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GENETIC AND ENVIRONMENTAL PREDICTORS OF CHRONIC KIDNEY DISEASE IN PATIENTS WITH TYPE 2 DIABETES AND DIABETIC FOOT ULCER: A PILOT STUDY

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Chronic kidney disease (CKD) is often observed among patients with type 2 diabetes mellitus (T2DM) and diabetic foot (DF) leading to end stage renal disease. The aim of this pilot study was to determine genetic and environmental factors involved in the etiology of CKD among patients with DF. The following polymorphisms were studied: rs1800469, rs759853, rs1553005, rs1799983, rs1801133, rs3134069, rs2073618, rs8192678, rs6330, rs11466112, rs121917832 in terms of alleles distribution in patients with DF and T2DM, with or without CKD. The study includes 101 patients with T2DM and DF. Studied groups were divided into 39 individuals with CKD (cases) and 62 controls, depending on the presence of kidney failure defined as eGFR < 60ml/min/1.73m^2 and coexistence of microalbuminuria > 30 mg/dl in at least 3 urine samples. Cases and controls were matched according to mean age, gender, mean duration of T2DM, mean duration of insulin therapy, mean duration of DF cholesterol levels and smoking frequencies. The study showed that CKD risk factors were the following variables: creatinine level, body weight, hips circumference, ischemic heart disease, hypertension and diabetic retinopathy. Moreover, the results suggest the protective role of the allele C of rs3134069 polymorphism in CKD development in patients with T2DM and DF in the following allelic variants: [AA] vs. [AC] and [AA] vs. [AC + CC]. The allele C was observed to be less frequent than the allele A in patients with T2DM and DF. None of the other following polymorphisms was observed to be a potential risk factor of CKD in T2DM and DF population: rs6330, rs759853, rs1553005, rs1799983, rs1801133, rs1800469, rs8192678, rs11466112, rs121917832. We concluded that the rs3134069 polymorphism seems to be the most likely protective genetic factor in CKD development in patients with T2DM and DF.

Key words: *polymorphism, osteoprotegerin, chronic kidney disease, diabetes mellitus, diabetic foot, glomerular filtration rate, hypertension*

INTRODUCTION

Each year in Poland about 4000 new patients require dialysis due to end stage renal disease (ESRD). In 33% of cases, the main reason of dialysis is type 2 diabetes mellitus (T2DM), but not in all patients with chronic kidney disease (CKD) T2DM is its causative factor (1). Many studies confirmed significant association between the presence of diabetic foot (DF) and decrease of glomerular filtration rate (GFR) (2). Margolis *et al*. investigated a correlation between amputation and decrease of GFR in patients with T2DM. Study on 90,617 individuals with T2DM show that the risk of amputation was 7 fold higher when GFR has been below 30 ml/min/1.73m^2 than in patients with GFR within normal limits (3). Stacey *et al*. conducted a study on 2098 diabetic patients with a lower extremity amputation and 2206 controls. The results showed that kidney failure is an early predictor of amputation in this population (4).

The pathophysiology of DF is multifactorial with 60% of patients suffering from neuropathy, deformation and injury (5). According to the International Working Group on the Diabetic Foot, DF is defined as the ulceration, infection and/or destruction of deep tissues below ankles in patients with diabetes and/or peripheral arterial disease (6). Complications affecting the lower limbs are among the most common manifestations of diabetes. It was reported that 15% of T2DM patients will eventually suffer from foot ulceration during their lifetime, and these complications are the frequent cause of hospitalization and disability (7).

Diabetic complications do not always develop in all patients with long lasting diabetes and poor glycemic control. This statement has been proven in an epidemiologic study, conducted by Klein *et al*. in diabetic retinopathy population with T2DM (8). Familial clustering of the condition and the ethnic background have a substantial influence on the development of microangiopathic complications (9, 10). We may presume that

genetic factors may play a relevant role. There is a need for early identification of patients with a particular genetic predisposition for CKD among patients with T2DM and DF.

Kidney failure is a result of metabolic dysregulation, leading to basal membrane hypertrophy and proliferation of extracellular matrix. Risk factors like hyperlipidemia, hypertension, hyperglycemia, high BMI (body mass index), low levels of HDL (high density lipoprotein), high levels of LDL (low density lipoprotein), smoking, low socioeconomic status, man gender and also genetic polymorphisms (many of which are still unknown) influence the development of CKD (11). In the group of potential genes that modify the risk of CKD development are: genes of renin-angiotensin system, glucose metabolism, growth factors, oxidative stress, lipid metabolism and inflammation (12). Due to multifactorial pathophysiology of CKD, many different genes may be involved in its development (13).

The etiology of CKD and DF has many common pathogenetic pathways. Both depend on vasculopathy and microangiopathy. In kidneys, they lead to endothelial dysfunction, activation of RAS and TGFβ, inflammation with podocyte and mesangial cell death. All of them finally cause renal vasoconstriction, fibrosis leading to glomerulosclerosis and tubular degeneration. It results with decrease of renal blood flow, glomerular filtration rate and decrease of renal function (14).

Micro- and macroangiopathy underlies the pathogenetic factors of diabetic foot which are neuropathy and peripheral arterial disease. Diabetic neuropathy depends on blood flow abnormalities, caused by activation of polyol, hexosamine, AGE, PKC pathways and oxidative stress. PAD in diabetes represents exacerbated form of arteriopathy. It combines two patterns of vascular calcification - atherosclerosis and arteriosclerosis. Both neuropathy and PAD finally decrease oxygen supply to microcirculation and cause vasoconstriction of arterial tree in lower limbs (15, 16).

