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Associations between Genetic Variants in DAB, PRKAG, and DACH Genes and Gender in Chronic Kidney Disease

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Abstract: Background: Recent genome-wide association studies demonstrated the association between the prevalence of chronic kidney disease (CKD) and rs11959928, rs626277, and rs7805747 polymorphisms. Materials and Methods: In this study, we investigated the association between CKD and these polymorphisms in patients and controls according to gender. High-resolution melting analysis was performed to detect DAB2 rs11959928, DACH1 rs626277, and PRKAG2 rs7805747 single nucleotide polymorphisms. Genomic DNA was extracted from the buffy coat of 163 patients with chronic renal disease and 218 control individuals. Ten percent of the results were also randomly confirmed by direct DNA sequencing. Results: Multivariable logistic regression analysis with adjustment for confounders showed rs7805747 (dominant model) has a statistically significant protective effect in females, and rs11959928 (additive and dominant models) was significantly associated with the prevalence of CKD in males. rs7805747 (recessive model) was significantly associated with the prevalence of CKD in males. Conclusion: The very same genetic variants have different effects in males and females separately. Our results warrant the need for similar studies in larger cohorts.

Keywords: chronic kidney disease; genetic polymorphism; high-resolution melting analysis; gender

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Citation: Kecskemétiné, G.; Zsóri, K.S.; Kőmives, S.; Sohajda, M.; Csiki, Z.; Mátyus, J.; Újhelyi, L.; Balla, J.; Nagy, A.; Shemirani, A.H. Associations between Genetic Variants in DAB, PRKAG, and DACH Genes and Gender in Chronic Kidney Disease. *Appl. Sci.* **2023**, *13*, 6633. https://doi.org/10.3390/app13116633

Academic Editor: Manousos Makridakis

Received: 7 May 2023
Revised: 25 May 2023
Accepted: 29 May 2023
Published: 30 May 2023

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1. Introduction

Chronic kidney disease (CKD), with a high morbidity and mortality ratio and considerable financial burden to health care systems, is a multifactorial disease with an important genetic component. As in other complex disorders, identifying the role of genetic variation in CKD progression improves our knowledge about its etiology, treatment, and prognosis. There has been a great interest in investigating the genetic contribution to the variation of kidney function with the aim of improving our understanding of its regulatory processes [1].

In recent years, genome-wide association studies (GWAS) have been used for the identification of common, low-penetrance alleles influencing kidney function. Many of the loci identified by GWAS harbor genes not previously implicated in kidney function and require extensive follow-up studies.

Globally, the prevalence of CKD is higher in women (14.9% vs. 12.3%) [2]. Atherosclerosis Risk in Communities study revealed, as others, higher CKD incidence in women (11 vs. 9.6 per 1000 person-years) [3]. However, men are more likely to acquire end-stage

renal disease, while women have a lower risk of CKD progression and mortality [4]. Polymorphisms associated with gender and CKD have been determined [5].

DAB2 (Dab, mitogen-responsive phosphoprotein, homolog 2) encodes a protein as a tumor suppressor. Genes believed to be causal were found to have a higher expression in the proximal tubule and play a role in endolysosomal function. DAB2, an adaptor protein in the Transforming Growth Factor-β pathway, was identified as a key central node in that region [6]. Chromatin-associated protein encodes by DACH1 (dachshund homolog 1) regulates gene expression. DACH1 is abundantly expressed in podocytes, and its function in dysfunctional podocytes has previously been discussed [7]. It is well known as a tumor suppressor protein whose decreased expression closely correlates with a poor prognosis in a number of malignancies [8]. DACH1 protein regulates the expression of the target gene either by binding to chromatin or by combining with other transcription factors [9,10]. DACH1 DNA-specific binding results in transcriptional repression of the target gene [11]. Lower DACH1 expression was linked to a GWAS risk variant in human renal tubules [12]. PRKAG2 (protein kinase, AMP-activated, gamma 2 non-catalytic subunit) encodes for AMP-activated protein kinase, which regulates cellular energy status and functions. Computational methods demonstrated that PRKAG2 rs7805747 is associated with CKD and serum creatinine. Its expression was found to be different in CKD cases compared with the control group [13].

