentration of these proteins was correlated with an increased risk of developing stage 3 CKD [1]. In plasma of patients at risk of developing diabetic nephropathy, an elevated concentration of plasma kininogen and its fragments was reported [2]. In patients with lipid disorders, who were treated with steroids after renal transplantation – cyclosporine A (CyA) or tacrolimus, and mycophenolate mofetil (MMF) or azathioprine – higher concentrations of proteins such as high-molecular fragments of kininogen (bradykinin) and C4 complement were found [3].

Most studies of protein markers of acute kidney injury, chronic kidney disease, nephrotoxicity of immunosuppressive drugs, and renal graft rejection were carried out using mass spectrometry technique involving renal tissue and urine, [4,5] but not plasma.

The present study examined the long-term (6 months) effect of the most commonly used immunosuppressive drugs in typical combinations used in patients after organ transplantation on the concentration of plasma proteins of Wistar rats via electrophoresis technique.

Material and Methods

Study design

We used 36 male Wistar rats obtained from a licensed breeder (the Institute of Occupational Medicine in Lodz, Poland). At the beginning of the study, the rats were 14 weeks old. The animals had genetic and health certificates issued by a veterinarian. The study was approved by the local Ethics Committee for Experiments on Animals in Szczecin (No. 06/08, dated 04 Feb. 2008, and No. 24/08, dated 24 Nov. 2008).

The animals were housed in cages, with 6 rats per cage, in the Animal Facility of Pomeranian Medical University. The room humidity was approximately 55%, and the air temperature was $22\pm 2^\circ\text{C}$. The lighting, on a 12/12-h cycle, was controlled by automatic timers. Before the study, all animals were weighed, and their mean weight was 305 ± 9 g. During 2 weeks of adaptation, the animals were fed with the specialized LSM diet (1474 kJ/100 g, 17.6% “Agropol” Motycz, Lublin, Poland) and ad libitum drinking water. All animals survived the adaptation period.

The study was performed using the pharmaceutical form of each drug. The animals received drugs orally in a ball of bread. The drug doses were based on data found in the literature [6–10]. The doses used in the study were: tacrolimus (Prograf, Astellas Pharma, Warsaw, Poland): 4 mg/kg/day; MMF (CellCept, Roche Registration Limited, Welwyn Garden City, Great Britain): 20 mg/kg/day; CyA (Sandimmune Neoral, Novartis Pharma GmbH, Nürnberg, Germany): 5 mg/kg/day; rapamycin (Rapamune, Wyeth, New Lane Havant Hants, Great Britain): 0.5 mg/kg/day; and prednisone (Encorton, Polfa, Pabianice, Poland): 4 mg/kg/day. The animals received medication every 24 h for 6 months. After 3 months the animals were weighed again, and each medication dose was adjusted based on body weight. The 6 rats in the control group did not receive treatment. A diagram of the study design is presented in Table 1.

Table 1. Description of the study design. The abbreviations of drugs used for giving groups in brackets.

Group	Glucocortico-steroids (G)	Tacrolimus (T)	Cyclosporine A (C)	Rapamycin (R)	Mycophenolate mofetil (M)
Control (n=6)	–	–	–	–	–
TRG (n=6)	+	+	–	+	–
CRG (n=6/4)	+	–	+	+	–
MRG (n=6)	+	–	–	+	+
CMG (n=6)	+	–	+	–	+
TMG (n=6)	+	+	–	–	+

Collection of material for the study

Thirty-four rats completed the study and 2 rats from the CRG (cyclosporine A, rapamycin, glucocorticosteroids) group died before the end of the study. After 6 months, the animals were anesthetized by intraperitoneal ketamine hydrochloride injection (50 mg/kg) and blood samples were collected. At the same time, tissue samples (kidneys) of rats were taken for analysis in a separate study.

Determination of drug concentrations in whole blood of rats

The concentration of drug was determined after 4 h of enteral drug administration in accordance with the literature data [10,11]. Rapamycin concentration was determined in the whole blood of rats collected into EDTA tubes by HPLC-UV method with extraction of 1-chlorobutane and 32-desmethoxyrapamycin as an internal standard. A Hewlett-Packard 1050 instrument equipped with Alltech Alltima C18 column 150×2.1 mm, 5 µm, maintained at 50°C, was used. The flow rate of the mobile phase containing 60% acetonitrile and 40% water was 0.5 mL/min, and the UV detector wavelength was established at 278 nm. The limit of quantitation was 1 ng/mL.

The concentration of tacrolimus and CyA was determined in the whole blood of rats using IMx assay based on microparticle enzyme immunoassay (MEIA). The assay was performed using Abbott equipment (Abbott Laboratories, Park, USA). The concentration of CyA was determined with the use of Abbott AxSYM assay, based on a fluorescence method (FPIA, fluorescence polarization immunoassay). The concentrations of the drugs were: cyclosporin A (ng/ml) 785.2±83.3 (ng/ml) in the CMG group, tacrolimus 14.1±13.1 (ng/ml) in the TMG group, tacrolimus 15.3±9.2 (ng/ml) and rapamycin 6.5±2.4 (ng/ml) in the TRG group, cyclosporin A 1272±556.7 (ng/ml) and rapamycin 6.3±4.4 (ng/ml) in the CRG group, and rapamycin 2.3±2.1 (ng/ml) in the MRG group.

Polyacrylamide gel electrophoresis of rat plasma proteins

Separation of proteins was carried out using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions, according to the procedure described by Laemmli [12]. Plasma was suspended in a solution containing 0.25 M TRIS-HCl buffer, pH 6.8, 10% SDS, and 0.5 M DTT, incubated at 94°C for 5 min. The acrylamide concentration of “stacking” and “resolving” gels were 4% and 10%, respectively. The separation was carried out at ambient temperature and at a voltage of 60 V for the “stacking” gel and 120 V for the “resolving” gel. Subsequently, the gels were stained with 0.1% Coomassie Brilliant Blue suspended in 10% acetic acid and 50% methanol. The separation of proteins was conducted

using the Mini-PROTEAN® (Bio-Rad) system, and the analyses of gels were performed using KTE Gelscan (Kucharczyk TE) program. The results are shown as proportions.