The aim of this pilot study was to determine genetic and environmental factors involved in the etiology of CKD among patients with DF. Basing on the literature review, we have chosen the most promising single nucleotide polymorphisms (SNPs), proved to be involved in etiopathogenesis of vasculopathy and microangiopathy: *TGFB1* (transforming growth factor b1 gene, rs1800469), *AKR1B1* (aldose reductase gene, rs759853), *CALCA* (calcitonin gene-related peptide gene, rs1553005), *NOS3* (nitric oxide synthase 3 gene, rs1799983), *MTHFR* (methylenetetrahydrofolate reductase (NAD(P)H) gene, rs1801133), *TNFRSF11B* (osteoprotegerin (OPG), also known as osteoclastogenesis inhibitory factor (OCIF), rs3134069, rs2073618), *PPARGC1A* (peroxisome proliferator-activated receptor gamma gene, rs8192678), NGF (nerve growth factor gene, rs6330, rs11466112), *CDKN1B* (cyclin-dependent kinase inhibitor 1B gene, rs121917832).

SNP rs1800469 in the *TGFB1* gene is implicated in the pathogenesis in many forms of progressive renal disease, by promoting renal hypertrophy and the accumulation of extracellular matrix (17-19). Another researched polymorphism that was proven to be implicated in the development of diabetic chronic renal insufficiency is rs759853 in *AKR1B1* gene (20). Also SNP rs1799983, within *NOS3* gene, influences cardiovascular homeostasis through maintenance of vascular tone and regulation of blood pressure. Variations in this gene may be involved in the development of both kidney failure and cardiovascular disease in patients with T2DM (21). Polymorphisms in the *MTHFR* gene are associated with increased homocysteine levels. The risk TT genotype of rs1801133 within *MTHFR* gene may increase the mortality in patients with ESRD (22). SNP rs8192678 in *PPARGC1A* gene is involved in the regulation of genes involved in glucose and lipid metabolism (23). SNP rs8192678 is associated with hypertension in male subjects with T2DM, atrial fibrillation in elderly patients, gestational diabetes and risk of T2DM (24-27).

Vasodilation of arterial tree is associated with the SNP rs1553005 of *CALCA* gene. Product of *CALCA* gene dilates a variety of vessels, including the coronary, cerebral and systemic vasculature. An association between *CALCA* gene and essential hypertension in Japanese subjects was confirmed (28).

Recent studies indicate that osteoprotegerin (OPG) acts as an important regulatory molecule in the vasculature (29). Moreover, an association was observed between it and microvascular complications as diabetic retinopathy (30). *TNFRSF11B* gene rs2073618 is associated with DF, but this relation was not proved in reference to SNP rs3134069 (31).

The *NGF* gene SNPs rs6330 and rs11466112 encodes a secreted protein which has nerve growth stimulating activity and the complex is involved in the regulation of growth and the differentiation of sympathetic and certain sensory neurons (32).

CDKN1B gene SNP rs121917832 encodes a cyclin-dependent kinase inhibitor. This protein controls the cell cycle progression at G1 stage. The degradation of this protein is required for the cellular transition from quiescence to the proliferative state (33).

MATERIAL AND METHODS

The study was conducted in the Department of Gastroenterology and Metabolic Diseases and the Department of Medical Genetics, Medical University of Warsaw, Poland between December 2010 and September 2013.

The study was approved by Medical University of Warsaw Bioethics Committee.

Patients

The presented pilot study includes 101 individuals with T2DM and DF (39 with CKD - cases and 62 patients without CKD - controls). The groups were matched due to mean age, gender, mean duration of T2DM, mean duration of insulin therapy, mean duration of DF, cholesterol levels and smoking frequencies. Cases were classified by having glomerular filtration rate below 60 ml/min/1.73m^2 of body surface area (at least stage 3 of chronic kidney disease due to WHO classification) and having microalbuminuria (results above 30 mg/dl in at least 3 urine samples). Each patient underwent assessment of blood glucose level, GFR estimated using the modified diet and renal disease equation, urine examination with assessment of proteins concentration (34). Patients with urinary tract infection and prerenal acute kidney injury (i.e. dehydration, vomits, diarrhoea) were excluded.

Experimental procedures

Before being enrolled into the study, each patient was treated in the Diabetic Foot Outpatient Clinic at Medical University of Warsaw and laboratory markers of CKD were assessed. Patients were subsequently hospitalized in the Department of Gastroenterology and Metabolic Diseases at Medical University of Warsaw, where the biochemical analyses were done, using routine methods. The period prior to hospitalization was at least 3 months. The markers of CKD were classified by authors as increasing risk of ESRD (35).

DF diagnosis criteria were based on the International Consensus on the Diabetic Foot and Practical Guidelines (6). Patients with T2DM and foot lesions (ulceration, infection, or destruction of deep tissues located in the lower limbs below the ankles) resulting from neuropathy and/or peripheral arterial

disease were included in the study. In the physical examination, we assessed arterial perfusion (the pulse on the tibial posterior and dorsal pedis arteries were assessed, the ankle-brachial index measured using mini-Doppler (Bidop Hadeco ES) and the presence of sense abnormalities (senses of touch, temperature, pain and vibration, knee and Achilles tendon reflexes).

According to the Toronto Clinical Neuropathy Score, the stage of neuropathy was assessed using monofilament, thermotip, neurotips and Semmes-Weinstein tunnel fork. If the ankle-brachial index was below the normal limits in mini-Doppler examination, a full Doppler ultrasound examination was performed and other diagnostic procedures were ordered to assess the severity of peripheral arterial disease.

DNA was isolated from the whole blood samples, using the salting-out Miller's method (36). Genotyping of selected SNPs: *TGFB1* (rs1800469), *AKR1B1* (rs759853), *CALCA* (rs1553005), *NOS3* (rs1799983), *MTHFR* (rs1801133), *TNFRSF11B* (rs3134069, rs2073618), *PPARGC1A* (rs8192678), *NGF* (rs6330, rs11466112), *CDKN1B* (rs121917832), was performed by SEQUENOM (GmbH Mendelssohnstrasse 15D, D-22761 Hamburg, Germany), with the Sequenom MassArray system (Sequenom iPLEX assay, CA). All genetic tests were performed with negative control.

Statistical analysis

Statistical calculations were performed using the STATISTICA 10 software (StatSoft Inc.). The frequencies of alleles in cases and controls were compared using the $\chi 2$ test. The obtained distribution of polymorphisms genotypes were statistically analyzed with an online associations test (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl).