High-resolution melting (HRM) analysis is shown to be a sensitive and cost-effective diagnostic method. The closed-tube system reduces the risk of contamination. We developed a simple and cost-effective triplex HRM assay to scan for variations in the aforementioned genes. In this study, we investigated the single nucleotide polymorphisms (SNPs), which were found to be associated with the estimated glomerular filtration rate (eGFR) among men and women.

2. Materials and Methods

2.1. Patients

A total of 200 unrelated and consecutive patients were included in the study. Patients with acute kidney disease, acute deterioration of kidney function, or who refused to consent were excluded from the study. A total of 163 patients (68 male, 95 female) were enrolled in the study. Glomerular filtration rate was estimated with the use of the Chronic Kidney Disease Epidemiology Collaboration equation (eGFR) [14]. CKD is categorized according to stages: eGFR between 60 and 80 mL/min/1.73 m^2, eGFR between 30 and 60 mL/min/1.73 m^2, eGFR between 15 and 30 mL/min/1.73 m^2, and eGFR less than 15 mL/min/1.73 m^2 [15]. We included patients with eGFR less than 60 mL/min/1.73 m^2. The control population comprised 218 (89 male, 129 female) individuals recruited from outpatient clinics for annual checkups. Hyperlipidemia was defined when serum triglyceride level was more than 1.7 mmol/L and/or cholesterol level was more than 5.2 mmol/L, and/or the individual was receiving regular administration of lipid-lowering agents. Hypertensive individuals were defined as those subjects having either blood pressure exceeding systolic 140 mmHg and/or diastolic 90 mmHg, and/or receiving antihypertensive treatment. Diabetes mellitus (DM) was defined as a fasting glucose $\geq$ 7 mmol/L (minimum of 8 h fasting), non-fasting glucose $\geq$ 11.1 mmol/L, or treatment for diabetes. Cigarette smoking history was based on self-report. Height and body weight were measured with participants standing without shoes and other heavy garments. Body mass index (BMI) was calculated as weight (kg)/height^2 (m). All laboratory measurements were carried out by technicians unaware of the subjects' history of pathological conditions. The Ethics Committee of the University of Debrecen, Hungary, approved the study (DEOEC RKEB/IKEB 3936-2013). The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. All patients and controls provided written informed consent.

2.2. Biochemical Analyses

Fasting blood was collected at venipuncture in Vacutainer tubes (Dade Behring, Marburg, Germany) for determination of plasma concentrations of total cholesterol, triglyceride, creatinine, and high-sensitivity C-reactive protein (CRP) (AU600, Beckman Coulter, Chaska, MN, USA).

2.3. Genotyping

DNA was extracted with the Genomic DNA Mini Kit (Central European Biosystems). Briefly, genomic DNA was isolated from peripheral blood lymphocytes. This procedure consisted of five steps: red blood cell lysis, cell lysis, DNA binding, washing, and elution.

First, we centrifuged the samples for 10 min at 800× *g*. Then, the buff-colored layer was pipetted, being careful not to disturb the other blood components, and up to 200 µL was transferred into a microcentrifuge tube. We discarded supernatant after 10 min incubation with RBC Lysis Buffer and centrifugation. We repeated the previous step and resuspended the pellet by adding 200 µL RBC Lysis Buffer. For cell lysis step, we added 250 µL of GB Buffer to the tube and incubated it at 70 °C for 30 min. For DNA binding step, we added 250 µL absolute ethanol to the sample and transferred all of the mixture to the GD Column. GD column was then placed in a new Collection Tube after centrifugation. We performed the washing sequentially by adding W1 Buffer, centrifuging, adding Wash Buffer, and centrifuging again. Finally, we added 100 µL preheated Elution Buffer to GD Column and incubated it at 37 °C for 10 min. Samples containing DNA were collected by centrifugation at 16,000× *g* for one minute.

All oligonucleotide primers and probes were obtained from Roche (Mannheim, Germany). We selected three functional SNPs: DAB2 (A/T) rs11959928 (minor allele frequency (MAF): A = 0.33), DACH1 (C/A) rs626277 (MAF: A = 0.354), and PRKAG2 (A/G) rs7805747 (MAF: A = 0.182).