Determination of proteins with the use of ELISA

The following ready-to-use kits were used for determination:

1. TIM-1/KIM-1/HAVCR Immunoassay Rat (catalogue number RKM100, R&D Systems, Minneapolis, USA).
2. Rat MCP-1 Instant ELISA (catalogue number BMS631INST, eBioscience, Vienna Austria).

Statistical analysis

The values of continuous variables were compared between groups using nonparametric tests (U-Mann-Whitney test) because most of the variables were non-normally distributed (as evidenced by Shapiro-Wilk test). The mean, standard deviation, median, minimum, and maximum values were calculated for each group.

To evaluate the correlation between continuous variables, nonparametric Spearman's rank correlation coefficient (RS) was used.

The cut-off level of statistical significance was set at $p < 0.05$ for bilateral statistical hypothesis tests. Calculations were performed using Statistica 10 software.

Results

As shown in our previous studies (Kedzierska et al.) the immunosuppressive drugs used in popular regimens induce a series of changes in protein expression in target organs (e.g., kidneys) [13]. Our team (Kedzierska et al.) found that the apoptosis in nephron tubules caused by immunosuppressive therapy in the same study group is not accompanied by any histopathological changes (e.g., fibrosis, inflammation, tubular atrophy, and vacuolation of the tubular cells) seen in light microscopy [14].

In a recent study, plasma proteins were subjected to 1-dimensional electrophoretic separation under denaturing conditions. The 22 plasma proteins occurring in the highest quantities were divided into 7 fractions (Figures 1 and 2; Tables 2, 3A, 3B), with molecular mass given in kDa as the division criterion. Statistically significant differences were observed between protein concentrations within the studied groups. The differences involved the proteins with masses of 195 kDa, 170 kDa, 103 kDa, and 58 kDa (ANOVA $p = 0.05$, $p = 0.01$, $p = 0.02$, and $p = 0.04$, respectively).

The 48-kDa protein was present in higher concentrations in the control group compared to the MRG (mycophenolate mofetil, rapamycin, glucocorticosteroids) and CMG (cyclosporine A, mycophenolate mofetil, glucocorticosteroids) groups ($p < 0.05$ and $p < 0.05$). In the control group, concentration of 58-kDa protein was significantly lower compared to TRG (tacrolimus, rapamycin, glucocorticosteroids) ($p < 0.05$), CMG ($p < 0.01$), and TMG (tacrolimus, mycophenolate mofetil, glucocorticosteroids) ($p < 0.05$) groups. The 88-kDa protein was found in lower concentrations in the TMG group as compared to the control group ($p < 0.05$), as was 66-kDa protein ($p < 0.05$ TMG vs. control). Presence of 103-kDa protein was found in higher concentrations in the control group as compared to the MRG group ($p < 0.05$). Presence of 108-kDa protein was found in higher concentrations in the CRG group compared to the control group ($p < 0.05$) (Table 3A).

The 146-kDa protein was found in lower concentrations in the control group as compared to the CRG study group ($p < 0.05$). Presence of 154-kDa protein was found in higher concentrations in the control group as compared to the TRG, MRG, CMG,

and TMG groups ($p < 0.05$). The 170-kDa protein was found in lower concentrations in the control group compared to the CRG ($p < 0.05$), CMG, and TMG (both $p < 0.01$) groups. The 195-kDa protein was present in higher concentrations in the control group compared to the CMG group ($p < 0.05$) (Table 3B).

In the next stage of the analysis, a comparison was made of the immunosuppressive regimens applied and their effect on the concentration of plasma protein fractions. The comparison excluded the control group, which did not receive any drugs.

The use of regimens based on CyA (CRG and CMG) was associated with the occurrence of a higher concentrations of 203-, 170-, and 108-kDa plasma proteins ($p < 0.04$, < 0.01 and < 0.05 , respectively), whereas the amount of 54-kDa protein was significantly reduced compared to the groups treated with regimens devoid of CyA (TRG, TMG, and MRG) ($p < 0.05$) (Table 4).

In the plasma of rats collected from the groups treated with tacrolimus (the TRG and TMG groups), we found decreased levels of the 108-kDa protein and increased levels of the 103-kDa protein ($p < 0.02$ and 0.03 , respectively) (Table 5) compared to the groups without drug treatment (CRG, CMG, and MRG).

Compared to other regimens, treatment with a set of drugs including rapamycin (CRG, MRG, and TRG) had the greatest effect on the change of the parameters studied. The content of plasma proteins in the groups receiving rapamycin was higher compared to the groups without rapamycin (CMG and TMG) in terms of proteins size 195 kDa, 88 kDa, 66 kDa, and 37 kDa ($p < 0.04$, $p < 0.03$, $p < 0.03$ and $p < 0.04$, respectively), and was lower in terms of proteins size 170 kDa, 103 kDa, 79 kDa, and 58 kDa ($p < 0.001$, $p < 0.01$, $p < 0.05$, and $p < 0.001$, respectively) (Table 6).

Treatment based on the administration of MMF (TMG, CMG, and MRG) along with other regimens had the lowest effect on the change of the parameters studied.