RESULTS

The univariate logistic regression analysis showed that CKD risk factors were the following variables: mean creatinine level, mean body weight, mean hips circumference, ischemic heart disease, hypertension and diabetic retinopathy. Each mg/dl more in creatinine serum level was increasing the risk of CKD development by 4.5%. The risk of CKD development was increased by 3.7% per each additional kg in body mass. There was also observed an increase in CKD development risk by 6.3% for each additional centimeter in hips circumference. Moreover, CKD risk was increased by the coexistence of ischemic heart disease, hypertension and diabetic retinopathy, over 2.7-fold, 7.3-fold and 4.4-fold, respectively (*Table 1*).

There were no statistically significant differences between cases and controls in the terms of: mean age, gender, mean T2DM duration, mean age of T2DM diagnosis, mean duration of insulin therapy, mean DF duration, mean glycated hemoglobin percentage (HbA_{1C}%), dyslipidaemia and smoking frequencies. The characteristics of studied groups are shown in *Table 2*.

SNP analyses showed a potential association of rs3134069 of *TNFRSF11B* gene with CKD in the group of patients with DF in the following allelic variants: [AA] vs. [AC] (OR = 0.12, 95% CI = 0.02 – 0.99, P = 0.021) and [AA] vs. [AC + CC] (OR = 0.12, 95% CI = 0.02 – 0.99, P = 0.021). The C allele of the rs3134069 was less frequent in cases in the allelic variant [A] vs. [C] (OR = 0.13, 95% CI = 0.02 – 1.06, P = 0.031). In contrast, being a carrier of the C allele of the rs2073618 of *TNFRSF11B* gene was not a risk factor in the following allelic variants: [CC] vs. [CG] (OR = 1.5; 95% CI = 0.58 – 3.94; P = 0.4) and [CC] vs. [CG + GG] (OR = 1.59, 95% CI = 0.64 – 3.97, P = 0.32). None of the other studied following SNPs was associated with the risk of CKD in T2DM and DF patients: rs6330, rs759853, rs1553005, rs1799983, rs1801133, rs1800469, rs8192678, rs11466112, rs121917832 (*Table 3*).

Furthermore, we observed that the patients with SNP rs11466112 of *NGF* gene presented only the allele C in CKD and control group. A similar result was observed in the SNP rs121917832 of *CDKN1B* gene for the allele G.

SNP-SNP and SNP-environment interactions were verified by logistic regression. No important statistic correlations were found.

DISCUSSION

CKD is the life-threatening condition resulting from progressive loss of renal excretory function leading to ESRD requiring renal replacement therapy (dialysis or kidney transplant). CKD may result from various factors such as hypertension, alteration of lipid and glucose metabolism, decreased perfusion to the kidneys, infections, mechanical obstructions and genetic susceptibility. Finally, pathophysiology of advanced stadium of CKD is similar regardless of its cause. Although T2DM is the most common reason of dialysis (37), 30 – 40% patients with T2DM develop nephropathy irrespective of glycemic control (38). In this group, hypertension is the leading cause of progressive renal dysfunction. Hypertension is the second cause of ESRD (39). Both diabetic and non-diabetic nephropathy depend on vasculopathy and microangiopathy. They are developing on the basis of genetic predisposition when favorable environmental factors like dyslipidemia,

Table 1. Characteristics of the chronic kidney disease group compared to controls, *t* Student test, *U* Mann-Whitney test, χ^2, and logistic regression analysis.

	Chronic kidney disease	S.D.	Control group without CKD	S.D.	P value	OR	95% CI
hips circumference** cm	165.3	22.2	107.7	10.7	0.014	1.063	1.01 – 1.12
mean weight** kg	101.1	18.1	90.5	17.0	0.004	1.037	1.01 – 1.06
creatinine level** mg/dl	1.51	1.1	0.87	0.18	0.00001	1.045	1.02 – 1.07
retinopathy/without***	31/8	-	29/33	-	0.001	4.410	1.75 – 11.11
hypertension/without ***	38/1	-	52/10	-	0.02	7.308	0.90 – 59.54
ischemic heart disease and/or heart failure/ without ***	27/12	-	27/34	-	0.02	2.732	1.18 – 6.35

t* Student test; ** *U* Mann-Whitney test; * χ^2
S.D., standard deviation; CKD, chronic kidney disease; OR, odds ratio, CI, confidence interval

Table 2. Characteristics of the CKD group compared to controls, *t* Student test, *U* Mann-Whitney test and χ^2.

Variable	Chronic Kidney Disease	S.D.	Control group without CKD	S.D.	P value
total number	39	-	62	-	-
female/male***	27/12 (69.3% / 30.7%)	-	43/19 (69.4% / 30.6%)	-	> 0.05
mean age**, years	63.6	8.3	64.5	10.6	0.9
mean age of T2DM diagnosis**, years	46.0	10.1	48.2	11.8	0.3
mean T2DM duration*, years	17.2	8.5	16.1	8.3	0.5
mean time from T2DM diagnosis to DF diagnosis*, years	10.6	8.1	10.6	7.4	0.9
mean time from T2DM diagnosis to insulin therapy*, years	5.8	6.1	7.09	5.9	0.3
mean age of insulin therapy **, years	51.0	11.5	55.0	10.2	0.1
mean duration of insulin therapy*, years	11.4	9.4	9.3	7.3	0.2
mean time from insulin therapy to DF diagnosis*, years	6.0	8.5	3.8	6.7	0.1
mean age at DF diagnosis**, years	57.0	8.7	59.0	11.3	0.2
mean DF duration*, years	6.2	4.3	5.1	4.1	0.2
mean waist circumference**, cm	108.5	16.5	101.8	14.6	0.2
WHR**	0.90	0.2	0.95	0.1	0.2
mean height**, m	1.74	0.1	1.72	0.1	0.1
BMI**, km/m^2	33.17	5.5	30.75	4.9	0.07
HbA_{1C}%**	7.94	1.8	7.73	1.9	0.8
dyslipidaemia/without***	32/5	-	39/17	-	> 0.05
smoking/without***	20/17	-	32/26	-	> 0.05