PCR was performed on a LightCycler 480 (Roche) in 20 µL of reaction volume. PCR reactions contained 10 µL 2× master mix including ResoLight high-resolution melting dye (Roche), 175 nM primers (100 nM in case of PRKAG2) (Table 1), 4 mM MgCl2, and 1 µL (≈20 ng/µL) of genomic DNA. All amplifications were initiated with a 5 min hold at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and an extension at 72 °C for 30 s. The melting program included denaturing at 95 °C for 1 min, annealing at 40 °C for 1 min, and subsequent melting that included a continuous fluorescent reading of fluorescence from 60 °C to 90 °C, and fluorescence values were acquired at 0.025 °C intervals. Melting curves were evaluated using Light Cycler 480 software release 1.5.0 SP4 (Roche). Different normalization regions of 71.8–79.5, 79.2–84.6, and 84.1–88.4 were used for analysis of amplicons of DACH1, PRKAG2, and DAB2, respectively. In the case of DAB2, to differentiate between homozygous variants, a known genotype was added to the unknown sample before PCR amplification [16]. Distilled water was used as a blank in each experiment.

Table 1. The genes under study and primers.

Target Gene	NCBI SNP	Primer	Sequence (5′ to 3′)
PRKAG2	rs7805747	Forward	CTAAGAGGGCACCGCTCAC
		Reverse	GACATCTCCATGTGTGTATCTGG
DAB2	rs11959928	Forward	CATGTTGGCTCTGCCAGTAA
		Reverse	ACATGTCCGAGTCCCTTCAC
DACH1	rs626277	Forward	GACTTTTTAAACCAAAGCACCAA
		Reverse	GGTTGAAAGTACTATGAGATCATTACA

2.4. Sequencing

To confirm the results of HRM analysis, 10% of samples were randomly sequenced using ABI Prism 310 DNA sequencer (Applied Biosystems). Primers for real-time PCR were also used for sequencing analysis. A 5-mL aliquot was analyzed on an agarose gel to verify

the specificity of the product, and the remaining material was used for sequencing. All PCR products were purified with Amicon Ultra (Merck Millipore, Schwalbach, Germany) and directly sequenced from both sides using the BigDye terminator V1.1 (Applied Biosystems, Foster City, CA, USA). The obtained sequences were aligned to the expected target sequences manually.

2.5. Statistical Analysis

The characteristics of the patients and controls are presented in Table 2. There was no deviation from Hardy–Weinberg equilibrium. Data with normal distribution were analyzed by Student's T test, and data with deviated distribution by Mann–Whitney U test. Logistic regression analysis was used to include environmental effects such as clinical variables and epidemiological risk factors. For all SNPs with significant p values per genotype (p value < 0.05), the best model was calculated (additive, dominant, or recessive models), and they were analyzed further, adjusting the data for age, CRP, body mass index (BMI), diabetes mellitus (DM), smoking, and hyperlipidemia. Multivariate logistic regression was used to calculate unadjusted and adjusted ORs as well as 95% confidence intervals (CIs). The gene counting method was used for allele frequency estimations. The selection of parameters in the logistic model was based on the method of forward likelihood ratio and clinically significant risk factors of CKD. Genotype associations were analyzed using a dominant model (major-allele homozygotes vs. minor-allele homozygotes plus heterozygotes), a recessive model (minor-allele homozygotes vs. heterozygotes plus major-allele homozygotes) and an additive model (minor-allele homozygotes vs. major-allele homozygotes).

Table 2. Demographic features and baseline clinical characteristics of study population with chronic kidney disease and control individuals.

	Controls			Patients		
	Total	**Female**	**Male**	**Total**	**Female**	**Male**
No. of subjects	218	129	89	163	95	68
Age (years)	49 ± 12.2	52 (42.5–56.5)	46.3 ± 13.3	69 ± 11.4 *	50 ± 11.2 *	69.1 ± 11.7
Hyperlipidemia, n (%)	155 (71.1)	100 (77.5)	56 (62.9)	103 (63.2)	64 (67.4)	42 (61.8)
Smoking, n (%)	67 (30.7)	33 (25.6)	43 (48.3)	19 (9.2) *	5 (5.2) *	14 (20.6) *
Hypertension, n (%)	72 (33)	62 (48)	13 (14.6)	80 (52.1)	54 (56.8)	26 (38.2) *
Diabetes mellitus, n (%)	21 (9.6)	15 (11.6)	10 (11.2)	61 (37.4) *	30 (31.6) *	32 (47) *
hs-CRP (mg/L)	2.2 (1.3–3.4)	2.7 (1.3–5.5)	1.5 (0.7–3.4)	3.6 (1.9–6.9) *	3.5 (1.5–7.6)	3.9 (1.8–8.7)
Creatinine (µmol/L)	72 ± 14.6	65.6 ± 12.5	82.3 ± 11.6	142 (122–163) *	132 (102–172) *	161 (128–213)
eGFR (mL/min/1.73 m^2)	85 (78–91.5)	83 (71.5–93.5)	90 ± 17	36 ± 13.7 *	35 ± 14 *	37.5 ± 13.4
BMI (kg/m^2)	27.2 ± 6.4	27.4 ± 7.1	26.9 ± 5.2	30 ± 6.5 *	30 ± 6.6 *	30 ± 6.2