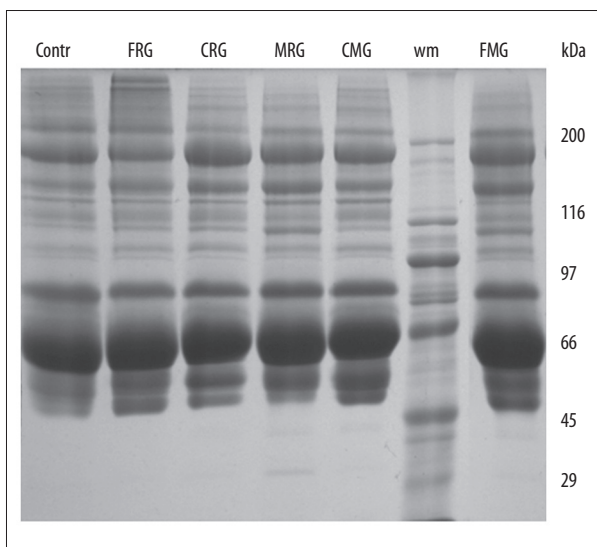


Figure 1. Electrophoretic separation of plasma proteins in rats.

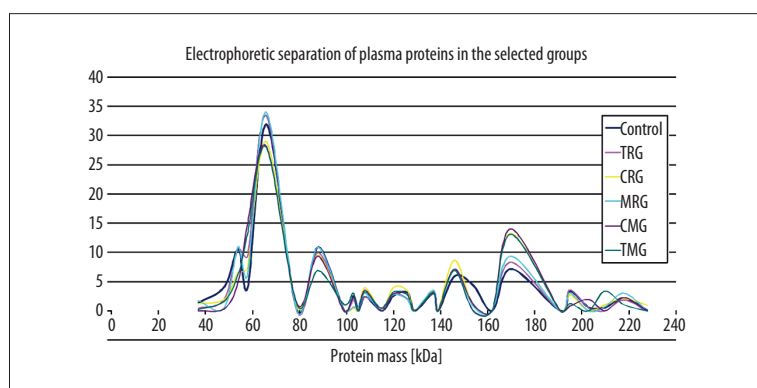


Figure 2. Electrophoretic separation of plasma proteins in the selected groups.

Table 2. Electrophoretic fractions of plasma proteins in the studied groups.

Molecular mass of electrophoretic fraction [kDa]	Examples of proteins included in fractions	Theoretical molecular mass [kDa]	Actual molecular mass [kDa]	Anova		
190–270	Fibronectin (monomer)	270	218	NS		
	Factor VIII	240				
	Apolipoprotein B48	218				
	Unidentified plasma protein	?				
	Unidentified plasma protein	?				
	C4 complement (whole molecule)	194			195	0.05
160–189	C3 complement (whole molecule)	186	170	0.01		
	α_1 -makroglobulin – heavy chain	165				
	α_2 -makroglobulin (monomer)	163				
	Unidentified plasma protein	?			154	NS
130–159	Unidentified plasma protein	?	146	NS		
	Unidentified plasma protein	?	137	NS		
	CRP (entametr)	125	126	NS		
100–129	Ceruloplasmin	120	120	NS		
	Bone morphogenetic protein 1 (BMP-1)	111	108	NS		
	Endothelial lipase (homodimer)	110				
	Inter a-H4P inhibitor (heavy chain)	103			103	0.02
	MMP-9	92			99	NS
	Fibrinogen alpha chain (monomer)	86			88	NS
KIM-1	85					
HSP 90 (homodimer)	84					
Factor XIII	82					
Haptoglobin (tetramer)	82					
HGF	82					
60–79	MMP-2	72	79	NS		
	Precallicrein	71	66	NS		
	Prothrombin	70				
	Kininogen (high-molecular-weight isoform)	70				
	Cholinesterase	68				
	Albumin	65				

Table 2. Electrophoretic fractions of plasma proteins in the studied groups.

Molecular mass of electrophoretic fraction [kDa]	Examples of proteins included in fractions	Theoretical molecular mass [kDa]	Actual molecular mass [kDa]	Anova		
50–59	TGF beta 2	55	58	0.04		
	C1 protease inhibitor	55				
	Coagulation factor X	54				
	Fibrinogen beta chain (monomer)	54				
	Chromogranin A	52				
	Haemopexin	51				
	Coagulation factor IX	51			54	NS
	Fibrinogen gamma chain	50				
	Factor VII	50				
	KIM-1	50				
30–49	α_1 -proteinase inhibitor (serpine1)	46	48	NS		
	Kininogen (low-molecular-weight isoform)	46				
	α_1 -makroglobulin light chain	45				
	AMBIP protein (lipocain)	38			37	NS
	Fetuin-A	37				
	Apolipoprotein E	35				

There was no correlation between morphology, creatinine, e-GFR, or histology of rat kidneys and plasma protein, and plasma protein was not correlated with serum calcium or urine test results.

In the next stage of the study we analyzed the concentration of KIM and MCP-1 in rat plasma as well as the correlation between rat plasma proteins and markers of kidney damage.

KIM-1 (*kidney injury molecule-1*) concentration was determined in the plasma of rats, and significant differences between the groups ($p=0.05$) were found (Table 7). The highest value of KIM-1 was found in the CMG group and it was significantly higher than in the control group (Mann-Whitney test, $p<0.05$). The concentration of MCP-1 (*Monocyte Chemoattractant Protein-1*) in the plasma of rats was also significantly different between the groups ($p<0.001$). In the MRG group, it was significantly lower compared to the control group ($p<0.05$), whereas in the TMG group, the concentration was significantly higher in comparison to the control group ($p<0.01$). The concentration of KIM-1 was inversely correlated with 66 kDa and 137 kDa proteins and positively correlated with 170 kDa protein (Figure 3). Numerous correlations were observed between plasma levels of MCP-1 and plasma proteins. MCP-1 was inversely correlated with 37

kDa, 120 kDa, and 146 kDa proteins ($p<0.001$). MCP-1 was positively correlated only with the 210 kDa protein (Figure 4).