* *t* Student test; ** *U* Mann-Whitney test; *** χ^2.
S.D., standard deviation; CKD, chronic kidney disease; OR, odds ratio; CI, confidence interval; WHR, waist-hip ratio; BMI, body mass index.

hyperglycemia, obesity, and hypertension occur. In diabetes, hyperglycemia promotes microangiopathic changes before renal vasoconstriction occurs. They are related with excessive generation of NADH and leads to oxidative stress, mitochondrial dysfunction, DNA damage, poly(ADP-ribose) polymerase (PARP) activation and production of invalid proteins. Additive influence of oxidative stress and hyperglycemia activate glucose changes to sorbitol (polyol pathway) and AGE formation. AGE, polyol, hexosamine and PKC pathways lead to diabetic neuropathy, which is the main cause of diabetic foot (40). Because kidney failure was found as early predictor of amputation in the population of patients with diabetic foot we decided to investigate genetic polymorphisms engaged into pathways leading to both CKD and DF (4).

The presented study confirms the high prevalence of diabetic retinopathy, which still exists in population with DF. It is consistent with earlier studies where the correlation between CKD and diabetic retinopathy in population with T2DM was investigated (41). Also, this correlation exist in population of type 1 diabetes mellitus patients (42). In meta-analysis, in which 26 papers with 2012 patients were investigated, it was proved that a diabetic retinopathy may be a highly specific indicator for CKD (43). So far researches focused on the relation of CKD and diabetic retinopathy without taking into consideration patients with DF.

The protein binding micro- and macroangiopathy pathogenesis in CKD and DF is OPG. OPG is a protein encoded by a gene located on chromosome 8 and it is a member of TNF family proteins. The main role of OPG is connecting itself to the RANK ligand (RANKL). While RANKL is increasing bone resorption and loss of bone tissue leading to osteoporosis, the OPG has the opposite effect (44). RANKL is a main cytokine playing a role in differentiation, activation and survival of osteoclasts, and regulates the balance between osteoblasts and osteoclasts and thus bone homeostasis (45). OPG is related with calcification of tunica media layer of the arteries leading to stiffness of its wall. This phenotype of vascular calcification, named as arteriosclerosis, occurs typically in patients with diabetic neuropathy. In patients with CKD serum levels of OPG were elevated, but the explanation of this relations is still not clear (46). There is data that smooth muscle cells transform into osteoblast-like cells. In calcified plaques, there were found osteoclast differentiation factors (RANK/RANKL system) (47). Deficiency of OPG concentration in serum increases the risk of osteoporosis and vascular calcification in the aortic and renal arteries in mice (48). Morena *et al*. found that OPG is independently associated with coronary artery calcification in non-dialysis chronic kidney disease patients (49). It was proved that this group of patients is of high risk of cardiovascular death (50). Lis *et al*. proved that serum levels of OPG are elevated in patients with calcific aortic valve stenosis, showing that circulating OPG can influence the processes occurring in the calcifying valves (51).

Hofbauer and Schoppet demonstrated a relationship between *TNFRSF11B* gene polymorphisms and the development of osteoporosis and vascular damage (52). Chung *et al*. in 2015 published a paper showing a functional role of rs2073618 in *TNFRSF11B* gene among 152 other candidate genes in patients with rheumatoid arthritis (RA). They found that this polymorphism is associated with coronary atherosclerosis. Among patients with RA, those with CC genotype of rs2073618 had higher coronary calcium as compared with CG and GG genotypes. This observation documents the role of OPG as a factor combining atherosclerosis and inflammation (53). The object of our study was the population of patients extremely exposed to inflammation and atherosclerosis, who suffered from DF and CKD.

In our previous study polymorphisms of *TNFRSF11B* gene for OPG was analysed in relation to the population of patients with DF (31). Our current study suggests correlation between

Table 3. Frequency of alleles of the selected SNP in patients with CKD (n = 39) compared with controls (n = 62).

Study groups	Genotypes			OR (95% CI) p		
	% n/N	% n/N	% n/N	heterozygous	homozygous	allele carriers
SNP rs6330	CC	CT	TT	Risk allele T		
				CC vs. CT	CC vs. TT	CC vs. CT+TT
CKD	33.33 13/39	46.15 18/39	20.51 8/39	0.76 (0.3-1.92) 0.56	0.75 (0.24-2.31) 0.61	0.76 (0.32-1.8) 0.53
Without CKD	27.42 17/62	50.00 31/62	22.58 14/62			
SNP rs759853	AA	AG	GG	Risk allele G		
				AA vs. AG	AA vs. GG	AA vs. AG+GG
CKD	20.51 8/39	38,46 15/39	41,03 16/39	1.3 (0.45-3.8) 0.63	1.39 (0.48-4.02) 0.54	1.34 (0.51-3.53) 0.54
Without CKD	25.81 16/62	37.10 23/62	37.10 23/62			
SNP rs1553005	CC	CG	GG	Risk allele G		
				CC vs. CG	CC vs. GG	CC vs. CG+GG
CKD	10.26 4/39	38.46 15/39	51.28 20/39	2.32 (0.63-8.53) 0.2	2.32 (0.66-8.18) 0.18	2.32 (0.7-7.72) 0.16
Without CKD	20.97 13/62	33.87 21/62	45.16 28/62			
SNP rs1799983	GG	GT	TT	Risk allele T		
				GG vs. GT	GG vs. TT	GG vs. GT+TT
CKD	48.72 19/39	38.46 15/39	12.82 5/39	0.97 (0.41-2.28) 0.95	2.1 (0.5-8.82) 0.3	1.12 (0.5-2.5) 0.78
Without CKD	51.61 32/62	41.94 26/62	6.45 4/62			
SNP rs1800469	CC	CT	TT	Risk allele T		
				CC vs. CT	CC vs. TT	CC vs. CT+TT
CKD	56.41 22/39	33.33 13/39	10.26 4/39	0.7 (0.3-1.7) 0.44	0.78 (0.2-2.99) 0.72	0.72 (0.32-1.62) 0.43
Without CKD	48.39 30/62	40.32 25/62	11.29 7/62			
SNP rs1801133	CC	CT	TT	Risk allele T		
				CC vs. CT	CC vs. TT	CC vs. CT+TT
CKD	56.41 22/39	38.46 15/39	5.13 2/39	0.59 (0.26-1.37) 0.22	0.39 (0.07-2.15) 0.27	0.56 (0.25-1.25) 0.16
Without CKD	41.94 26/62	48.39 30/62	9.68 6/62			
SNP rs2073618	CC	CG	GG	Risk allele G		
				CC vs. CG	CC vs. GG	CC vs. CG+GG
CKD	23.08 9/39	53.85 21/39	23.08 9/39	1.5 (0.58-3.94) 0.4	1.81 (0.56-5.92) 0.32	1.59 (0.64-3.97) 0.32
Without CKD	32.26 20/62	50.00 31/62	17.74 11/62			
SNP rs3134069	AA	AC	CC	Risk allele C		
				AA vs. AC	AA vs. CC	AA vs. AC+CC
CKD	97.44 38/39	2.56 1/39	0.00 0/39	0.12 (0.02-0.99) 0.021	1.34 (0.03-68.93) 1.0	0.12 (0.02-0.99) 0.022
Without CKD	82.26 51/62	17.74 11/62	0.00 0/62			
SNP rs8192678	AA	GA	GG	Risk allele G		
				AA vs. GA	AA vs. GG	AAvs. GA+GG
CKD	7.69 3/39	43.59 17/39	48.72 19/39	1.35 (0.28-6.47) 0.7	0.88 (0.19-4.09) 0.87	1.05 (0.24-4.68) 0.95
Without CKD	8.06 5/62	33.87 21/62	58.06 36/62			

