Diabetic nephropathy : pathology, genetics and carnosine metabolism

Mooyaart, A.L.

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CNDP1 POLYMORPHISM PREDISPOSES FOR PROGRESSION TO END STAGE RENAL DISEASE AFTER GLOMERULAR DAMAGE

[1]Antien Mooyaart, [2]Dina J de Jager, [2]Diana C Grootendorst, [1]Nina Daha, [4]Fathali Farnoosh, [3]Elizabeth W Boeschoten, [1]Ingeborg Bajema, [5]Verena Peters, [4]Christine Fischer, [1]Jan A Bruijn, [2]Friedo W Dekker, [4]Bart Janssen, [1]Hans J Baelde, [1]Emile de Heer on behalf of the Netherlands Cooperative Study on the Adequacy of Dialysis (NECOSAD) Study Group and the PREDICTIONS study group

Depts of [1]Pathology and [2]Clinical Epidemiology, Leiden University Medical Center, Netherlands, [3]Hans Mak Institute, Naarden, The Netherlands, [4]Dept. of Human Genetics, [5]Dept. of Pediatrics, University of Heidelberg, Germany

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Abstract

A polymorphism in the number of CTG-repeats in exon 2 of the *CNDP1* (carnosinase) gene correlates with the development of nephropathy in diabetes patients. Carnosinase degrades carnosine, which protects glomeruli against oxidative and hemodynamic damage. This study investigated whether the *CNDP1* polymorphism is associated with occurrence of ESRD and mortality risk in patients with vascular kidney diseases other than diabetic nephropathy, such as renal vascular disease and chronic glomerulonephritis.

Included were 97 Caucasian end stage chronic glomerulonephritis patients, 143 end stage renal vascular disease patients and 732 healthy Caucasian controls. Furthermore, 104 end stage polycystic kidney disease patients and 95 end stage tubulointerstitial nephritis were included as disease controls. Prevalence of genotypes was compared by calculating odds-ratios, survival of patients by Kaplan-Meier techniques.

Compared to patients with genotypes resulting in lower carnosinase activity, genotypes with higher carnosinase activity were associated with an increased risk of developing ESRD in patients with renal vascular disease (OR [95% CI] 2.47 [1.02, 5.98]) and chronic glomerulonephritis (5.08 [2.15, 11.99]). These chronic glomerulonephritis patients also had an increased mortality risk (p log rank 0.01). In patients with other primary kidney diseases the genotype was not associated with ESRD or mortality.

In conclusion, in patients with glomerular damage the *CNDP1* polymorphism predisposes for development of ESRD. In chronic glomerulonephritis this was also reflected by increased mortality. These findings support the hypothesis that there is a common genetic basis for progression to ESRD after glomerular damage.

Introduction

End stage renal disease (ESRD) has reached epidemic proportions and the number of ESRD patients is still increasing [1]. These patients require renal function replacement therapy by either dialysis or transplantation. This leads to loss of quality of life, high mortality rates and has become a great economic burden [1]. Identifying risk factors for progression to ESRD are therefore of great interest and might be able to prevent susceptible individuals from developing ESRD by earlier and more aggressive treatment. One of the most important risk factors for developing ESRD seems to be a positive family history of ESRD [2]. Lei *et al.* found that familial aggregation of renal disease could not be fully explained by familial clustering of diabetes and hypertension. Therefore it is likely that a separate genetic susceptibility factor exists for progression of ESRD [3].

A possible susceptibility locus for diabetic nephropathy on chromosome 18q has been identified in earlier studies [4;5]. We identified the responsible modifying gene within this locus as the *CNDP1* gene [6]. These findings were further confirmed in 963 American patients of European descent with type 2 diabetes-induced nephropathy [7], but not in patients with type 1 diabetes [8]. Type 2 diabetes patients with the homozygosity for the Mannheim allele (5 copies of a trinucleotide repeat encoding for leucine in the leader peptide on exon 2) of the *CNDP1* gene demonstrated a 2.56-fold reduced risk for developing diabetic nephropathy (DN) compared to individuals with more leucine repeats (6-8 repeats) [6]. The presence of more than 5 leucine repeats has been shown to lead to higher serum carnosinase secretion [9] and more serum carnosinase activity [6;10]. Serum carnosinase is known to degrade histidine-containing dipeptides called carnosines, which function as scavengers of reactive oxygen species [11] and as inhibitors of angiotensin converting enzyme (ACE) [12].