Discussion

In a separate study by our team (Kędzierska et al.) [13], we analyzed the impact of the most commonly used immunosuppressive drugs on protein expression in the native kidneys of Wistar rats. We found that the immunosuppressive drugs used in popular regimens induce a series of changes in protein expression in target organs. The expression of proteins involved in drug, glucose, amino acid, and lipid metabolism was pronounced. To a lesser extent, we also observed changes in nuclear, structural, and transport protein synthesis. Very slight differences were observed between the group receiving cyclosporine, mycophenolate mofetil, and glucocorticoids (CMG) and the control group. In contrast, compared to the control group, animals receiving tacrolimus, mycophenolate mofetil, and glucocorticoids (TMG) exhibited higher expression of proteins responsible for renal drug metabolism and lower expression levels of cytoplasmic actin and the major urinary protein. In the TMG group, we observed higher expression of proteins responsible for drug metabolism and a decrease in the expression

Table 3A. Electrophoretic fractions of plasma proteins in the studied groups.

Protein [MW]/group	Control	TRG	CRG	MRG	CMG	TMG	p (Anova)	
37 kDa	Mean ±SD	1.09±1.71	0.44±1.07	1.19±1.38	1.68±1.38	0±0.00	0.21±0.51	NS
	Median	0	0	1.08	2.13	0	0	
	Min-max	0-3.75	0-2.63	0-2.57	0-3.34	0-0	0-1.24	
48 kDa	Mean ±SD	3.38±2.21	1.98±3.70	1.87±2.61	0.45±1.10	0.29±0.65	1.65±3.15	NS
	Median	3.71	0	0.98	0.0**	0.0**	0	
	Min-max	0-6.03	0-9.23	0-5.53	0-2.7	0-1.45	0-7.87	
54 kDa	Mean ±SD	11.5±4.84	10.8±6.73	6.45±3.68	10.9±4.19	4.9±0.72	6.22±1.3	NS
	Median	12.28	9.41	6.05	11.24	4.94	6.15	
	Min-max	3.49-16.1	4.04-20.4	2.64-11.1	4.14-15.8	3.99-5.73	4.43-7.87	
58 kDa	Mean ±SD	4.4±2.26	9.36±5.98	7.4±5.99	6.06±5.60	14.9±1.74	13.2±6.56	0.04
	Median	4.98	9.95**	8.5	4.27	14.7*	15.8**	
	Min-max	0-6.06	0-16.44	0-12.59	0-15.02	12.61-17.46	0-16.86	
66 kDa	Mean ±SD	31.5±4.04	33.4±5.87	28.9±3.08	33.9±6.89	28.1±1.94	27.9±3.19	NS
	Median	29.88	34.23	28.53	31.44	27.6	26.6**	
	Min-max	28-38.02	24.4-42.1	26.2-32.6	26.6-44.7	25.9-30.8	26.1-34.3	
79 kDa	Mean ±SD	0±0.00	0±0.00	0.67±1.34	0±0.00	1.2±1.70	1.09±1.70	NS
	Median	0	0	0	0	0	0	
	Min-max	0-0	0-0	0-2.68	0-0	0-3.59	0-3.68	
88 kDa	Mean ±SD	10.7±1.22	9.96±1.37	9.46±0.89	10.8±0.88	9.3±1.40	6.9±3.05	NS
	Median	10.31	9.78	9.65	10.54	9.66	7.2**	
	Min-max	9.73-13	8.32-12.2	8.28-10.3	9.85-12.3	7.48-10.9	3.43-11	
99 kDa	Mean ±SD	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	1.15±2.8	NS
	Median	0	0	0	0	0	0	
	Min-max	0-0	0-0	0-0	0-0	0-0	0-6.88	
103 kDa	Mean ±SD	2.09±1.1	1.85±1.15	0.44±0.88	0.63±1.05	2.22±0.51	3.03±2.06	0.02
	Median	2.52	2.33	0	0.0**	2.51	2.31	
	Min-max	0-3.01	0-2.83	0-1.76	0-2.5	1.5-2.65	1.84-7.18	
105 kDa	Mean ±SD	0±0.00	0±0.00	0±0.00	0.61±1.50	0±0.00	0±0.00	NS
	Median	0	0	0	0	0	0	
	Min-max	0-0	0-0	0-0	0-3.66	0-0	0-0	
108 kDa	Mean ±SD	2.52±1.35	2.41±1.0	3.88±0.62	3±1.5	3.5±0.46	3.27±0.53	NS
	Median	2.72	2.69	3.80**	3.57	3.55	3.19	
	Min-max	0-4.04	0.66-3.57	3.25-4.66	0-4.06	2.77-3.95	2.72-3.92	

*p<0.01 vs. K; **p<0.05 vs. K.

Table 3B. Electrophoretic fractions of plasma proteins in the studied groups.