CI, confidence interval; CKD, chronic kidney disease; N, total number of individuals in a group; OR, odds ratio; S.D., standard deviation; n, number of individuals; SNP, single nucleotide polymorphism

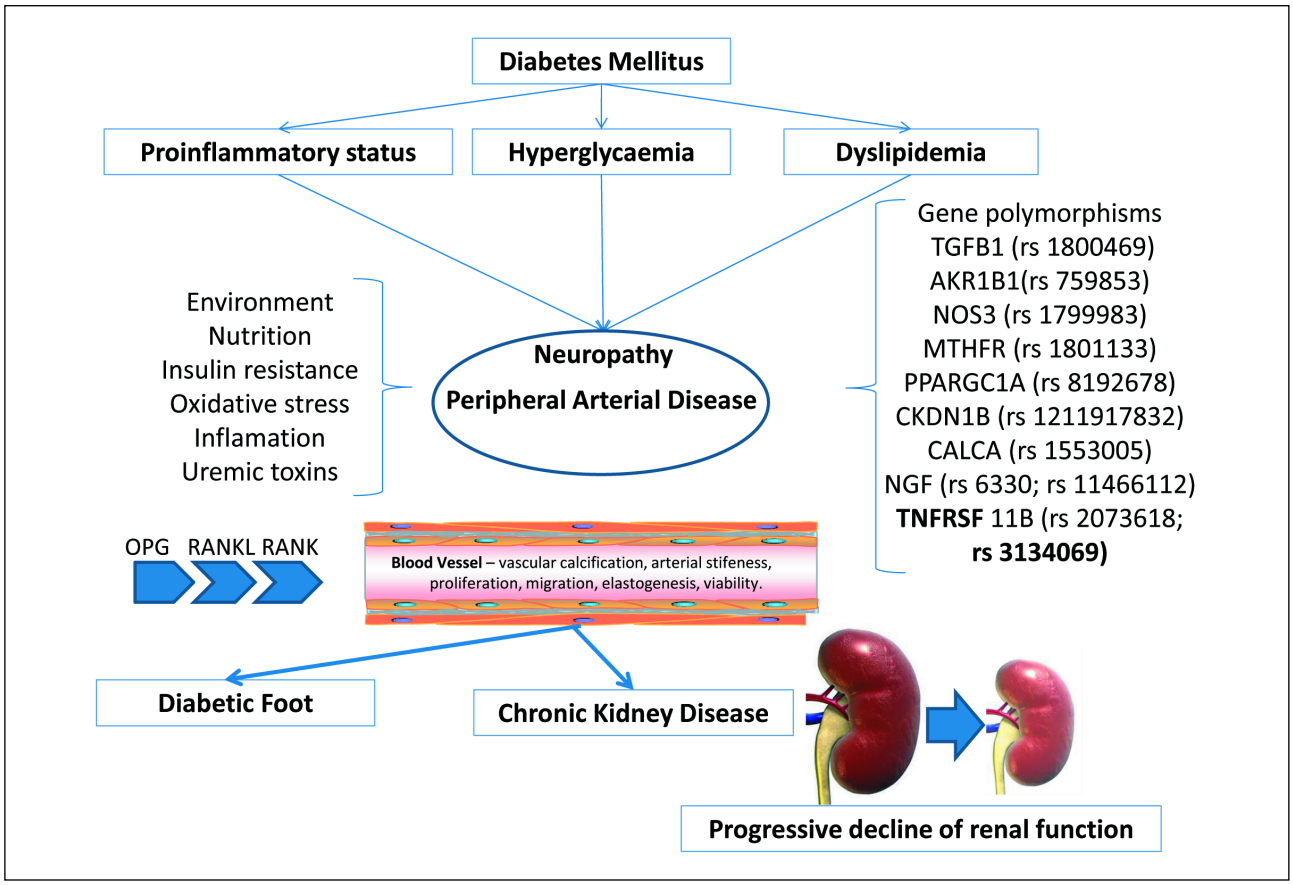

Fig. 1. The possible interactions between selected gene polymorphins and CKD in patients with DF.