Data are expressed as mean ± SD or median (inter-quartile range). * $p < 0.05$ for each group of patients compared to its relevant control group. WBC—white blood cell, hs-CRP—high-sensitivity C-reactive protein, eGFR—estimated glomerular filtration rate, and BMI—body mass index.

3. Results

Ten patients had an eGFR of less than 15 mL/min/1.73 m^2. We analyzed our patients according to gender, and Table 3 shows the genotype and allele frequencies of polymorphisms under study in the participants. Among three polymorphisms, allele frequency and homozygosity of rs11959928 were significantly increased in male patients with CKD. rs7805747 heterozygosity was significantly increased in both sexes, and homozygosity was significantly lower among female patients compared to female controls.

Table 3. Prevalence of the genotypes and alleles of the rs11959928 (DAB2), rs626277 (DACH1), and rs7805747 (PRKAG2) polymorphisms related to chronic kidney disease (CKD) in controls and patients.

Gene Symbol (Locus) and Gender	Groups	Wild-Type	Heterozygous	Homozygous	Carrier F	Allele F
DACH1 (rs626277)						
Female, n (%)	Patient	37 (38.9)	45 (47.4)	13 (13.7)	61.4%	30.2%
	Control	48 (37.2)	56 (43.4)	25 (19.4)	62.8%	41.1%
	p value, OR		0.9, 1.0 (0.6–1.9)	0.3, 0.7 (0.3–1.5)	0.8, 0.9 (0.5–1.6)	0.4, 0.9 (0.6–1.3)
Male, n (%)	Patient	27 (39.7)	31 (45.6)	10 (14.7)	60.3%	37.5%
	Control	36 (40.4)	36 (40.4)	17 (19.1)	59.5%	39.3%
	p value, OR		0.7, 1.1 (0.6–2.3)	0.6, 0.8 (0.3–1.98)	0.9, 1.0 (0.5–1.96)	0.5, 0.9 (0.6–1.5)
PRKAG2 (rs7805747)						
Female, n (%)	Patient	43 (45.3)	45 (47.4)	7 (7.4)	54.8%	31.1%
	Control	67 (51.9)	30 (23.3)	32 (24.8)	48.1%	36.4%
	p value, OR		0.006, 2.3 (1.3–4.3) *	0.019, 0.3 (0.1–0.8) *	0.3, 1.3 (0.8–2.2)	0.2, 0.8 (0.5–1.2)
Male, n (%)	Patient	26 (38.2)	29 (42.6)	13 (19.1)	61.7%	40.4%
	Control	49 (55.1)	26 (29.2)	14 (15.7)	44.9%	30.3%
	p value, OR		0.041, 2.1 (1.03–4.3) *	0.22, 1.7 (0.7–4.3)	0.038, 1.98 (1.0–3.8) *	0.06, 1.6 (0.98–2.5)
DAB2 (rs11959928)						
Female, n (%)	Patient	16 (16.8)	48 (50.5)	31 (32.6)	83.1%	57.9%
	Control	30 (23.3)	66 (51.2)	33 (25.6)	76.8%	52.7%
	p value, OR		0.4, 1.4 (0.7–2.8)	0.1, 1.8 (0.8–3.8)	0.2, 1.5 (0.8–2.9)	0.1, 1.3 (0.9–1.96)
Male, n (%)	Patient	10 (14.7)	28 (41.2)	30 (44.1)	85.3%	64.7%
	Control	25 (28.1)	47 (52.8)	17 (19.1)	71.9%	45.5%
	p value, OR		0.37, 1.5 (0.6–3.55)	0.002, 4.4 (1.7–11.3) *	0.049, 2.3 (1.0–5.1) *	0.001, 2.1 (1.3–3.4) *

