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PCSK9 and inflammatory biomarkers in the early post kidney transplantation period

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Abstract. – OBJECTIVE: Various biomarkers have been studied in the early post-kidney transplantation (post-KTx) period in order to identify potential therapeutic targets for improving long-term graft survival. Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a biomarker that has recently gained interest in cardiovascular disease but its role still remains to be defined post-KTx.

PATIENTS AND METHODS: We prospectively evaluated the levels of PCSK9, interleukin (IL)-6, WBC and C-reactive protein in seventy-three hemodialysis patients undergoing KTx, at 3 time-points; pre-transplantation (day 0) and at 1 and 6-months post-KTx. All data were also analyzed according to donor-type (living or deceased) and compared with hemodialysis patients on transplant waiting list.

RESULTS: At Day 0 there was no difference in WBC, CRP, IL-6 and PCSK9 levels between patients scheduled for transplantation and those who remained on hemodialysis. In transplanted patients WBC, CRP and IL-6 levels were significantly reduced early post-KTx [logIL-6 Day 0: 0.68 (0.33, 0.85) *vs*. 1-month: 0.57 (0.37, 0.75) *vs*. 6-months: 0.50 (0.32, 0.69) pg/ml, p=0.01], while PCSK9 levels were significantly increased (Day 0: 199.8±63.0 *vs.* 1-month: 276.2±79.4 *vs.* 6-months: 245.9±62.5 ng/ml, p<0.001). In contrast, no change of WBC, CRP, IL-6 and PCSK9 levels was observed in hemodialysis patients on follow-up (p=NS for all). Between living-donor and deceased-donor recipients, analysis showed reduced CRP and increased PCSK9 levels in both groups (p<0.05 for all), while IL-6 levels were reduced in living-donor and increased in deceased-donor recipients 1-month post-KTx. PCSK9 levels were not correlated with renal function, delayed graft function, rejection episodes or inflammatory biomarkers.

CONCLUSIONS: PCSK9 levels were increased post-KTx independently from renal function and inflammatory biomarkers, in both living and deceased-donor recipients.

Key Words:
Kidney transplantation, PCSK9, C-reactive protein, IL-6, Inflammation, Allograft vasculopathy.

Introduction

Despite the recent advancements in immunosuppressive agents and patient care, long term graft survival has minimally improved[1]. Late graft failure is the consequence of events occurring in the early post-transplantation period[2]. Various biomarkers and methods have been studied during the post-transplantation period aiming to characterize the structural and functional changes that happened in renal allograft and identify potential therapeutic targets[3,4]. Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a biomarker that has recently gained interest in the field of cardiovascular diseases[5]. PCSK9 is an important player in hypercholesterolemia and may also be involved in the inflammatory pathway of atherosclerosis[6,7].

In this prospective study we evaluated the changes of PCSK9 and inflammatory biomarkers in renal transplant recipients from living and deceased donors, in the early post-transplantation period.

Patients and Methods

Study Population

We performed a single-center, prospective, observational study that included consecutive kidney graft recipients who underwent transplantation in our center between May 2015 and December

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2016. We additionally investigated 10 hemodialysis patients of our dialysis unit who remained on transplant waiting list at the same period and served as control group (Group C). From a total of 80 transplanted patients, 73 were finally included in the study. We excluded patients in peritoneal dialysis and patients who lost renal allograft within the first month of transplantation. Additionally, patients not completing the follow-up visits at 1 and 6-months post-transplantation were not analyzed. Transplanted patients were separated into 2 groups. Group A (n=36) included patients who received a living-donor graft and group B (n=37) those who received a deceased-donor graft.

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional research committee and with the 2000 Helsinki declaration. This study was approved by the Ethical Committee of our hospital. Informed consent was obtained from all individual participants included in the study.

Immunosuppression

At the time of renal transplantation, all patients had a negative CDC T-cell crossmatch and a negative flow cytometry crossmatch. Induction therapy comprised intravenous methylprednisolone 500 mg intraoperatively and basiliximab 20 mg (day 0 and 4), with 14 patients (19%) additionally receiving rituximab. Maintenance immunosuppression consisted of mycophenolate sodium, a calcineurin inhibitor (tacrolimus in 95.9% of kidney recipients and cyclosporine in the remaining 4.1% of them) and corticosteroids.

Sample Collection and Analysis

Serum samples were collected before transplantation and at 1 and 6 months post-transplantation. Similarly, for kidney transplant candidates who remained on the waiting-list, blood samples were collected at the study entry and on the follow up period. Upon arrival at the laboratory, the blood samples were centrifuged at 3500 rpm for 15 min, aliquoted, and stored at −80°C until analysis.

PCSK9 levels were measured using a Quantikine Enzyme-linked immunosorbent assay (ELISA) Human Proprotein Convertase 9/PCSK9 (R & D Systems, Inc.), while IL-6 levels were measured using a Quantikine® High Sensitivity ELISA Human IL-6 Immunoassay (R&D systems, Inc, Minneapolis, MN, USA).

Biochemistry data which were collected during the scheduled visits were extracted from the electronic medical files (i.e. serum creatinine, urea, total cholesterol (TC), low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C). White blood cells (WBC) and C-reactive protein (CRP) were investigated as inflammatory biomarkers. Renal function was assessed using the Modification of Diet in Renal Disease (MDRD) equation.