Since injury to glomerular cells by oxidative stress and hemodynamic factors is not confined to development of diabetic nephropathy, we hypothesize that lower number of leucine repeats in the *CNDP1* play a protective role in progression to ESRD in underlying renal diseases due to glomerular and microvascular injury, such as chronic glomerulonephritis and renal vascular disease. In line with this hypothesis, one would expect that within these vascular disease groups, survival would differ in patients with different *CNDP1* genotypes.

Methods

Patient and control subjects

Patients were selected from the Netherlands Cooperative Study on the Adequacy of Dialysis (NECOSAD), a multicenter, prospective follow-up study of ESRD patients who were included at the time of the initiation of dialysis [13]. Data on ethnic background, gender, primary kidney disease, comorbidities, and modality were collected between four weeks prior and two weeks after the start of dialysis. Patients were at least 18 years of age with no previous renal replacement therapy and were followed till death or censoring. Reasons for censoring included transplantation, recovery of renal function or loss to follow-up. All local medical ethics committees approved of the study and patients gave informed consent before inclusion.

For the current study, data from chronic glomerulonephritis, polycystic kidney disease, renal vascular disease and tubulointerstitial nephritis were selected. These diseases were classified according to the European Renal Association-European Dialysis and Transplantation Association (ERA-EDTA) codes (http://www.era-edta-reg.org/files/annualreports/pdf/AnnRep2006.pdf.). Based on these ERA-EDTA codes, primary kidney disease groups were defined: chronic glomerulonephritis (cGN, codes 9 to 20), tubulointerstitial nephritis (TIN, codes 20 to 40), polycystic kidney disease (PKD, codes 40 to 50) and renal vascular disease (RVD, codes 70 to 73, or code 79). Patients that were not of Caucasian origin were excluded, as the distribution of *CNDP1* is dependent of ethnic origin [10]. Comorbidity was defined according to the risk criteria of Khan *et al.* [14]. The Khan index is a combination of age and co-morbidity that divides risk groups into three degrees of severity as low, medium, or high. Three months after beginning dialysis, a blood sample and 24-hour urine sample were collected on the same day. Serum albumin, plasma creatinine, and plasma urea levels were determined. Urea and creatinine levels were also analyzed in the urine sample. Genomic DNA was isolated from peripheral blood with the Puregene® DNA isolation kit (Gentra, Minneapolis, USA). The genotype frequencies in NECOSAD patients were compared to those in 732 healthy controls above 18 years of age. To eliminate ethnic differences, we only included Caucasian subjects who confirmed that all grandparents were of Northwest European origin.

Genotyping

CNDP1 genotyping was performed as described elsewhere [6]. In brief, a standard PCR protocol was used with the primers GCGGGGAGGGTGAGGAGAAC (forward) and GGTAACAGACCTTCTTGAGGAATTTGG (reverse). The denaturating, annealing and extension temperatures were 94°C, 60°C, and 72°C, respectively. Fragment analysis was performed on the ABI-3130 analyzer (Perkin Elmer) to determine the number of leucine repeats on each allele. A product length of 157, 160, and 163 bp corresponded to 5, 6, or 7 CTG (Leu) codons, respectively.

Determination of serum-carnosinase activity

Serum-carnosinase activity was determined according to the method described by Teufel *et al*. [15]. Briefly, the reaction was initiated by addition of substrate (L-carnosine) to a serum sample and stopped after 10 minutes of incubation at 30°C by adding 1% trichloracetic acid. Liberated histidine was derivatized with *o*-phtaldialdehyde (OPA). Fluorescence was measured by excitation at 360 nm and emission at 460 nm. Serum samples were obtained from 60 healthy controls.

Mortality

Causes of death were determined by treating physicians and classified according to the codes of the ERA-EDTA which can be found at: http://www.era-edta-reg.org/files/annualreports/pdf/AnnRep2006.pdf. The following codes were classified as cardiovascular mortality: 0 (cause of death uncertain/not determined), 11 (myocardial ischemia and infarction), 14 (other causes of cardiac failure), 15 (cardiac arrest, cause unknown), 18 (fluid overload), 22 (cerebrovascular accident), 26 (hemorrhage from ruptured vascular aneurysm, not code 22 or 23), or 29 (mesenteric infarction).