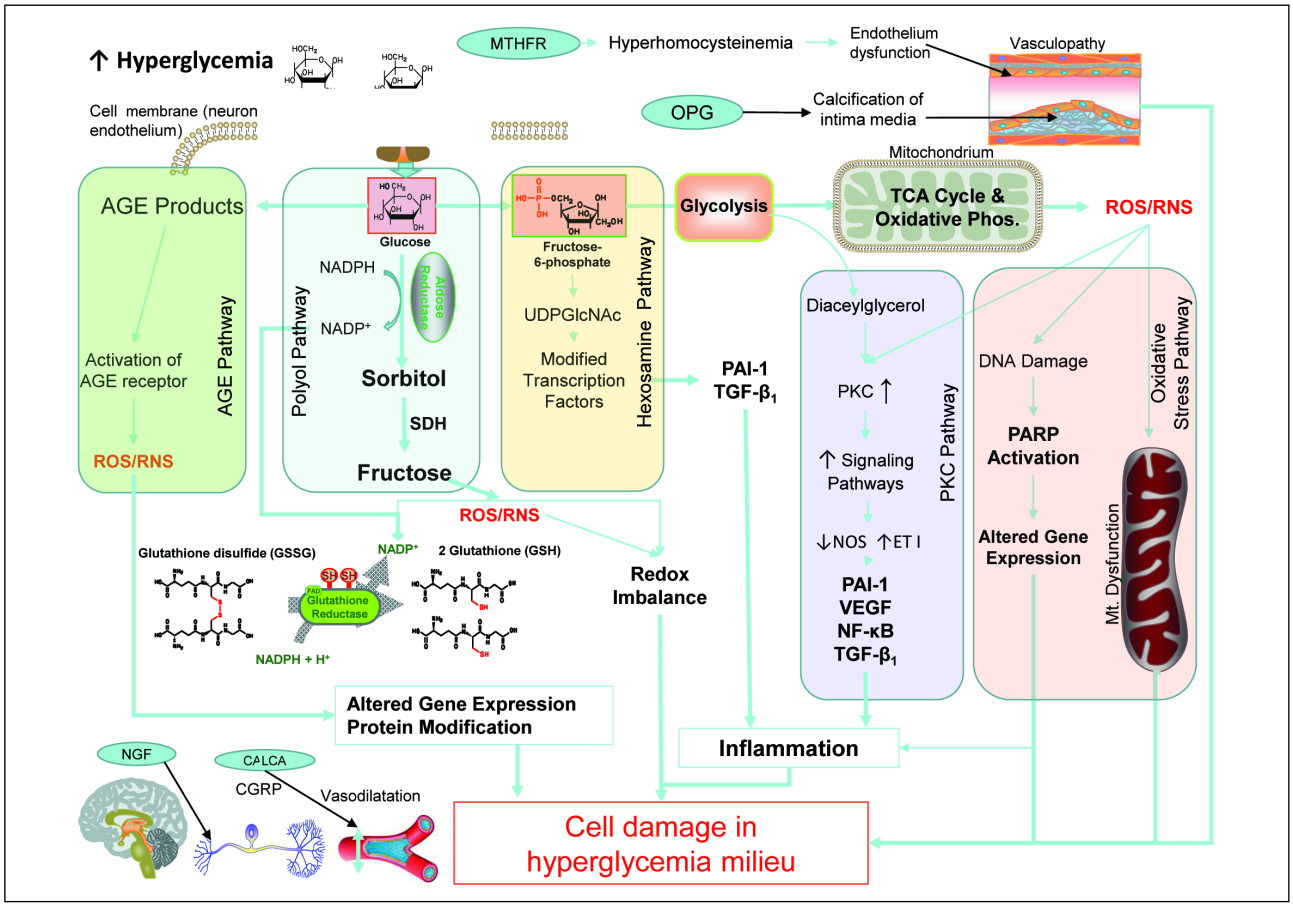

Fig. 2. The pathways describing the roles of studied genes products.

SNP rs3134069 of *TNFRSF11B* gene and CKD in group of patients with T2DM and DF, SNP rs2073618 showed no correlation. Currently, the results of previous studies of OPG genes in DF have been presented in few publications (31, 54, 55). The Italian study with 59 consecutive Caucasian subjects conducted in 2009 showed a significant correlation between rs3134069 and rs2073618 gene polymorphisms and rare T2DM complication called Charcot neuroarthropathy (54). This study suggested a protective role for the alleles C and T of rs2073618 and rs3134069 SNPs. Similar results were obtained in a later study of 54 Charcot's patients from Poland performed by Korzon-Burakowska *et al*. (55). Moreover, Nehring *et al*. proved an association between the rs2073618 polymorphism and all DF types risk. Allele A of the rs2073617 polymorphism had a protective role in diabetic foot in women. Authors did not find a correlation of SNP rs3134069 with any type of DF risk (31). In the presented study we observed that SNP rs3134069 is associated with CKD, which suggests that this polymorphism is more specific for CKD than for DF, while rs2073618 seems to be related to diabetic complications of lower limbs. This observation may explain why not all T2DM patients with the presence of DF develop CKD. Scialla *et al*. found that higher serum OPG levels were associated with lower eGFR and higher aortic pulse wave velocity but not with measures of abnormal bone or mineral metabolism. Authors are wondering if this association result from a role of OPG in the vascular wall or if the observed associations are secondary to the effects of OPG in modulating bone turnover and mass, or if OPG rises in response to vascular injury (56). There is a suggestion that OPG signaling pathway may be involved in the vascular disease associated with CKD independent of adynamic bone disease (chronic kidney disease - mineral and bone disorder). The mechanism might be secondary to the expression and up regulation of endothelial OPG, which is among the tumor necrosis factor alpha (TNF alpha) super family, and act as a proinflammatory molecule and as an inductor of vascular calcification and atherosclerosis in CKD (57). Therefore, the potential link of CKD and DF should be sought in proinflammatory and proatherogenic activity of OPG, which is modified by OPG gene alterations. Some weaknesses of our study lack of measurements of vessel calcification in renal and lower limb arteries, OPG serum levels and bone turnover metabolism.

Our study did not show any correlation between other examined polymorphisms (rs6330, rs759853, rs1553005, rs1799983, rs1801133, rs1800469, rs8192678, rs11466112, rs121917832) and CKD in T2DM population with DF. These results are coherent with other researches.