Protein [MW]/group	Control	TRG	CRG	MRG	CMG	TMG	p (Anova)	
115 kDa	Mean ±SD	0±0.00	0.52±1.28	0±0.00	0±0.00	0±0.00	0.43±1.06	NS
	Median	0	0	0	0	0	0	
	Min-max	0-0	0-3.15	0-0	0-0	0-0	0-2.6	
120 kDa	Mean ±SD	2.54±0.77	2.98±1.86	3.91±1.08	2.75±2.16	2.87±0.39	3.21±0.70	NS
	Median	2.34	2.97	4.06	3.82	2.79	3.17	
	Min-max	1.79-3.99	0-5.17	2.45-5.06	0-4.74	2.36-3.37	2.24-4.22	
126 kDa	Mean ±SD	3.02±1.15	2.01±1.2	3.37±1.57	1.9±1.52	2.73±1.08	2.72±0.25	NS
	Median	2.68	2.7	2.6	2.55	2.81	2.77	
	Min-max	2.3-5.33	0-2.88	2.56-5.73	0-3.59	1.68-4.4	2.29-2.96	
137 kDa	Mean ±SD	3.02±0.46	2.57±1.72	3.31±0.57	3.51±0.73	3.04±0.71	3.24±0.82	NS
	Median	3.01	3.06	3.19	3.66	2.74	3.22	
	Min-max	2.33-3.74	0-4.3	2.77-4.11	2.18-4.29	2.22-3.95	2.3-4.62	
146 kDa	Mean ±SD	5.96±0.89	6.2±2.71	8.59±1.03	6.9±2.01	7.11±0.78	6.93±0.10	NS
	Median	5.77	6.64	8.91**	7.59	7.01	6.95	
	Min-max	5.14-7.45	1.52-9.49	7.11-9.45	4.04-9.16	5.96-7.84	6.77-7.04	
154 kDa	Mean ±SD	5.98±4.75	0.73±1.80	0.83±1.02	0.74±1.16	1.06±0.98	0±0.00	NS
	Median	5.17	0.0**	0.6	0.0**	1.65**	0.0**	
	Min-max	0-11.71	0-4.4	0-2.1	0-2.42	0-1.99	0-0	
170 kDa	Mean ±SD	6.04±4.68	8.34±1.87	13.2±0.97	9.35±3.56	14±1.74	13.1±1.28	0.01
	Median	4.47	7.85	13.02**	10.11	13.9*	12.8*	
	Min-max	0-12.3	6.09-11.53	12.22-14.54	4.8-13.21	11.38-15.95	11.64-14.84	
195 kDa	Mean ±SD	3.15±1.67	3.59±1.46	1.17±1.35	2.59±2.34	0.76±1.70	1.25±2.03	0.05
	Median	2.54	3.71	1.17	3.04	0.0**	0	
	Min-max	1.68-5.53	1.45-5.08	0-2.36	0-4.98	0-3.81	0-4.7	
203kDa	Mean ±SD	0.47±1.16	0.56±1.38	0.52±1.04	0.43±1.05	1.42±0.86	0±0.00	NS
	Median	0	0	0	0	1.75	0	
	Min-max	0-2.84	0-3.39	0-2.09	0-2.56	0-2.22	0-0	
210 kDa	Mean ±SD	0.46±1.12	0±0.00	0.98±1.96	0.67±1.65	0.5±1.12	3.38±3.71	NS
	Median	0	0	0	0	0	3.21	
	Min-max	0-2.74	0-0	0-3.91	0-4.05	0-2.51	0-7.01	
218 kDa	Mean ±SD	2.23±1.18	1.87±1.55	2.01±0.32	3±0.73	2.22±0.46	1.07±1.24	NS
	Median	2.73	2.22	2.07	2.97	2.21	0.87	
	Min-max	0-3.05	0-3.49	1.63-2.28	2.18-3.85	1.71-2.86	0-2.85	

*p<0.01 vs. K; **p<0.05 vs. K.

Table 4. Comparison of the results of analysis of selected plasma proteins in the groups where regimens based on cyclosporin A and without it, were used.

Parameter	Cyclosporin A "yes" – CRG, CMG					Cyclosporin A "no" – TMG, TRG, MRG					p
	Max	Min	Median	±SD	Mean	Max	Min	Median	± SD	Mean	
Protein 203 kDa	2.2	0.0	1.3	1.0	1.0	3.4	0.0	0.0	1.0	0.3	0.04
Protein 170 kDa	16.0	11.4	13.5	1.4	13.6	14.8	4.8	11.3	3.1	10.3	0.01
Protein 108 kDa	4.7	2.8	3.6	0.5	3.7	4.1	0.0	3.1	1.1	2.9	0.05
Protein 54 kDa	11.1	2.6	4.9	2.5	5.6	20.4	4.0	8.4	4.9	9.3	0.05

Table 5. Comparison of the results of analysis of selected plasma proteins in the groups where regimens based on tacrolimus and without it, were used.

Parameter	Tacrolimus "yes" – TRG, TMG					Tacrolimus "no" – CRG, CMG, MRG					p
	Max	Min	Median	±SD	Mean	Max	Min	Median	± SD	Mean	
Protein 108 kDa	3.9	0.7	2.8	0.9	2.8	4.7	0.0	3.6	1.0	3.4	0.02
Protein 103 kDa	7.2	0.0	2.3	1.7	2.4	2.7	0.0	1.3	1.1	1.1	0.03

Table 6. Comparison of the results of analysis of selected plasma proteins in the groups where regimens based on rapamycin and without it, were used.

Parameter	Rapamycin „yes" – CRG, MRG, TRG					Rapamycin „no" – CMG, TMG					p
	Max	Min	Median	±SD	Mean	Max	Min	Median	± SD	Mean	
Protein 195 kDa	5.1	0.0	2.5	2.0	2.6	4.7	0.0	0.0	1.8	1.0	0.04
Protein 170 kDa	14.5	4.8	10.1	3.1	9.9	16.0	11.4	13.5	1.5	13.5	0.002
Protein 103 kDa	2.8	0.0	0.4	1.2	1.0	7.2	1.5	2.5	1.6	2.7	0.01
Protein 88 kDa	12.3	8.3	10.1	1.2	10.2	11.0	3.4	8.6	2.7	8.0	0.03
Protein 79 kDa	2,7	0.0	0.0	0.7	0.2	3.7	0.0	0.0	1.6	1.1	0.05
Protein 66 kDa	44.7	24.4	31.4	5.8	32.5	34.3	25.9	27.1	2.6	28.0	0.03
Protein 58 kDa	16.4	0.0	6.8	5.6	7.6	17.5	0.0	15.2	4.9	14.0	0.002
Protein 37 kDa	3.3	0.0	0.0	1.3	1.1	1.2	0.0	0.0	0.4	0.1	0.04

Table 7. Concentrations of kidney damage markers in rat plasma.