Statistical analysis

In all analyses subjects carrying the *CNDP1* Mannheim variant were used as reference group and contrasted to all other possible genotypes (5-0, 5-6, 5-7/6-6, 6-7/6-8/7-7). Hardy-Weinberg equilibrium was calculated using the gene-counting method, and differences between NECOSAD patients and the control group were assessed by chi-square test. In NECOSAD patients, differences in baseline characteristics between the different *CNDP1* genotype groups were tested with the chi-square test for dichotomous and categorical variables and analysis of variance for continuous variables. The Armitage

Trend Test was used to test for genetic association, as the genotypes could be ranked according to the respective enzyme activities.

Survival of patients with different *CNDP1* genotypes was analyzed by means of Kaplan-Meier survival curves. Log rank tests were used to determine survival differences. All statistical analyses were performed with SPSS statistical software (version 12; SPSS, Chicago, IL) and SAS (Version 9.1; SAS, Heidelberg, Germany).

Results

A total of 439 dialysis patients were selected from the NECOSAD, consisting of 97 chronic glomerulonephritis patients, 143 renal vascular disease patients, 104 polycystic kidney disease patients and 96 tubulointerstitial nephritis patients.

Baseline characteristics of these 439 patients and these patients grouped by allelic variant of *CNDP1* are seen in Table 1.

Carnosinase activity

The carnosinase activity was measured in 60 healthy controls with genotypes 5-5, 5-6, 6-6, 5-7. The genotype 5-0 was estimated on theoretical enzyme activity and the 5-8 was extrapolated from a different study done by Riedl *et al.* [9]. A clear correlation was found between carnosinase enzyme levels and the number of leucine repeats in the *CNDP1* gene. The various genotypes were ranked according to the corresponding enzyme activities (Figure 1).

Table 1. Baseline characteristics of patients under study (N=439), grouped by allelic variant of *CNDP1*

	Total	***CNDP1* genotype**			
		5-5	**5-6**	**5-7/6-6**	**6-7/6-8/7-7**
	N=439	**N=163**	**N=164**	**N=84**	**N=28**
Age (yrs)	59.5 (14.0)	59.7 (13.8)	59.2 (13.9)	59.0 (15.0)	61.6 (14.3)
Gender (% female)	35.1	38.7	32.3	32.1	39.3
Chronic Therapy (% HD)	62.9	65.0	59.8	67.9	53.6
Primary kidney disease (%)					
Glomerulonephritis	22.1	17.2	22.0	27.4	35.7
Interstitial nephritis	21.6	27.6	17.7	15.5	28.6
Polycystic kidney disease	23.7	27.0	24.4	21.4	7.1
Renal vascular disease	32.6	28.2	36.0	35.7	28.6
Khan co-morbidity score (%)					
low	49.7	51.5	50.0	48.8	39.3
moderate	28.2	30.7	28.0	23.8	28.6
high	22.1	17.8	22.0	27.4	32.1
DM co-morbidity (%)	5.1	4.4	4.9	6.0	7.1
CVD co-morbidity (%)	35.3	31.0	36.5	36.8	48.1
Smoking habit (%)					
never	27.1	27.6	28.7	28.6	10.7
ever, >3 mo ago	43.1	45.4	41.5	41.7	42.9
ever, <=3 mo ago	4.8	3.7	5.5	3.6	10.7
current	25.1	23.3	24.4	26.2	35.7
GFR (ml/min/1.73m^2)†	5.3 (3.1)	5.3 (2.7)	5.4 (3.4)	5.3 (3.1)	5.1 (3.5)
Blood pressure (mmHg)					
systolic	149.1 (24.8)	149.6 (26.2)	147.6 (24.7)	151.6 (24.1)	147.3 (19.8)
diastolic	83.6 (12.8)	84.0 (12.8)	82.5 (13.1)	84.8 (13.2)	83.2 (10.7)

Mean values ± SD are given for continuous variables. For categorical variables percentages are shown. DM, diabetes mellitus; GFR, glomerular filtration rate; CVD, cardiovascular disease; *Exclusive vasculitis; †GFR = glomerular filtration rate, measured between four weeks prior to and two weeks after start of dialysis (for 5-5 [N=91], 5-6 [N=100], 5-7/6-6 [N=48] and 6-7/6-8/7-7 [N=19]).