Dysregulation of TGF-β1 in diabetes was found as leading pathophysiological factor in diabetic and non-diabetic nephropathy, while it was not proved its role of the same significance in diabetic retinopathy or neuropathy. TGF-β1 is responsible for renal fibrosis in both experimental and human kidney diseases. TGF-β1 is highly upregulated in the kidney with severe renal fibrosis (58-60). In diabetes and DFU there were observed elevated TNF and reduced TGF-beta1 levels, what may result from low generation of growth factors by poorly oxygenated tissues (61). McKnight *et al*. investigated the rs1800469 role of *TGF-β1* gene in an Irish type 1 diabetic patients and did not detect significant association between kidney failure and SNP rs1800469 (18). Bazzaz *et al*. found in type1 diabetics some differences in distribution of allele and genotype frequencies of TGF-β1 gene polymorphism in diabetes microvascular complications, but the differences were not statistically significant (62). In this paper, patients with diabetic nephropathy formed the group with the highest frequency of the allele T in the total group of diabetics, which can be explained by the most prominent role of TGF-β1 in the development of DN, but it was still lower than in healthy controls, which is explicable by the pre-selection of the allele C by the preceding diabetes. However, when diabetic subjects were compared with each other according to the presence or absence of DN, the allele distribution showed no significant difference. The lack of association between CKD and non-CKD and DF in rs1800469 in our study could be explained either by relatively low population or comparison of patients with DF among each other. Depletion of growth factors in DF could be under unknown genetical regulatory factors which interfere with *TGF-β1* gene, but this gene-gene interaction (*TGF-β1* gene and *NGF* gene) was not found in our study.

Another influence on vessel wall constriction potentially affecting CKD and DF could appear due to dysfunctions of homocysteine to methionine metabolism. It depends on *MTHFR*, which decreases levels of methionine and glutathion, while higher of homocysteine. Hyperhomocysteinemia leads to abnormal endothelial function with depletion of NO production (63, 64).

The *MTHFR* gene also known as C677T, Ala222Val, and A222V can be represented by CC CT and TT allels configuration. Zhong *et al*. examined 4855 individuals with T2DM and 5242 healthy controls from 15 countries and showed that *MTHFR* SNP rs1801133 was not associated with the risk of T2DM (65). The allele T of SNP rs1801133 was associated with higher mortality risk in patients with ESRD, patients with CKD presented a similar association, but without statistical significance (22). Yigit *et al*. investigated possible association between *MTHFR* gene C677T mutation and diabetic peripheral neuropathy (DPN) in 230 patients with DPN and 282 controls. The distributions of the genotype and allele frequencies of the *MTHFR* gene C677T mutation were statistically different between the patients with DPN and the control group ($P = 0.003$ and $P = 0.002$, odds ratio = 1.59, 95% confidence interval =1.19 – 2.13 (66). The major finding of Yigit's study is the demonstration of an association between the *MTHFR* gene C677T mutation and DPN as well as history of retinopathy. Hyperhomocysteinemia affected nervous function by direct cytotoxic effects or by oxidative damage of endothelial cells, leading to occlusive arteriosclerosis in small vessels. Therefore, macro- and microvascular damage associated with higher hyperhomocysteinemia plasma values could be associated with nerve damage and would explain Yigit's results.

The lack of correlation in our study may be explained by different allele distribution in the group of patients without and with CKD. Our results were 41.94% of CC, 48.39 of CT and 9.68% for TT genotypes, where in Yigit's study the frequency of the CC, CT, and TT genotypes of the C677T mutation in the patients were 53.5%, 37.0%, and 9.5%, respectively, and in the controls, the frequency were 63.8%, 33.0%, and 3.2%, respectively. The negative correlation in our study was also due to fact that we compared results between two groups with diabetes which can be treated as homogenous against the *MTHFR* gene distribution. Aldose reductase (AR) belongs to aldo-keto reductase superfamily and is the first level-limiting enzyme in the polyol pathway. AR catalyses NADPH-dependent reduction of glucose to sorbitol (67). The pathway activation is under hyperglycemia condition and leads to sorbitol accumulation in the cell (68). Sorbitol is a hyperosmotic compound and in the renal glomeruli cells and mesangial cells is found under hyperglycemic condition of streptozotocin-induced diabetic rats (69). C-106 T single nucleotide polymorphism (rs759853) is a mutation of C to T at nucleotide 106 in the promoter of AR gene. Variants in the gene encoding aldose reductase (AKR1B1) and diabetic nephropathy were proved in American Indians. Association analysis of *ADPRT1, AKR1B1, RAGE, GFPT2* and *PAI-1* gene polymorphisms with chronic renal insufficiency were investigated among Asian Indians with T2DM (70). In our study,

we observed no correlation between *AKR1B1* gene SNP rs759853 polymorphism and CKD. Similar conclusions were found by Wolford *et al*. (71). In some studies the association between rs759853 polymorphism in the promoter of aldose reductase gene and risk of diabetic nephropathy were positive (72-76), but other publications find it as a neutral factor (71, 77). Because of controversy around rs759853 polymorphism in AR gene and the risk of DN, a meta-analysis was performed to evaluate the overall evidences (78). The conclusion from this meta-analysis shows that the AR rs759853 polymorphism may correlate with the susceptibility of DN. However, data do not support the association between this DNA variation and the progression of DN.

In diseases where arterioslerosis in underlying as a primary deffect an excess of reactive peroxide, ion was observed in mitochodrial endothelial cells. In those cells biochemical processes leading to diminish synthesis of vasodilating factors and increase of vasocontrictive predominate (hexosamine, poliol, PKC, AGE pathways). Activation of PKC influences on diminishing of NO production and increase of endothelin I, which finally decrease blood flow in microcirculation (79). The results of presented study suggest no association of *NOS3* gene SNP rs1799983 with the CKD, but without evidence from previous studies. Mollsten *et al*. described that tubular dysfunction was common in patients with macroalbuminuria (70% of patients) and the GG genotype of SNP rs1799983 (80). Another study on type 1 diabetes patients showed that SNP rs1799983 GG genotype was a protective factor in normoalbuminuric patients, but not in patients with macroalbuminuria and suggests that the *NOS3* gene may be involved in the development of kidney failure in patients with type 1 diabetes (21). Moreover, Zintzaras *et al*. conducted meta-analysis proving that SNP rs1799983 was associated with kidney failure in T2DM in East Asians population (81). These conclusions are not corresponding to our study. It may be explained by differences in genetic factors between type 1 and type 2 diabetes, and also differences between the East Asians and European populations.