Statistical Analysis

Normal distribution of continuous variables was tested for normality using the Kolmogorov-Smirnov test and by visual inspection of Q-Q plots. Not normally distributed continuous variables were logarithmically transformed to improve normality. Continuous variables are expressed as mean ± standard deviation if normally distributed otherwise as median with interquartile range. Categorical variables are expressed as valid percentages. Differences between categorical variables were tested by forming contingency tables and performing chi square tests. For continuous variables *t*-test was used for between groups differences and repeated measures analysis of variance (Friedman's ANOVA) for changes over the follow up period. Repeated measures *t*-test was applied to test for differences between different examined days. Correction for multiple comparisons was applied when indicated. To test for interaction of the over-time changes of the examined variable according to type of treatment a general linear model was applied. A linear mixed model was used to test for the different impact of treatment (transplantation *vs.* hemodialysis continuation) on the examined parameters during the study period. Linear mixed models that were applied included fixed effects (type of treatment) and random effect (subjects' identity with unstructured covariance structure). Correlation coefficient between normally distributed continuous variables was assessed using Pearson correlation coefficient. Differences were considered statistically significant when the *p*-value was less than 0.05. The statistical software SPSS version 25 (IBM, Armonk, NY, USA) was used for all analyses.

Results

Patient Characteristics

Baseline characteristics of the patients are shown in Table I. In the entire study population glomerular diseases were the most common cause of end-stage renal disease (ESRD) ($p<0.001$) **(Supplementary Figure 1)**. The age of the par-

Table I. Drug-drug interaction of concern when COVID-19-positive patients take opioids.

	All patients N=83	Living-donor recipients Group A N = 36	Deceased-donor recipients Group B N = 37	Hemodialysis patients Group C N = 10	*p*
Age, years	45.3±13.6	40.9±12.9	49.9±12.8*	44.3±15.1	0.02
Male sex, n (%)	50 (60)	24 (67%)	20 (54)	6 (60)	0.55
Time on dialysis, years	5.3±5.0	2.0±1.9	8.1±4.4	7.0±7.7*	<0.001
Causes of chronic renal diseases					
Diabetes mellitus, n (%)	3 (4)	2 (6)	0 (0)	1 (10)	0.56
Arterial hypertension, n (%)	1 (1)	1 (3)	0 (0)	0 (0)	-
Glomerular disease, n (%)	26 (31)	13 (36)	10 (27)	3 (30)	0.05
ADPKD, n (%)	15 (18)	5 (14)	8 (22)	2 (20)	0.17
Obstructive uropathy, n (%)	10 (12)	3 (8)	5 (14)	2 (20)	0.50
Genetic causes, n (%)	5 (6)	3 (8)	2 (5)	0 (0)	0.66
Unknown, n (%)	23 (28)	9 (25)	12 (32)	2 (20)	0.03
Cardiovascular risk factors					
Arterial hypertension(%)	37 (45)	16 (44)	16 (43)	5 (50)	0.93
Hyperlipidemia, n (%)	18 (21)	10 (28)	6 (16)	2 (20)	0.17
Statin use, n	14 (17)	9 (25)	3 (8)	2 (20)	0.15
Diabetes mellitus, n (%)	4 (5)	3 (8)	0 (0)	1 (10)	0.17
Smoking					
Active/Former/Never	18/9/56	8/5/23	9/1/27	1/3/6	0.14
Atrial fibrillation, n (%)	4 (5)	0 (0)	4 (11)	0 (0)	0.07
History of coronary artery disease, n (%)	11 (13)	4 (11)	3 (8)	4 (40)†	0.03
Peripheral artery disease (%)	7 (8)	3 (8)	3 (8)	1 (10)	0.98
Family history of CVD, n (%)	13 (16)	6 (17)	6 (16)	1 (10)	0.87
Body mass index, Kg/m^2	23.9±3.8	22.9±3.3	24.9±4.2	23.2±3.2	0.08
Inflammatory markers					
WBC, K/μl	7.84±2.34	8.34±2.56	7.55±2.11	7.12±2.15	0.21
CRP, ng/ml	3.26±0.28	3.25±0.29	3.25±0.29	3.29±0.14	0.92
LogIL-6, pg/ml	0.70 [0.34, 0.86]	0.76 [0.32, 0.90]	0.59 [0.33, 0.79]	0.85 [0.60, 0.96]	0.11
PSCK, ng/ml	203.3±65.1	213.0±64.3	187.0±59.9	228.5±77.3	0.11
Transplantation data					
Donor age		57 (39-72)	47 (16-69)		
Number of transplantation>1		2	9		
HLA mismatch		3.2±1.5	3.2±1.4		
PRAs (<10%/≥10%)		35/1	20/17*		
Cold ischemia time, hours		0	17.7±5.5*		