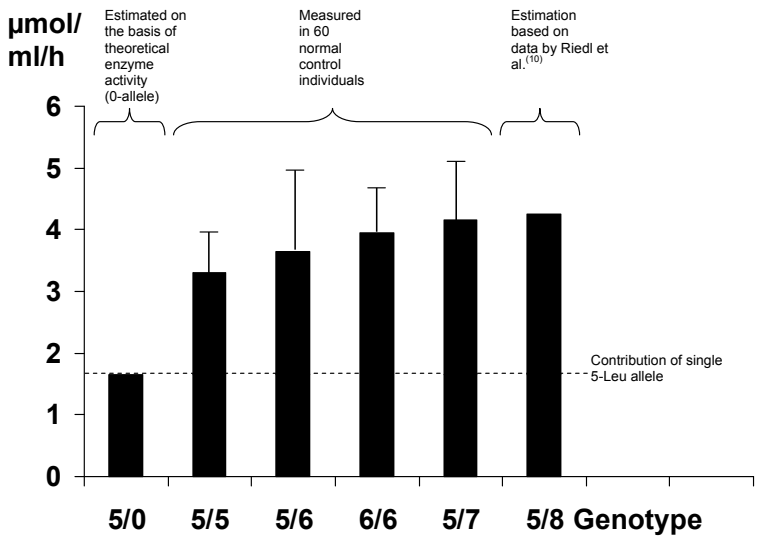

Figure 1. Estimated and measured serum-carnosinase activity in compound heterozygosity with the Mannheim allele (5-Leu) in normal Caucasian individuals.

Analysis of CNDP1 genotype frequencies by comparison of disease subgroups. Patients with chronic glomerulonephritis had significantly more leucine repeats than controls (Table 1, p= 0.0006). ESRD patients with the highest number of leucine repeats in the *CNDP1* gene have a 5.1 (95% CI 2.15, 11.99) higher risk of developing ESRD due to glomerulonephritis compared to glomerulonephritis patients with lower number of leucine repeats (Table 2). The renal vascular disease patient group also showed significant association with *CNDP1* repeat length (p=0.011) (Table 1). ESRD patients with the highest number of leucine repeats in the *CNDP1* gene have a 2.5 (95% CI 1.02, 5.98) higher risk of developing ESRD due to renal vascular disease compared to renal vascular disease patients with lower number of leucine repeats (Table 2). Patients who became dialysis-dependent due to polycystic kidney disease or tubulointerstitial nephritis, as well as normal controls, all had similar distributions of the *CNDP1* genotypes (Table 2).

Table 2. Distribution of *CNDP1* leucine repeats of NECOSAD patients grouped by primary kidney disease compared to controls.

	NECOSAD grouped by primary kidney disease									
	cGN		RVD		TIN		PKD		Control group	
CNDP1[#]	(n=97)		(n=143)		(n=95)		(n=104)		(n=732)	
	n	%	n	%	n	%	n	%	n	%
5/0, 6/0, 7/0	0	0	0	0	0	0	0	0	13	1.8
5-5	28	28.9	46	32.2	45	47.4	44	42.3	270	36.8
5-6	36	37.1	59	41.3	29	30.5	40	38.5	303	41.4
5/7, 6/6	23	23.7	30	21.0	13	13.7	18	17.3	127	17.3
6/7, 6/8, 7/7, 7/8	10	10.3	8	5.6	8	8.4	2	1.9	19	2.6
p*	**0.0006**		**0.011**		0.55		0.74			

GN indicates chronic glomerulonephritis; TIN, tubulointerstitial nephritis; PKD, polycystic kidney disease; RVD, renal vascular disease; DN, diabetic nephropathy; and OMD, other multisystem diseases.
*) NECOSAD patients vs. the control group as determined with Armitage Trend Test.
#) Genotypes grouped according to enzyme activity (Table 1).

Mortality

Survival probabilities for patients with different *CNDP1* genotypes were not different (log rank test, p=0.62). A stratified survival analysis revealed that the survival probability in patients with glomerulonephritis (log rank test, p<0.01) as primary renal disease were significantly different between the leucine repeat groups (5-5, 5-6, 5-7/6-6, 6-7/6-8/7-7) (Figure 2). Patients with glomerulonephritis and a high number of leucine repeats had higher mortality rate compared to glomerulonephritis patients with lower number of leucine repeats. Survival probabilities in ESRD patients due to polycystic kidney disease, tubulointerstitial nephritis and renal vascular disease did not differ between the leucine repeat groups.