Parameter/group	Control	TRG	CRG	MRG	CMG	TMG	p (Anova)
KIM-1 (pg/mg protein)	Mean ±SD	67.51±25.3	102.7±62.2	94.48±45.9	61.2±29.3	172.5±105.3	71.99±34.15
	Median	60.39	82.24	98.51	49.55	149.4**	70.94
	Min-max	46.1–113.9	48.34–220	34.7–146.2	36.3–110.7	50.4–324.6	34–128.4
MCP-1 (pg/mg protein)	Mean ±SD	197.2±22.9	215.7±51	163±22.85	143.55±6.68	165.9±30.4	330.2±45.6
	Median	200.4	235.9	153.9	143.8**	156.5	311.8*
	Min-max	167–221	157.4–263.5	146.1–189	136.2–150.4	137.7–217.7	284.9–405.3

* p<0.01 vs. K; ** p<0.05 vs. K.

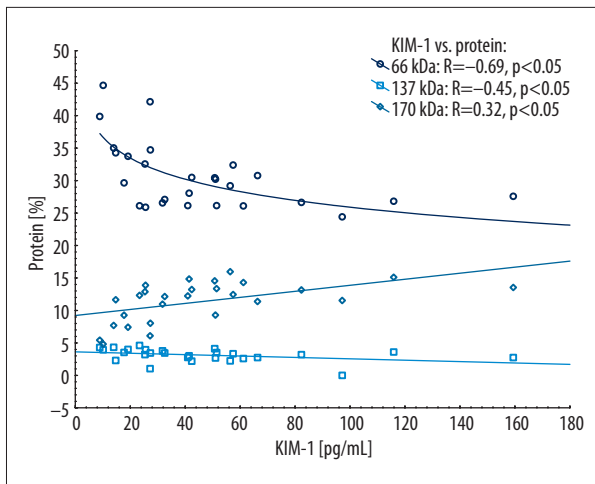


Figure 3. Correlations between plasma levels of KIM-1 and plasma proteins.

of respiratory chain enzymes (thioredoxin-2) and markers of distal renal tubular damage (heart fatty acid-binding protein) compared to expression in the CMG group [13].

In the present study, 22 proteins were isolated from rat plasma from both the study groups and the control group, which were further divided into 7 fractions, depending on the molecular mass [kDa]. Because of the large number of proteins found in the plasma (several thousand), which would impair the readout of hard-to-observe differences between proteins, 2-dimensional (2D) electrophoresis could not be performed.

Statistically significant differences were observed between concentrations of proteins within all the studied groups and the control group. These differences were related to 195 kDa, 170 kDa, 103 kDa, and 58 kDa proteins.

A protein with a mass of 195 kDa may correspond to C4 complement (whole molecule). A 103-kDa protein may correspond to the inter α -H4P inhibitor (heavy chain), the 170-kDa protein is most probably a α_2 -macroglobulin, and the 58-kDa protein may correspond to a β chain of TGF or C1 protease inhibitor.

The concentration of 195-kDa protein (probably a C4 complement) was higher in the control group; however, its concentration in the CMG and TMG study groups was very low.

The 170-kDa protein, which is most likely an α_2 -macroglobulin, which is an acute-phase protein in rats, relative to CRP, was at significantly higher concentration in the rat plasma collected from the study groups compared to the control group. In older rats, the concentrations of α_2 -macroglobulin and other parameters of the acute phase were increased, and the concentration of albumin was decreased [15]. In the study groups, no differences in the concentration of protein corresponding to

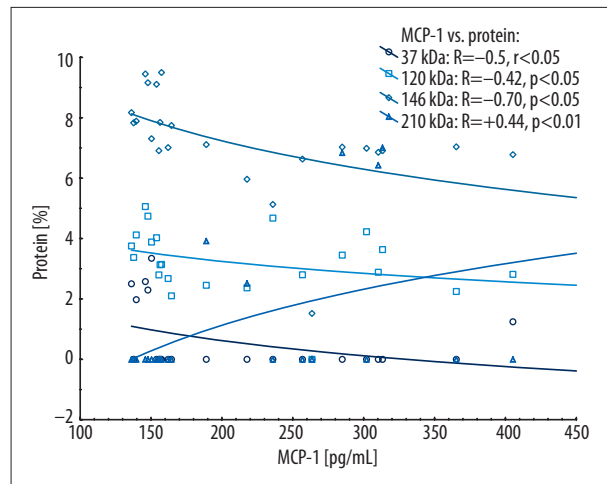


Figure 4. Correlations between plasma levels of MCP-1 and plasma proteins.

albumin (66 kDa) were observed, except for the TMG group, in which the concentration was significantly decreased compared to the control group.

There was a significant increase in the concentration of 58-kDa protein in almost all the studied groups except for the MRG and control groups. The 48-kDa protein was reported in the highest concentration in the control group, while in the remaining groups it was found at a very low level; however, due to the large variation, statistical significance was only observed between the control group and the MRG and CMG groups.

Merchant et al. reported an elevated concentration of plasma kininogen and its fragments in patients with microalbuminuria during the course of type I diabetes [2].

These are very small proteins (~3 kDa), which were detected using mass spectrometry technique; unfortunately, the electrophoresis method, which was used in this study, is not sensitive enough to detect such small proteins. However, proteins with masses of 70 kDa and 46 kDa, which may correspond to low- and high-molecular-weight kininogen in rats, were detected in our study. The concentration of 48-kDa protein (possibly a low-molecular-weight kininogen) was significantly different between the experimental groups and the control group.

Levitzy et al. observed the correlation of apolipoprotein H, α_1 microglobulin, β_2 microglobulin of factor VII, and chromogranin A with increased risk of chronic kidney disease development in the third stadium. Moreover, elevated levels of these proteins were found in patients who received tacrolimus and CyA in monotherapy after liver transplantation [1].

In another study, the effect of atorvastatin on lipid profile and plasma protein composition in patients after kidney

transplantation was observed. It was found that treatment with statins may decrease the concentration of certain serum proteins, and patients receiving steroids, CyA, tacrolimus, MMF, and azathioprine exhibit higher concentrations of high-molecular-weight kininogen fragment (bradykinin) and C4 complement [16].