ADPKD: autosomal dominant polycystic kidney disease; CVD: cardiovascular disease; CRP: C reactive protein; IL-6: interleukin 6; PCSK9: Proprotein convertase subtilisin/kexin type 9; HLA: human leukocyte antigen; PRA: panel-reactive antibody.
Values are expressed as mean values ± standard deviation; LogIL-6 is presented as median with interquartile range.
p-values in the last column of the table represent the differences between the three study groups (group A, group B and group C).
Differences between categorical variables were tested by forming contingency tables and performing χ^2-tests.
Between-group comparisons for continuous variables were performed using one-Way analysis of variance (ANOVA).
*denotes statistical significance (p<0.025) compared to group A, †denotes statistical significance (p<0.025) compared to group B

ticipants was 45.3±13.6 years without any difference between transplanted patients (Groups A and B) and patients on hemodialysis (Group C) (Groups A and B: 45.4±13.5 years *vs.* Group C: 44.3±15.1 years, p=0.81). Compared to deceased donors, living-donor graft recipients were younger (deceased-donor recipients 49.9±12.8 years *vs.* living-donor recipients 40.9±12.9 years, p=0.01) and had shorter time on hemodialysis (8.1±4.4 years *vs.* 2.0±1.9 years, p<0.001). Between the

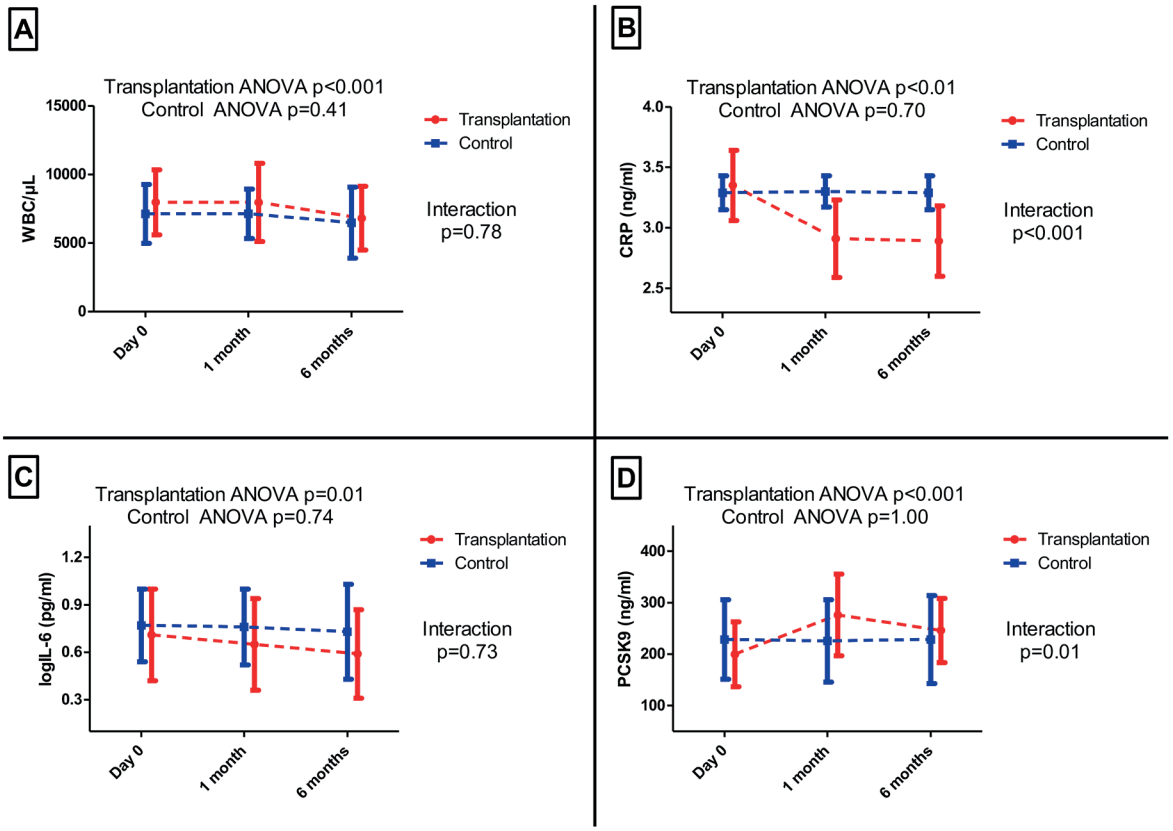

Figure 1. Changes over time of studied biomarkers following transplantation or hemodialysis continuation. Transplantation decreases WBC, CRP, IL-6 and increases PCSK9 levels. Graphs show variation over follow-up time of (**A**) WBC, (**B**) CRP, (**C**) logIL-6, (**D**) PCSK9. ANOVA: analysis of variance; WBC: white blood cells; CRP: C reactive protein; IL-6: Interleukin 6; PCSK9: Proprotein convertase subtilisin/kexin type 9

three study groups there was no difference in the prevalence of cardiovascular risk factors (i.e. arterial hypertension, diabetes mellitus, hyperlipidemia, and smoking) (Table I), while statins were rarely used in all groups. Human Leucocyte Antigen (HLA) mismatches were similar between the two transplanted groups, while the deceased-donor group comprised of more sensitized patients [Panel Reactive Antibodies (PRAs)≥10% in 17 patients (46%) *vs.* 1 (3%) in the living-donor group respectively, $p<0.001$].