The following molecule, PPARG, is similarly as PKC, related non only to diabetic complications. PPARG, despite its role in muscle fibre determination, is responsible for blood pressure control, body mass control and regulates lipid homeostasis *via* interaction with transcriptional factors, like NFκB leading to insulin resistance (82). Also, *PPARGC1A* is associated with DNA damage in patients with type 2 diabetes and increased risk of cardiovascular diseases. It is well-known that a hyperglycemia leads to increases in superoxide production in mitochondria. Overproduction of superoxide results in mitochondrial DNA damage (83).

PPARG in kidneys affects either podocyte, mesangial cell or endothel cell functions (84, 85). *PPARGC1A* gene SNP rs8192678, occurring in transcriptional pathways related to glucose and lipid metabolism, may be involved in weight regulation and development of hyperglycemia which is a main reason of diabetic complications (86). The meta-analysis conducted by Barroso *et al*., concluded that rs8192678 polymorphism was associated with increased risk development of type 2 diabetes (87). We showed no association of CKD and *PPARGC1A* gene SNP rs8192678. It is in opposition to the Prior *et al*. study, showing that allele A of rs8192678 was associated with kidney failure in 583 European subjects with T2DM (88). Authors observed a significant association between genotype rs8192678 and urinary albumin excretion. The different results in our study may arise due to smaller group of patients enrolled.

Franks *et al*. focused on a relationship between an accumulation of subcutaneous adiposity and an increase of insulin resistance and SNP rs8192678 of *PPARGC1A* gene indicating that this SNP may have an influence on the risk of diabetic complications including kidney failure by enhancing the insulin resistance or hyperlipidaemia (89). Results obtained by Jing *et al*. suggest that the allele A increases the risk of T2DM in the Chinese Han population but not European (90). Lai *et al*. propose that *PPARGC1A* influence on development of T2DM through DNA damage (83).

Our study delivered the first data on *NGF* gene SNPs rs6330 and rs11466112 assessing the potential role in the CKD development among patients with diabetic foot. The correlation for *NGF* and CKD in T2DM and DF patients was not observed in our study. Therefore, we were only presuming that NGF polymorphisms may play role in CKD, there is no proof.

Similarly, there is a lack of previous studies in T2DM population for SNP rs1553005 of *CALCA* gene and SNP rs121917832 of *CDKN1B* gene.

The product of *CALCA* gene is the calcitonin gene-related protein, which is involved in calcium regulation and acts to regulate phosphorus metabolism. This protein have a potent vasodilatory effect on vascular tone, dilates a variety of vessels including the coronary, cerebral and systemic vasculature (91). Association between *CALCA* gene and essential hypertension in Japanese subjects was confirmed (28). Also, *CALCA* has been demonstrated to be a growth factor that may be involved in several key steps of angiogenesis. *CALCA* stimulates proliferation of various cell types, including T lymphocytes, Schwann cells, and tracheal epithelial cells (91-93).

The product of *CDKN1B* gene is a multifunctional protein called p27 which counteracts in apoptosis, cell adhesion, migration and inhibits cyclin-CDK complexes. Retention of *CDKN1B* in the cytoplasm leads to the protein adopting an oncogenic role in the regulation of cytoskeletal dynamics and cell migration (94). One of the earliest structural renal manifestations following the onset of hyperglycemia is renal hypertrophy. It has been reported that high glucose-mediated G1 arrest was due to the up-regulation of the cyclin-dependent kinase inhibitor p27Kip1 (95). High glucose increases p27Kip1 protein stability by activating the mitogen-activated protein (MAP) kinases, extracellular signal-regulated protein kinase, that, in turn, directly phosphorylates p27Kip1 (96). Previously, studies conducted by Wolf *et al*., showed that p27Kip1 is necessary for hypertrophy in cultured mesangial cells exposed to high glucose concentrations (97). This effect was caused by p27Kip1 -mediated cell cycle arrest facilitating enlargement of mesangial cells (98).

Interesting results were found by Iciek *et al*., showing statistically significant difference in homozygous and heterozygous frequency variants of *VEGF* SNPs rs699947 and rs35569394 between pregnant women with T1DM delivering children appropriate and small for gestational age indicting the field for further genetic researches (99).

We did not observe the presence of pathological variants in rs11466112 of *NGF* gene and rs121917832 of *CDKN1B* gene.

In the *Fig. 1*, we demonstrate the possible interactions between selected gene polymorphisms and CKD in patients with DF. In the *Fig. 2* there are demonstrated pathways describing the roles of studied genes products.

Our study has several limitations. The relatively small sample size could yield a false positive result, but the presented study has only preliminary character. In addition, low frequency of some alleles may lead to insufficient statistical power to detect positive associations. A further limitation of this study was the heterogeneity in the DF types foot including neuropathic and ischemic ones.

More studies should be performed on a larger population to confirm the influence of the *OPG* polymorphisms in the CKD development in T2DM and DF population.

Our study suggests the correlation of rs3134069 with CKD. According to our knowledge, the presented study was the first to investigate the impact of various genetic polymorphisms on the development of CKD in T2DM and DF population up to date. Understanding the pathophysiology and molecular mechanisms leading to CKD and DF may help to elaborate new specific methods to identify patient who are more prone to development of CKD. In the future, it may allow to initiate early intensive therapeutic intervention aimed at delaying the development of these complications.

Probably, pathological SNPs are not fully responsible for the development of vascular complications in T2DM patients, but rather in combination with other risk factors. Differences in selected SNPs may contribute to faster progression of CKD in patients with DF and T2DM.

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