We also examined the effect of drugs used in immunosuppressive regimens on the concentration of plasma proteins, but we excluded the control group from this part of the study. The available literature lacks any data against which our results can be compared. Most studies on the protein markers of acute kidney injury, CKD, nephrotoxicity of immunosuppressive drugs, and acute rejection of renal transplant were conducted using mass spectrometry technique in renal tissue and urine, but not in plasma.

In the present study, we observed that among all the drugs tested, only MMF used in various regimens usually had no effect on the concentrations of proteins analyzed using SDS-PAGE technique. The remaining immunosuppressive drugs affected the protein concentration to a greater or lesser extent.

In the rat plasma collected from the groups in which tacrolimus was administered, both decreased and increased proportions of 108-kDa and 103-kDa proteins, respectively, were observed. Regimens based on CyA had higher concentrations of plasma proteins with masses of 203 kDa, 170 kDa, and 108 kDa, whereas the concentration of 54-kDa protein was significantly decreased compared to the groups treated with regimens devoid of CyA.

The strongest effect on plasma proteins was in regimens with rapamycin. The concentrations of proteins with masses of 195 kDa, 88 kDa, 66 kDa, and 37 kDa were higher, while the concentrations of proteins with masses of 170 kDa, 103 kDa, 79 kDa, and 58 kDa were lower compared to the groups devoid of rapamycin.

Three proteins, with masses of 170 kDa, 108 kDa, and 103 kDa, were interesting in terms of their mobility. A 170-kDa protein (possibly an α_2 -macroglobulin) was found at significantly higher concentrations in the groups treated with CyA, and the 103-kDa protein (possibly an inter α -H4P inhibitor, heavy chain) had elevated levels in the groups in which tacrolimus was administered. In the groups in which rapamycin was administered, decreased concentrations of 170- and 103-kDa proteins were observed.

We found a positive correlation between KIM and 170-kDa protein, which could correspond to alpha-2 macroglobulin in rats. Alpha-2-macroglobulin is a typical acute-phase protein in rats [17,18]. In our study groups, KIM-1 levels were higher in rats treated with CyA and lower in rats treated with rapamycin.

KIM-1 in plasma is very sensitive and is a specific marker of acute kidney damage in many rat models (e.g., streptozotocin-induced diabetic nephropathy) [19]. KIM-1 is a marker of kidney damage, especially for the proximal tubule, and is useful in the monitoring of acute kidney damage, as well as in renal diseases characterized by interstitial fibrosis and primary and secondary glomerulonephritis [20].

We found that higher release of 170-kDa protein and KIM-1 concentration in rat plasma was associated with cyclosporine A treatment compared to rapamycin. Although histopathology of kidneys did not show significant differences between study groups, we think our finding is interesting. The limitation of this is that alpha-2 macroglobulin synthesis also increases as rats age [21]. Our experiment was terminated when our rats were 9 months old; the average lifetime of a Wistar rat is 24–36 months.

Another interesting finding in our study was the multiple negative correlations between MCP-1 and 37-kDa, 120-kDa, and 146-kDa protein ($p < 0.001$). MCP-1 positively correlated only with the 210-kDa protein ($p < 0.01$). This negative correlation may indicate the strong effect of rapamycin on decreased MCP-1 synthesis and increased production of other proteins in our study groups of rats compared to rats treated with tacrolimus.

The meaning of this finding is unknown because there are no relevant studies for comparison.

MCP-1 influences the activation and migration of monocytes, macrophages, and T lymphocytes into the tubulo-interstitial space of kidneys and plays an important role in renal fibrosis [22]. Wu et al. observed a decrease in the concentration of MCP-1 after treatment with MMF (mycophenolate mofetil) [23]. In our study, in the experimental groups of rats, no effect of MMF on the activity of MCP-1 was observed. The level of MCP-1 in rats treated with regimens based on rapamycin was significantly lower in comparison to the groups without rapamycin. In rats treated with regimens based on tacrolimus, significantly higher MCP-1 was observed in comparison to the rats that did not receive tacrolimus.

As in our study, Lui et al. demonstrated the influence of rapamycin on the inhibition of MCP-1 expression in the kidneys of mice, and Lee et al. experimentally confirmed, on endothelial cells of the proximal tubules, that rapamycin inhibits stress in the endoplasmic reticulum and indirectly reduces the expression of MCP-1 [24,25]. Moreover, Oliveira et al. found that rapamycin inhibits the production of cytokines, including MCP-1, much more strongly in comparison to MMF [26].

A 108-kDa protein (bone morphogenetic protein BMP-1, probably endothelial lipase) was present at both increased and

decreased concentrations in rats treated with regimens based on CyA and tacrolimus, respectively.

Shu et al. conducted an experiment in which CyA at a low (5 mg/kg) or high (100 mg/kg) dose was administered to rats. As compared to the control group, changes were only reported in rats receiving higher dose of CyA. The authors found an increased concentration of 2 plasma proteins: clusterin (51 kDa) and alpha-1 acid glycoprotein (23 kDa). As compared to the control group, a decreased concentration of haptoglobin, a plasma protein with a mass of 38 kDa, was reported [27].

Taking into account all correlations detected in the studied groups of rats, we consider proteins with masses of 48 kDa, 146 kDa, and 170 kDa as the most promising to become novel markers of toxicity in plasma during the course of immunosuppressive therapy in rats.