Time Course of inflammatory biomarkers (Table II)

Transplanted Patients

At day 0 there was no significant difference in WBC counts between transplanted (Groups A and B) and patients on hemodialysis (Group C) (Group A+B: 7.94±2.36 K/µl *vs.* Group C: 7.12±2.15 K/µl, $p=0.30$). WBC counts were significantly reduced in the post transplantation period (Groups A and B) (Day 0: 7.94±2.36 K/µl *vs.* 1 month: 7.96±2.85 K/µl *vs.* 6 months: 6.76±2.34 K/µl, $p<0.001$) while in the hemodialysis group (Group C) there were no significant changes in WBC counts during the follow-up period (Day 0: 7.12±2.15 K/µl *vs.* 1 month: 7.13±1.80 K/µl *vs.* 6 months: 6.48±2.59 K/µl, $p=0.41$) (Table II and Figure 1A).

At Day 0 there was no significant difference in CRP levels between transplanted (Groups A and B) and patients on hemodialysis (Group C) (Group A+B: 3.25±0.29 ng/ml *vs.* Group C: 3.29±0.14 ng/ml, $p=0.68$). CRP levels were significantly reduced in the post transplantation period (Groups A and B) (Day 0: 3.25±0.29 ng/ml *vs.* 1 month: 2.88±0.31 ng/ml *vs.* 6 months: 2.83±0.28 ng/ml, $p<0.001$) while in the hemodialysis group (Group C) there was no significant changes in CRP levels during the follow-up period (Day 0: 3.29±0.14 ng/ml *vs.* 1 month: 3.30±0.13 ng/ml *vs.* 6 months: 3.29±0.14 ng/ml, $p=0.70$) (Table II and Figure 1B). To further study how transplantation

affects CRP levels during the follow up period we proceeded to general linear model analysis. Accordingly, we found a significant interaction of transplantation over hemodialysis continuation (p<0.001), with significant improvement of CRP during follow up in the transplanted patients (Groups A and B) over the Group C of hemodialysis continuation (Figure 1B).

Concerning IL-6, no significant differences were noted between the transplanted (Groups A and B) and the patients on hemodialysis (Group C) at baseline (Day 0) [Groups A and B: logIL-6 0.68 (0.33, 0.85) pg/ml *vs.* Group C: 0.85 (0.60, 0.96) pg/ml, p=0.08). There was a significant decrease in IL-6 levels following transplantation [Day 0: 0.68 (0.33, 0.85) pg/ml *vs.* 1 month: 0.57 (0.37, 0.75) pg/ml *vs.* 6 months: 0.50 (0.32, 0.69) pg/ml, p=0.01]. In patients on hemodialysis (Group C) there was no change in the IL-6 levels at the follow up period [Day 0: 0.85 (0.60, 0.96) pg/ml *vs.* 1 month: 0.88 (0.52, 0.93) pg/ml *vs.* 6 months: 0.87 (0.34, 0.98) pg/ml, p=0.74] (Table II and Figure 1C).

There was no difference in PCSK9 levels in the transplanted patients (Groups A and B) and in the patients on hemodialysis (Group C) at baseline (Day 0) (Groups A and B: 199.8±63.0 ng/ml *vs.* Group C: 228.5±77.3 ng/ml, p=0.08). There was a significant increase in PCSK9 levels following transplantation (Groups A and B) (Day 0: 199.8±63.0 ng/ml *vs.* 1 month: 276.2±79.4 ng/ml *vs.* 6 months: 245.9±62.5 ng/ml, p<0.001). In patients on hemodialysis (Group C) there was no change in the PCSK9 levels at the follow up period (Day 0: 228.5±77.3 ng/ml *vs.* 1 month: 225.8±80 ng/ml *vs.* 6 months: 228.4±85.5 ng/ml, p=0.99) (Table II and Figure 1D). To further study how transplantation affects PCSK9 levels during the follow up period we proceed to a general linear model analysis. Accordingly, we found a significant interaction of transplantation over hemodialysis continuation (p=0.01), with significant increase of PCSK9 during follow up in the transplanted patients (Groups A and B) over the Group C of hemodialysis continuation (Figure 1D).

Inflammatory Biomarkers Change According to Type of Transplantation

With regards to the two transplanted subgroups A and B (living-donor and deceased-donor graft

Table II. Laboratory parameters of transplanted and hemodialysis patients at day 0 and at 1 and 6 months after study entry.

	All transplanted patients (Groups A+B) N=73				Hemodialysis patients (Group C) N=10			
Laboratory parameters	Day 0	1 month	6 month	p	Day 0	1 month	6 month	p
Hb, g/dl	12.1±1.4	12.6±1.5	13.3±1.6*†	<0.001	10.7±1.2	10.7±1.0	11.2±0.9	0.64
WBC, K/μl	7.94±2.36	7.96±2.85	6.76±2.34*†	<0.001	7.12±2.15	7.13±1.80	6.48±2.59*	0.41
Creatinine, mg/dl	10.1±3.1	1.4±0.4*	1.3±0.4*	<0.001	10.3±3.3	9.8±2.6	10.3±2.6	0.74
eGFR (MDRD), mL/min/1.73 m^2	6.5±2.3	58.7±20.4*	61.7±19.2*	<0.001	4.3±1.5	5.0±1.0	4.7±1.2	0.18
Total cholesterol, mg/dl	175±41	238±71*	229±61*	<0.001	156±38	158±37	148±42	0.50
LDL-C, mg/dl	95±35	144±63*	135±54*	<0.001	80±23	77±28	67±23	0.51
HDL-C, mg/dl	43±14	62±18*	58±18*	<0.001	38±18	49±13	39±22	0.28
Albumin, mg/dl	4.3±0.4	4.4±0.3	4.5±0.3*†	0.01	4.0±0.4	4.2±0.6*	4.2±0.3	0.03
Glucose, mg/dl	93±38	98±21	98±21	0.001	92±20	98±17	96±25	0.71
HbA1c, %	4.9±0.5	5.1±0.6	5.3±0.5*†	<0.001	5.1±0.9	5.1±0.8	5.1±0.7	0.80
CRP, ng/ml	3.25±0.29	2.88±0.31*	2.83±0.28*	<0.001	3.29±0.14	3.30±0.13	3.29±0.14	0.70
	0.68	0.57	0.50*		0.85	0.88	0.87	0.74
logIL-6, pg/ml	[0.33, 0.85]	[0.37, 0.75]	[0.32, 0.69]	0.01	[0.60, 0.96]	[0.52, 0.93]	[0.34, 0.98]	
PCSK9, ng/ml	199.8±63.0	276.2±79.4*	245.9±62.5*†	<0.001	228.5±77.3	225.8±80	228.4±85.5	1.00