OR—odds ratio with 95% confidence interval and F—frequency. * $p < 0.05$.

rs7805747 (additive and dominant models) was significantly associated with a lower prevalence of CKD in females, and rs11959928 (all three models) was significantly associated with the prevalence of CKD in males (Table 4). Multivariable logistic regression analysis with adjustment for age, CRP, BMI, the prevalence of diabetes mellitus, and smoking status showed rs7805747 (dominant model) was significantly associated with a lower prevalence of CKD in females, and rs11959928 (additive and dominant models) was significantly associated with the prevalence of CKD in males even after adjustment for confounders. rs7805747 (recessive model) was significantly associated with the prevalence of CKD in males, and multivariable logistic regression analysis with adjustment for confounders did not change its significance (Table 4).

Table 4. Multivariable logistic regression analysis of single nucleotide polymorphisms (SNP) related to chronic kidney disease.

	Gene	SNP	Adjustment [a]	Additive Model	Dominant Model	Recessive Model
Group				***p*-Value, OR (95% CI)**	***p*-Value, OR (95% CI)**	***p*-Value, OR (95% CI)**
Females	DACH1	rs626277	A	0.332, 0.821 (0.552–1.223)	0.264, 0.66 (0.318–1.369)	0.791, 0.929 (0.538–1.603)
			B	0.853, 0.944 (0.515–1.73)	0.837, 1.116 (0.392–3.175)	0.472, 1.344 (0.601–3.004)
	PRKAG2	rs7805747	A	0.019, 0.584 (0.372–0.917) *	0.001, 0.241 (0.101–0.574) *	0.324, 1.307 (0.768–2.224)
			B	0.382, 0.743 (0.381–1.447)	0.039, 0.299 (0.095–0.939) *	0.391, 1.41 (0.644–3.088)
	DAB2	rs11959928	A	0.155, 1.327 (0.899–1.96)	0.249, 1.409 (0.786–2.525)	0.242, 1.496 (0.762–2.938)
			B	0.444, 1.272 (0.687–2.355)	0.539, 1.317 (0.547–3.173)	0.485, 1.417 (0.533–3.765)
Males	DACH1	rs626277	A	0.607, 0.886 (0.557–1.408)	0.471, 0.73 (0.311–1.716)	0.925, 1.031 (0.542–1.965)
			B	0.607, 1.211 (0.584–2.508)	0.927, 1.059 (0.311–3.603)	0.627, 1.262 (0.494–3.224)

Table 4. *Cont.*

Group	Gene	SNP	Adjustment [a]	Additive Model	Dominant Model	Recessive Model
				***p*-Value, OR (95% CI)**	***p*-Value, OR (95% CI)**	***p*-Value, OR (95% CI)**
	PRKAG2	rs7805747	A	0.219, 1.323 (0.847–2.067)	0.578, 1.266 (0.551–2.907)	0.038, 1.979 (1.04–3.765) *
			B	0.211, 1.558 (0.777–3.125)	0.384, 1.758 (0.494–6.264)	0.014, 3.477 (1.287–9.391) *
	DAB2	rs11959928	A	0.002, 2.1 (1.31–3.368) *	0.001, 3.344 (1.639–6.822) *	0.049, 2.266 (1.003–5.118) *
			B	0.04, 2.125 (1.036–4.356) *	0.009, 4.199 (1.437–12.27) *	0.079, 2.8 (0.888–8.828)

SNP—single nucleotide polymorphism. [a] Multivariate logistic regression: A for unadjusted and B for adjusted for age, C-reactive protein, body mass index, diabetes mellitus, and smoking. * $p < 0.05$.

4. Discussion

There are some reports in the literature about the association between rs626277, rs7805747, and rs11959928 genetic variants and CKD. However, the effect of these genetic variants on CKD according to gender has not been revealed. We investigated these three SNPs in female and male patients with CKD. Our association study demonstrated that rs7805747 has a protective effect on females. However, this SNP and rs11959928 increased the risk of CKD in male patients. The present results suggest that some polymorphism affects the development of CKD in contrasting ways in males and females. Complex diseases have multiple genetic and environmental background risk factors. Genome-wide association study with its ability to scan many genetic variations has emerged recently to find such susceptibility variants.