References:

- Levitsky J, Salomon DR, Abecassis M et al: Clinical and plasma proteomic markers correlating with chronic kidney disease after liver transplantation. *Am J Transplant*, 2011; 11(9): 1972–78
- Merchant ML, Niewczasz MA, Ficociello LH et al: Plasma kininogen and kininogen fragments are biomarkers of progressive renal decline in type 1 diabetes. *Kidney Int*, 2013; 83(6): 1177–84
- Pérez V, Navarro-Muñoz M, Mas S et al: Proteomic approach to the study of statin pleiotropy in kidney transplant patients. *Pharmacology*, 2011; 87(3–4): 161–68
- Serkova NJ, Christians U: Biomarkers for toxicodynamic monitoring of immunosuppressants: NMR-based quantitative metabolomics of the blood. *Ther Drug Monit*, 2005; 27(6): 733–37
- Nishihara K, Masuda S, Shinke H et al: Urinary chemokine (C-C motif) ligand 2 (monocyte chemoattractant protein-1) as a tubular injury marker for early detection of cisplatin-induced nephrotoxicity. *Biochem Pharmacol*, 2013; 85(4): 570–82
- Westrhenen R, Aten J, Hajji N et al: Cyclosporin A induces peritoneal fibrosis and angiogenesis during chronic peritoneal exposure to a glucose-based, lactate-buffered dialysis solution in the rat. *Blood Purif*, 2007; 25(5–6): 466–72
- Joffe I, Katz I, Sehgal S et al: Lack of change of cancellous bone volume with short-term use of the new immunosuppressant rapamycin in rats. *Calcif Tissue Int*, 1993; 53(1): 45–52
- Jolicoeur EM, Qi S, Xu D et al: Combination therapy of mycophenolate mofetil and rapamycin in prevention of chronic renal allograft rejection in the rat. *Transplantation*, 2003; 75(1): 54–59
- Katz LA, Takizawa M, Joffe II et al: Comparison of the effects of FK 506 and Cyclosporine on bone mineral metabolism in the rat. *Transplantation*, 1991; 52(3): 571–74
- Ma Y, Kobayashi T, Kuzuya T et al: Is absorption profile of cyclosporine really important for effective immunosuppression? *Biol Pharm Bull*, 2006; 29(2): 336–42
- Schmitz V, Klawitter J, Bendrick-Pearl J et al: Metabolic profiles in urine reflect nephrotoxicity of sirolimus and cyclosporine following rat kidney transplantation. *Nephron Exp Nephrol*, 2009; 111(4): e80–91
- Laemmli UK: Cleavage of structural proteins during the assembly of the head of acteriophage T4. *Nature*, 1970; 227(5259): 680–85
- Kędzierska K, Sporniak-Tutak K, Sindrewicz K et al: Effects of immunosuppressive treatment on protein expression in rat kidney. *Drug Des Devel Ther*, 2014; 8: 1695–708
- Kędzierska K, Sporniak-Tutak K, Kolasa A et al: The effect of immunosuppressive therapy on renal cell apoptosis in native rat kidneys. *Histol Histopathol*, 2015; 30(1): 105–16
- Mayot G, Vidal K, Martin JF et al: Prognostic values of alpha2-macroglobulin, fibrinogen and albumin in regards to mortality and frailty in old rats. *Exp Gerontol*, 2007; 42(6): 498–505
- Pérez V, Navarro-Muñoz M, Mas S et al: Proteomic approach to the study of statin pleiotropy in kidney transplant patients. *Pharmacology*, 2011; 87(3–4): 161–68
- Van Westrhenen R, Westra WM, Van den Born J et al: Alpha-2- macroglobulin and albumin are useful serum proteins to detect subclinical peritonitis in the rat. *Perit Dial Int*, 2006; 26(1): 101–7
- Kuribayashi T, Tomizawa M, Seita T et al: Relationship between production of acute-phase proteins and strength of inflammatory stimulation in rats. *Lab Anim*, 2011; 45(3): 215–18
- Alter ML, Kretschmer A, Von Websky K et al: Early urinary and plasma biomarkers for experimental diabetic nephropathy. *Clin Lab*, 2012; 58(7–8): 659–71
- Edelstein C: Biomarkers of kidney disease. Academic press. Elsevier, 2011; 192–95
- Kuribayashi T, Seita T, Kawato K et al: Comparison of α_2 -macroglobulin synthesis by juvenile vs. mature rats after identical inflammatory stimulation. *Inflammation*, 2013; 36(6): 1448–52
- Viedt C, Orth SR: Monocyte chemoattractant protein-1 (MCP-1) in the kidney: does it more than simply attract monocytes? *Nephrol Dial Transplant*, 2002; 17(12): 2043–47
- Wu YG, Lin H, Qi XM et al: Prevention of early renal injury by mycophenolate mofetil and its mechanism in experimental diabetes. *Int Immunopharmacol*, 2006; 6(3): 445–53
- Lui SL, Yung S, Tsang R et al: Rapamycin prevents the development of nephritis in lupus-prone NZB/W F1 mice. *Lupus*, 2008; 17(4): 305–13
- Lee JY, Chang JW, Yang WS et al: Albumin-induced epithelial-mesenchymal transition and ER stress are regulated through a common ROS-c-Src kinase-mTOR pathway: effect of imatinib mesylate. *Am J Physiol Renal Physiol*, 2011; 300(5): F1214–22
- Oliveira JG, Xavier P, Sampaio SM et al: Compared to mycophenolate mofetil, rapamycin induces significant changes on growth factors and growth factor receptors in the early days post-kidney transplantation. *Transplantation*, 2002; 73(6): 915–20
- Shu Z, Pu X, Xiong X et al: Differential expression of plasma proteins in cyclosporine A-induced rat acute nephrotoxicity. *Biosci Biotechnol Biochem*, 2009; 73(3): 592–98

Conclusions

- Immunosuppressive drugs cause changes in the proteinogram of plasma proteins.
- In the groups with regimens based on CyA, higher KIM-1 concentration was found and KIM-1 was positively correlated to 170 kDa protein (alpha2-macroglobulin).
- The use of rapamycin was associated with decreased concentrations of MCP-1 in plasma and MCP-1 in plasma was negatively correlated to 37-kDa, 120-kDa, and 146-kDa proteins
- The strongest effect on plasma proteins in rats was shown by regimens based on rapamycin; intermediate, weak, and the weakest effects were reported for regimens based on CyA, tacrolimus, and MMF, respectively.