Hb: hemoglobin, WBC: white blood cells, eGFR: estimated glomerular filtration rate, LDL-C: low density lipoprotein cholesterol, HDL-C: high density lipoprotein cholesterol, TG: triglycerides, CRP: C-reactive protein, IL-6: interleukin-6, PCSK9: Proprotein convertase subtilisin/kexin type 9

Values are expressed as mean values ± standard deviation; LogIL-6 is presented as median with interquartile range.

Analysis of between-group changes at baseline and at 1 and 6-months post-transplantation were performed using independent sample t-test.

* denotes statistical significance (p<0.025) compared to day 0; † denotes statistical significance (p< 0.025) compared to 1 month.

recipients) we observed that WBC counts were significantly reduced in both group A (Day 0: 8.34±2.56 K/µl *vs.* 1 month: 8.98±2.93 K/µl *vs.* 6 months: 7.41±2.61 K/µl, $p<0.001$) and group B (Day 0: 7.58±2.13 K/µl *vs.* 1 month: 6.94±2.40 K/µl *vs.* 6 months: 6.13±1.86 K/µl, $p<0.001$) during the follow up period (Table III, Figure 2A). Also, in subgroups A and B (living-donor and deceased-donor graft recipients) we observe that CRP levels were significantly reduced in both group A (Day 0: 3.25±0.29 ng/ml *vs.* 1 month: 2.82±0.32 ng/ml *vs.* 6 months: 2.79±0.3 ng/ml, $p<0.001$) and group B (Day 0: 3.25±0.29 ng/ml *vs.* 1 month: 2.94±0.30 ng/ml *vs.* 6 months: 2.86±0.27 ng/ml, $p<0.001$) during follow up period (Table III, Figure 2B). In Group A, IL-6 levels were reduced significantly during follow up period [logIL-6 Day 0: 0.76 (0.32, 0.90) pg/ml *vs.* 1 month: 0.50 (0.21, 0.64) pg/ml *vs.* 6 months: 0.45 (0.18, 0.69) pg/ml, $p<0.001$]. In Group B, IL-6 levels were significantly changed during the follow up period [Day 0: 0.59 (0.33, 0.79) pg/ml *vs.* 1 month: 0.65 (0.48, 0.83) pg/ml *vs.* 6 months: 0.53 (0.38, 0.69) pg/ml, $p=0.02$] (Table III, Figure 2C). Interestingly, 1-month post transplantation IL-6 levels were significantly decrease in living donors graft recipient (Group A) (Table III, Figure 2C) compared to pre-transplantation levels (Day 0). In contrast in deceased donor graft recipients (group B) IL-6 levels were increased compared to pre-transplantation levels (Day 0) (Table III, Figure 2C). PCSK9 levels were significantly increased in both Group A (Day 0: 213.0±64.3 ng/ml *vs.* 1 month: 298.3±88.4 ng/ml *vs.* 6 months: 246.5±70.3 ng/ml, $p<0.001$) and Group B (Day 0: 187.0±59.9 ng/ml *vs.* 1 month: 254.8±63.7 ng/ml *vs.* 6 months: 245.2±54.8 ng/ml, $p<0.001$) during follow up period (Table III, Figure 2D).

Determinants of PCSK9 Levels in the Post-Transplantation Period

To assess how PCSK9 levels may be affected in ESRD patients following transplantation, we tested for associations of PCSK9 with several factors possibly implicated. In the transplanted patient population, there was no correlation of PCSK9 levels

Table III. LLaboratory parameters of transplanted patients receiving living donor and deceased donor grafts at day 0 and at 1 and 6 months after study entry.