Table 3. Genotypic odds ratios (OR) comparing genotype-related risks to the 5Leu / 5Leu reference and primary causes of development of ESRD.

CNDP1	cGN (OR, 95% CI)	RVD (OR, 95% CI)
5/5 (ref.)	1.00	1.00
5/6	1.14 (0.68, 1.92)	1.14 (0.75, 1.73)
5/7, 6/6	1.70 (0.95, 3.08)	1.35 (0.82, 2.25)
6/7, 6/8, 7/7, 7/8	**5.08 (2.15, 11.99)**	**2.47 (1.02, 5.98)**

cGN: chronic glomerulonephritis; RVD: renal vascular disease.

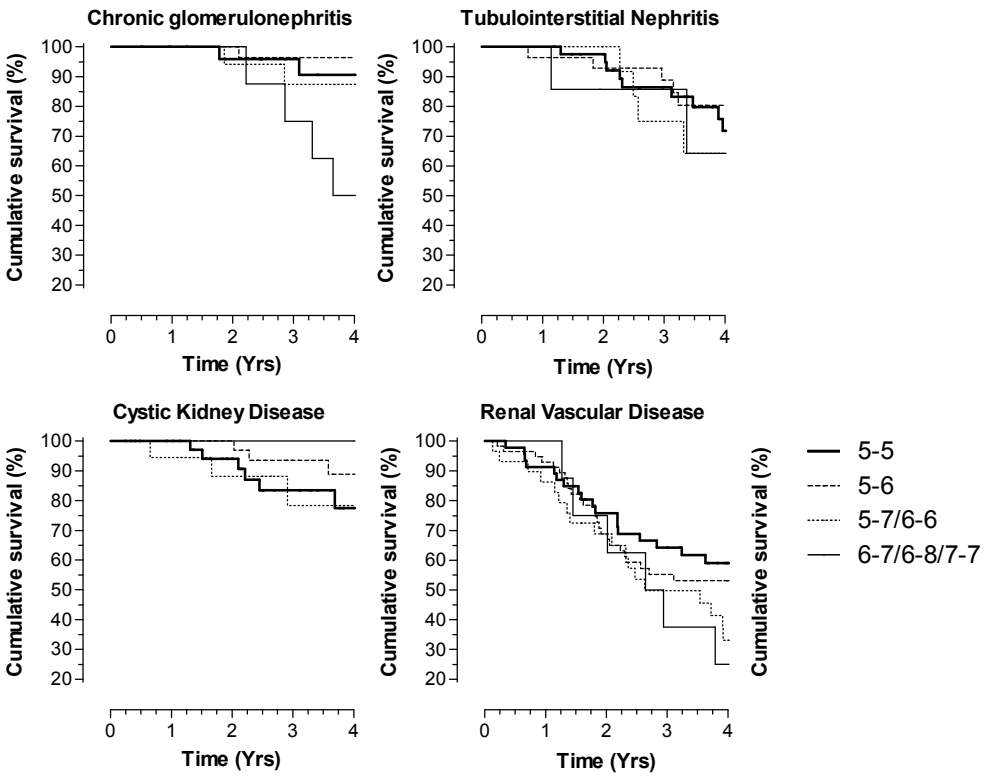

Figure 2. Survival within the first four years of follow-up of patients with chronic glomerulonephrits, tubulointerstitial nephritis, cystic kidney disease and renal vascular disease.

Discussion

Our results demonstrate that the higher number of leucine repeats in the *CNDP1* genotype is related to a faster progression to ESRD in patients with compromised kidney function due to chronic glomerular inflammatory renal diseases and the group of patients with renal vascular disease. In line with these results, the mortality risk is increased in chronic glomerulonephritis patients with higher number of leucine repeats in the *CNDP1* gene, which is associated with higher serum carnosinase levels. Our data show a correlation between the leucine repeat distribution of the *CNDP1* gene and renal vascular disease, however there was no relation with survival. As predicted, patients who developed ESRD due to either polycystic kidney disease or tubulointerstitial had a *CNDP1* genotype

distribution similar to that of healthy controls. These findings support the hypothesis that the leucine repeat in *CNDP1* may contribute to microvascular damage.