HRM offers a low-cost, sensitive, convenient, and closed-tube method for single-nucleotide discrimination and easy combination with real-time PCR [17]. Moreover, the close-tube approach decreases the risk of contamination.

Megalin plays a crucial role in the process of filtered low molecular weight protein endocytosis in the apical region of renal proximal tubular cells. DAB2, as an intracellular adaptor, regulates megalin trafficking [18]. Our investigation also showed a more than two-fold increase in CKD because of the presence of rs11959928 variation in males. In multiple GWAS, the rs11959928 variation has been linked to CKD [19]. However, neither the Genotype-Tissue Expression (GTEx25) nor the gene expression variation (eQTLs; https://www.eqtlgen.org/, accessed on 1 June 2017) datasets showed any correlation between this variation and changes in gene expression [20]. It is assumed that DAB2 in CKD plays a role in the renal tubule cells and the endolysosomal pathway [6]. They came to the conclusion that tubular fibrosis is caused by higher expressed levels of DAB2 in tubule cells, which is linked to the DAB2 locus in CKD. Reducing DAB2 expression in renal tubules protected mice from CKD, according to functional tests [6]. The degree of CKD was significantly lower in DAB2 knock-out mice compared with wild-type mice with folic acid-induced kidney injury. These studies have found rs11959928 to be associated with a higher CKD risk, but without accounting for sex heterogeneity.

In our study, the rs7805747 dominant model demonstrated more than four times the odds of kidney involvement in males even after adjustment for age, C-reactive protein, body mass index, diabetes mellitus, and smoking. This variant had the opposite effect on females. Köttgen et al. highlighted the role of PRKAG2 in the metabolic functions of the kidney [19]. It has also been mentioned that PRKAG2 may cause enlarged kidneys [21]. With a genome-wide significance level, PRKAG2 was found to be significantly linked with both serum creatinine and CKD [22]. Comprehensive functional analysis using multiple bioinformatics databases reported this variant to be associated with serum creatinine [13]. It has been reported that 5′-AMP-activated protein kinase (AMPK), as a metabolic enzyme, plays a role in regulating energy metabolism in response to cellular stress [23]. The presence

of missense mutations in the regulatory subunit, PRKAG2, causes AMPK activation and, consequently, cardiomyopathy [24].

DACH1, as a transcription factor, has a role in organogenesis, and it is expressed in the kidney [19]. It has been demonstrated that rs626277, rs7805747, and rs11959928 are associated with glomerular filtration rate and incidents of CKD [1]. A recent meta-analysis of these genetic variants did not show a significant association with CKD in East Asian populations [25]. Our study showed no significant effect of this variant.

These are novel findings, and the molecular mechanisms behind these gender-specific associations seem to be unclear. However, previous studies revealed heterogeneous associations of different polymorphisms only in one gender, or manifesting in opposite directions in the two genders. For example, the rs4235308 variant was associated with a higher risk of type 2 diabetes mellitus in females, while it played a protective role in males [26].

There are some limitations to this study. Firstly, the study was designed as a retrospective study. The results were not anticipated to be influenced by the estimator's bias because each variable was evaluated by an investigator who was blinded to the genotyping results. Secondly, a relatively small number of patients allows us to consider the results obtained as preliminary, requiring verification in larger studies.

In conclusion, rs7805747 has a statistically significant protective effect in females. rs11959928 and rs7805747 were significantly associated with the prevalence of CKD in males. Different polymorphisms have variable influences in different population groups and genders. These variables should be clarified in each case.

Author Contributions: Conceptualization, A.H.S. and K.S.Z.; methodology, A.H.S. and G.K.; formal analysis, A.H.S. and G.K.; investigation, Z.C., S.K., M.S., J.M., L.Ú. and A.N.; data curation, A.H.S. and G.K.; writing—original draft preparation, A.H.S., G.K., K.S.Z. and J.B.; writing—review and editing, A.H.S. and G.K.; supervision, J.B. and A.N.; project administration, A.H.S., Z.C. and G.K.; funding acquisition, A.H.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Új Szécsényi Terv Gazdaságfejlesztési Operatív Program (GOP-2.1.1-11/M-2013-2529).

Institutional Review Board Statement: The study was approved by the Debrecen University Ethics Committee.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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