	Living donor graft recipients (Groups A) N = 36				Deceased donor graft recipients (Group C) N = 37			
Laboratory parameters	Day 0	1 month	6 month	*p*	Day 0	1 month	6 month	*p*
Hb, g/dl	11.8±1.3	13.0±1.5	13.6±1.6*†	<0.001	12.4±1.5	12.2±1.4	12.9±1.6†	0.09
WBC, K/µl	8.34±2.56	8.98±2.93	7.41±2.61†	0.001	7.58±2.13	6.94±2.40	6.13±1.86*	0.001
Creatinine, mg/dl	10.5±3.1	1.3±0.3*	1.3±0.3*	<0.001	9.8±3.2	1.5±0.5*	1.3±0.4*†	<0.001
eGFR (MDRD), mL/min/1.73 m^2	6.9±7.4	65.3±17.4*	63.4±14.9*	<0.001	6.0±2.5	52.2±21.4*	60.7±22.9 *	<0.001
Total cholesterol, mg/dl	166±41	243±65*	226±52*	<0.001	187±37	224±73*	230±67*	0.03
LDL-C, mg/dl	87±37	141±54*	138±46*	<0.001	104±29	139±68*	134±62*	0.04
HDL-C, mg/dl	40±12	67±17*	61±22*	<0.001	45±16	56±17*	55±14*	<0.001
Albumin, mg/dl	4.2±0.5	4.4±0.3*	4.5±0.3*†	0.002	4.5±0.4	4.3±0.3*	4.4±0.3†	0.04
Glucose, mg/dl	101±50	97±21	97±26	0.40	84±16	97±21*	98±14*	<0.001
HbA1c, %	4.9±0.7	5.1±0.6	5.2±0.6*	0.01	4.8±0.3	5.0±0.6	5.3±0.5*†	<0.001
CRP, ng/ml	3.25±0.29	2.82±0.32*	2.79±0.3*	<0.001	3.25±0.29	2.94±0.30*	2.86±0.27*	<0.001
logIL-6, pg/ml	0.76 (0.32, 0.90)	0.50* (0.21, 0.64)	0.45* [0.18, 0.69]	<0.001	0.59 (0.33, 0.79)	0.65 (0.48, 0.83)	0.53† [0.38, 0.69]	0.02
PCSK9, ng/ml	213.0±64.3	298.3±88.4*	246.5±70.3*†	<0.001	187.0±59.9	254.8±63.7*	245.2±54.8*	<0.001

Hb: hemoglobin, WBC: white blood cells, eGFR: estimated glomerular filtration rate, LDL-C: low density lipoprotein cholesterol, HDL-C: high density lipoprotein cholesterol, TG: triglycerides, CRP: C-reactive protein, IL-6: interleukin-6, PCSK9: Proprotein convertase subtilisin/kexin type 9

Values are expressed as mean values ± standard deviation; LogIL-6 is presented as median with interquartile range.

Analysis of between-group changes at baseline and at 1 and 6-months post-transplantation were performed using independent sample *t*-test.

*denotes statistical significance ($p<0.025$) compared to day 0; † denotes statistical significance ($p < 0.025$) compared to 1 month.