In chronic glomerulonephritis the higher number of leucine repeats in the *CNDP1* gene seems to be involved in progression to ESRD and mortality on dialysis. There are two pathways by which this association can be explained. Common to the development of ESRD in these patients is that the development of progressive glomerulosclerosis is accompanied by oxidative injury to glomerular cells from reactive oxygen species (ROS) and hemodynamic factors [16]. Histidine-containing dipeptides such as carnosines reportedly function as ROS scavengers [17], natural inhibitors of transforming growth factor beta (TGF-beta) production [6], anti-apoptotic compounds [18], and natural inhibitors of ACE [12]. The rapid degradation of these dipeptides by locally produced serum-carnosinase may impair a protective mechanism required for recovery after renal disease. Indeed, transfection experiments have shown that multiple leucine repeats in carnosinase results in increased secretion of the enzyme [9] and our results show an increased carnosinase activity with increasing number of leucine repeats. This current result is a replication, as this was found in both Caucasians [6] and South Asians [10]. Furthermore, carnosinase was expressed in the kidney, making a specific role in the kidney likely [10]. Carnosines are released from skeletal muscle after physical exercise [19], and the beneficial effects of exercise on diabetic nephropathy [20], hypertension-induced nephrosclerosis [21;22], and progressive renal disease in general [23] have been well documented. Thus, the protective effects of carnosine seem to be essential for natural recovery of the kidney after microvascular injury.

The second possibility regards the connection between the autonomic nervous system and the kidney. Homocarnosine, a particular substrate of serum carnosinase, is composed of gamma-amino-butyric acid-L-histidine (GABA-his) [24]. Cleavage of homocarnosine by serum-carnosinase releases the neurotransmitter GABA, resulting in GABA-receptor-mediated activation of the sympathetic innervations of the kidney [24;25]. In addition, carnosine has been shown to inhibit sympathetic nerve activity directly, resulting in reduction of systemic blood pressure [26]. The same research group also demonstrated the involvement of carnosine in the regulation of blood glucose levels via autonomic nerves [27]. Indeed, hyperactivity of the sympathetic nerves in both hypertension-induced renal insufficiency and progressive renal disease in general has been reported in both experimental settings and clinical studies [28-31].

Recently, we found that the relation between the *CNDP1* gene and DN is sex-specific, including patients with DN from the NECOSAD study [32]. We also stratified the data of this study for men and women. The frequency of the 5-5 homozygous genotype was lower in women than in men in chronic glomerulonephritis patients (23.1% vs. 31.0%) and renal vascular disease patients (29.7% vs. 33.0%). Although this is in line with the data in DN, no definite conclusion should be drawn as due to the stratification the sample size became too small.

Ideally, one would have preferred to compare the ESRD group with the diseased patients not progressing to ESRD to exclude the possible correlation between susceptibility for the disease. However we feel that such correlation is unlikely because of the aetiological heterogeneity within the disease groups. Furthermore, the different diseases can be seen to serve as disease control groups.

Our data have clinical implications. Carnosine and related compounds have already been used as therapies for cataracts [33] and gastric ulcers [34]. In rats induced with gentamicin, treatment with carnosine led to a significant improvement of the kidney function [35]. Furthermore, gentamicin-induced glomerular shrinkage was absent in those rats treated with carnosine [35]. In humans, carnosine seems to be hydrolyzed within 2 hours after carnosine is absorbed by the intestine and enters the bloodstream [36]. However, when Parkinson patients were treated with carnosine in addition to dopamine (L-dopa), they had a decreased level of protein carbonyls in their blood plasma and increased levels of red cell superoxide dismutase compared with patients treated with only dopamine (L-dopa) [37]. This suggests that supplementation with carnosine has a biochemical effect after oral intake and could, therefore, be of potential therapeutic value. Our study indicates that carnosine supplementation can also be considered in patients with chronic glomerulonephritis and renal vascular disease.

In summary, here we have provided evidence for a genetic predisposition for progression to ESRD due to chronic glomerulonephritis and renal vascular disease. In glomerulonephritis this was reflected in both genotype distribution and in mortality risk. Hereby we identified a genetic polymorphism that seems to be involved in the clinical course of renal disease after sustained glomerular injury.

Disclosure

I declare that none of the authors have conflicts of interest.

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