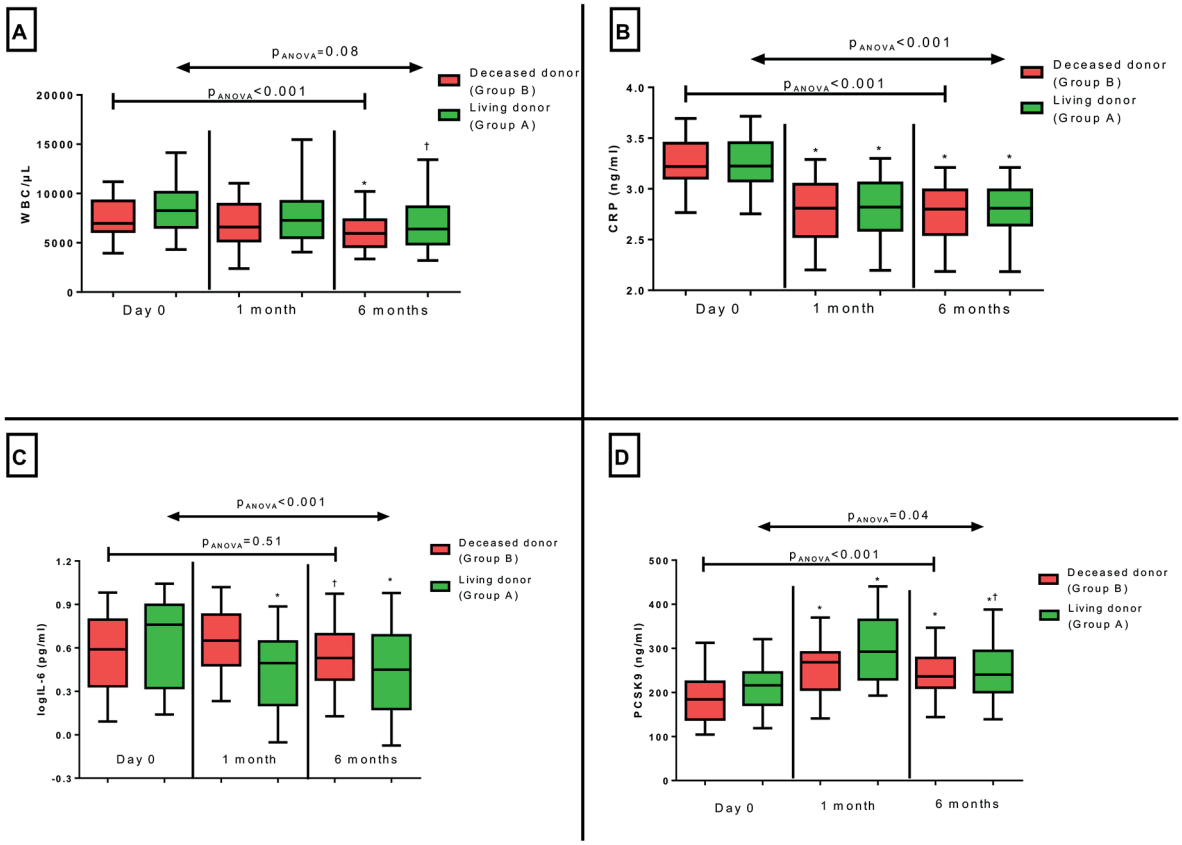

Figure 2. Boxplots indicating the changes over time of studied biomarkers according to type of graft donor (living *vs.* deceased). Graphs show changes over follow-up time of (**A**) WBC, (**B**) CRP, (**C**) logIL-6, (**D**) PCSK9. *p*-values are based on ANOVA. ANOVA: analysis of variance; WBC: white blood cells; CRP: C reactive protein; IL-6: Interleukin 6; PCSK9: Proprotein convertase subtilisin/kexin type 9; * denotes statistical significance ($p<0.025$) compared to day 0; † denotes statistical significance ($p<0.025$) compared to 1 month.

with renal function (creatinine and eGFR based on MDRD) during the early post-transplantation period ($r=0.203$, $p=0.09$ and $r=-0.027$, $p=0.82$ for eGFR-MDRD at 1- and 6-months post-transplantation respectively) **(Supplementary Table I)**. Total cholesterol, LDL cholesterol levels as well as CRP and IL-6 levels had no correlation with PCSK9.

Delayed Graft Function and Inflammatory Biomarkers

To test whether delayed graft function (DGF) could affect PCSK9 and inflammatory biomarkers post transplantation, we further stratified our transplanted population in subjects with and without DGF. We found that patients with DGF were older (DGF: 51.6±13.4 years *vs.* no DGF: 42.4±12.6 years, $p=0.01$) and had longer cold ischemia time (DGF: 16.7±7.9 hours *vs.* 5.2±8.2 hours, $p<0.001$). Concerning inflammatory biomarkers, IL-6 levels were significantly correlated with DGF during the first month post-transplantation. Non-significant differences were noted in PCSK9 and CRP levels between patients with and without DGF **(Supplementary Table II)**.

Clinical Outcomes of Transplanted Patients

During the 6-months of follow-up, cardiovascular events occurred in 5 patients receiving grafts from deceased donors and in none in the living-donor group ($p=0.02$) (Table IV). Moreover, five acute rejection episodes (6%) occurred. When analyzing patients with acute rejection, no difference was observed in the baseline levels of the CRP (Rejection: 3.47±0.12 ng/ml *vs.* no rejection: 3.24±0.29 ng/ml, $p=0.09$), IL-6 [Rejection: 0.75 (0.42, 0.94) pg/ml *vs.* no rejection: 0.62 (0.33, 0.84) pg/ml, $p=0.48$) or PCSK9 (Rejection: 216.9±58.1 ng/ml *vs.* no rejection 198.5±63.6 ng/ml, $p=0.53$).

Table IV. Clinical outcomes of transplanted patients at 6 months post-transplantation.

	All transplanted patients N=73	Living-donor recipients (Group A) N = 36	Deceased-donor recipients (Group B) N = 37	p
Cardiovascular events, n (%)	5 (7)	0	5 (14)	0.02
Acute rejection, n (%)	5 (6)	2 (6)	3 (8)	0.67
Biopsy, n (%)	24 (33)	9 (25)	15 (41)	0.16
Infections				
Bacterial, n (%)	20 (27)	10 (28)	10 (27)	0.76
Viral, n (%)	12 (16)	6 (17)	6 (16)	0.60

All values are expressed as mean ± standard deviation.

Discussion

This study is the first prospective study that evaluates the time course of the levels of PCSK9 and inflammatory biomarkers, in the early post kidney transplantation period. It is interesting that PCSK9 levels were increased at 1 and 6 months post transplantation, while IL-6 levels and conventional inflammatory biomarkers were decreased in the same period compared to the pre-transplant levels. When we tested PCSK9 levels for associations with factors that possibly implicated in the increased post-transplant levels, we didn't find any correlation with renal function, DGF or rejection episodes. Interesting were also the results from the subgroup analysis according to the type of donor (living-donor vs deceased-donor graft recipients). Although we did not find differences in PCSK9 levels and CRP between living-donor and deceased-donor recipients in the follow up period, IL-6 levels at one month post-transplant were reduced in living-donor recipients and increased in deceased-donor recipients. PCSK9 is a protease with pivotal role in cholesterol homoeostasis and atherosclerosis[8]. Circulating PCSK9 binds LDL-Receptor (LDL-R), driving its lysosomal degradation within cells, which leads to reduced LDL-R expression, decreased LDL catabolism, and increased plasma levels of LDL. Beyond its role in cholesterol metabolism, recent studies have shown increased levels of PCSK9 in cases of systemic inflammation[9]. Although ESRD is a state of chronic inflammation, studies focusing on hemodialysis patients and PCSK9 levels reported conflicting results. Abujrad et al[10] showed lower PCSK9 levels in hemodialysis patients without lipid-lowering therapy, in contrast to Konarzewski et al[11] that found increased levels before hemodialysis session.

In kidney transplant recipients there are very few studies focusing on PCSK9[11,12]. These studies have measured PCSK9 levels at least 1-year post-transplantation and data on patients' inflammatory status are lacking. Our study is the first that included and analyzed separately renal transplant recipients from both living and deceased donors during the early post-transplantation period (≤6 months) and studied simultaneously PCSK9 as well as other inflammatory biomarkers at three time points, pre- and in the early post-transplantation period. Konarzewski et al[11] measured PCSK9 levels in 20 transplanted patients and compared them with a group of ESRD patients without finding any difference. Eisenga et al[12] studied the association of PCSK9 levels with the risk of new-onset diabetes after transplantation and a significant association was detected but no data about other inflammatory markers were provided.

In our study, PCSK9 levels were increased post-transplantation in the total of transplant recipients. Although renal function was significantly improved after transplantation, PCSK9 levels were not reduced and no correlation between PCSK9 levels and renal function was observed. It seems that the clearance of PCSK9's LDL-R was not increased enough in the post-transplantation period, despite the substantially improved renal function. Perhaps, other pathways related to transplantation may have played a role in the overexpression of the PCSK9 gene.

There are experimental studies that report a PCSK9 gene overexpression in association with inflammation[9]. PCSK9 and inflammation is a new target pathway that has been studied in clinical trials with conflicting results. Le Bras et al[13] in a study of intensive care unit patients demonstrated

that plasma concentrations of PCSK9 were drastically increased after a severe multiple trauma. In contrast, randomized controlled trials failed to find a significant impact of PCSK9 inhibitors on plasma hs-CRP levels[14]. In our study no correlation between PCSK9 and IL-6 was confirmed early post-transplantation.

IL-6 is a cytokine with multiple functions and is often used as a marker of inflammation. Studies have shown increased levels of IL-6 in patients with ESRD[14,15]. The increased levels of IL-6 in dialysis patients have been associated with retention of uremic solutes, persistent infections and oxidative stress[16]. The chronic inflammatory state has also been associated with various dialysis-related factors[17]. In our study IL-6 levels decreased post-transplantation and this reduction was more pronounced in recipients from living-donors. In agreement with this finding is the study of Simmons et al[18] who observed a rapid and significant decline in IL-6 within 2 months after transplantation from living-donors.

Although inflammation is reduced one month post-transplantation, PCSK9 is increased and this is a very interesting finding. PCSK9 could be a new target pathway in the early post kidney transplantation period. Future studies focusing on an early intervention with PCSK9 inhibition could characterize its crucial role in long-term allograft survival. The pathogenesis of progressive graft dysfunction is a combination of immunological and non-immunological factors taking place soon after transplantation[19]. Dyslipidemia is a significant risk factor for allograft vasculopathy and graft dysfunction[20,21]. Renal transplant patients have an enhanced hyperlipidemic profile following transplantation, including total cholesterol and triglycerides[22]. The effect of immunosuppressive drugs in combination with pre-transplantation dyslipidemia and the presence of glucose intolerance or diabetes mellitus are some of the factors contribute to hyperlipidemia. Initiation of a lipid lowering therapy is of great importance to prevent cardiovascular complications. Statin therapy is essential but cannot reduce PCSK9 levels. PCSK9 inhibitors may contribute effectively to dyslipidemia with the reduction of PCSK9 activity.

Clinical Implications

Our findings may have important clinical implications in the era of new drugs that could potentially decrease renal allograft vasculopathy. PCSK9 inhibitors[23,24], and interleukin-6 inhibitors[25] have been tested in experimental and clinical settings aiming to reduce cardiovascular events and allograft vasculopathy with favorable outcomes. Already, the PCSK9 inhibitors have been used in heart transplanted patients with encouraging results[26]. Future studies assessing the role of PCSK9 inhibitors in relation with long-term renal graft function are needed.

Study Limitations

Although this study is the larger in renal transplant recipients in the early post-transplantation period, it still has, as a single-center study, a limited number of participants. Larger studies are needed to further define the role of PCSK9 as a predictor of vasculopathy events in this special patient population and the role of inflammatory biomarkers beyond six months, as our study was neither designed nor powered to examine such associations. Finally, our study did not attempt to elucidate the pathogenetic mechanism of the role of PCSK9 in renal transplant recipients and more studies are needed towards this direction.

Conclusions

Despite tremendous progress in the field of renal transplantation, chronic allograft vasculopathy remains the main cause of late graft failure. PCSK9 inhibition could be the key to this problem. Our study is the first that clearly describes the early rising of PCSK9 levels post-transplantation in both living and deceased-donor recipients independently from renal function and other inflammatory markers. Early intervention in this pathway could potentially affect allograft's survival. Aristotle a Greek philosopher said 'well begun is half done' means that an error at the beginning can be important for the future. Perhaps the long-term allograft survival depends on facts occurring early after transplantation. The role of early PCSK9 inhibition in late renal allograft dysfunction needs to be investigated more extensively in future trials.

Supplementary Appendix

Additional supporting information may be found online in the Supplementary Appendix section at the end of the article **(Supplementary Table I, Supplementary Table II, Supplementary Figure 1)**.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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