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The Relationship of Uremic Toxin Indoxyl Sulfate and Intestinal Elimination Mechanisms in Hemodialysis Patients

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Keywords
Hemodialysis · Indoxyl sulfate · Uric acid · Intestine · Polymorphism · ABCG2 · ABCC2

Abstract
Introduction: High indoxyl sulfate (IS) concentration is a serious problem for patients with CKD increasing the risk of cardiovascular diseases and CKD progression. Thus, the methods of decreasing the toxin concentrations are highly desired. The study aimed to discover the role of selected intestine-related factors on IS concentration. ***Methods:*** We evaluated the impact of ABCG2 and ABCC2 polymorphisms influencing activity and protein intake by normalized protein catabolic rate. Additionally, we examined the relation of IS and uric acid (UA) that can share common elimination transporters. A monocentric, prospective, open cohort pilot study was performed on 108 patients undergoing dialysis treatment. ***Results:*** The positive effect of residual diuresis on the reduction of IS levels was confirmed ($p = 0.005$). Also, an increase in IS depending on the dietary protein intake was confirmed ($p = 0.040$). No significant correlation between ABC gene polymorphisms was observed either, suggesting the negligible role of ABCG2 and ABCC2 in the elimination of IS in small bowel. The significant difference was observed for UA where ABCG2 421C>A (rs72552713) gene polymorphism was higher (505.3 μmol/L) in comparison with a wild-type genotype (360.5 μmol/L). ***Conclusion:*** No evidence of bowel elimination pathway via ABCC2 and ABCG2 transporters was found in renal replacement therapy patients.

Introduction

Classified as a small molecule, Indoxyl sulfate (IS) is a uremic toxin (M = 213 g/mol) bound to plasma proteins, especially albumin [1]. IS is created by the intestinal microflora from the amino acid tryptophan ingested in the diet [2]. Normally, IS is excreted by the kidneys. However, IS can accumulate in the body in cases of renal impairment, e.g., chronic kidney disease (CKD) [3]. IS retention can promote renal and nonrenal toxic effects causing direct damage to the structure of the kidneys and thus to their physiological function. Further, IS can also promote

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cardiovascular damage, renal and cardiac fibrosis, mineral and bone disorders, immune dysfunction [4–6]. It is difficult to remove IS effectively through hemodialysis because more than 90% of it is protein-bound in serum. Moreover, its elimination through dialysis is only partial [7]. Previous reports suggest that the clearance of IS through dialysis ranges between 25 and 30 mL/min whereas that simultaneous urea clearance exceeds 200 mL/min. The elimination of IS though this method is very low; therefore, patients with renal deficiency possess higher serum IS concentration on average [8]. The serum concentration of IS in healthy individuals is normally very low (1–2.9 μmol/L); however, patients with CKD can show IS values as high as 500 μmol/L according to some authors [9]. Conventional hemodialysis and online hemodiafiltration, using a dialysis bicarbonate solution with the addition of acetate, are equally effective in the removal of IS in a 4-h procedure by about half [10]. Regardless, the removal of IS from CKD patients using the current dialytic strategies is insufficient, resulting in a high incidence of cardiovascular complications that limit the long-term survival of patients on dialysis.

As previously mentioned, the levels of IS in the serum of CKD patients can be affected by several factors, some of which can be related to the digestive tract, i.e., microbiota variety and therein ABC transporter activity (present also in gut) and the amount of undigested protein entering the large intestine. The L-tryptophan found in these residual proteins is converted to indole by gastrointestinal bacteria. Once it reaches the liver, indole is transformed into indoxyl by hydroxylation followed by its sulfatation into IS [11]. Thus, the concentration of IS correlates with dietary protein intake and diet type. The concentration of IS under a vegetarian diet is lower, likely due to the smaller amount of residual protein reaching the colon and the different composition of the intestinal microbiome caused by the diet itself [12]. This protein intake may be estimated using a normalized protein catabolic rate (PCRn), determined by measuring the interdialytic appearance of urea in bodily fluids. Urea is the dominant catabolite of proteins; therefore, PCRn is widely recommended in the evaluation of dietary protein intake and its adequacy according to the needs of the patient in question [13]. A daily protein intake of ~1.2 g/kg body weight is recommended in patients under dialysis because of its increased loss during treatment and higher secondary catabolism [13]. However, limiting protein intake, in an effort to reduce residual protein entering the gut, is difficult due to the risk of malnutrition. Thus, other ways to decrease the IS concentration in plasma of CKD patients are needed.

Recent studies suggest that in addition to its elimination by the kidneys, IS could also be excreted by the small intestine. This observation could have a major impact on patient care should it be confirmed in future studies. Due to the beneficial effects of the end products of bacterial tryptophan metabolism on the gut, the possibility of excretion of IS by the gut should be explored, rather than reducing its production [14]. It is known that the expression of ABC transporters can be stimulated through some orally administered drugs. In this regard, the ABCG2 transporter has been involved with the excretion of IS through the small intestine [15]. This transporter protein is one of 48 members in the ABC group, which are linked with the cellular excretion of a wide variety of compounds [16, 17]. ABCG2 is expressed in the apical membrane of the small intestine and kidney cells [18]. Most interestingly, the gene encoding ABCG2 is highly polymorphic, with more than 80 polymorphisms identified so far. However, only those polymorphisms changing the transporting activity are of interest in this study. In addition to the presumed excretion of IS, the ABCG2 transporter also plays a significant role in the excretion of uric acid (UA) in the gut, i.e., urinary excretion accounts for 2/3 of total UA elimination whereas the remainder is excreted through the faces [19, 20]. Because both IS and UA could be excreted by the same transporter, the association between their concentrations should be inquired. It is known that the downregulated expression and function of ABCG2 can lead to hyperuricemia [20]. According to other reports, IS can inhibit UA secretion by the ABCG2 transporter. To date, however, only in vitro studies have been performed in colorectal adenocarcinoma cell line cells and in animal models [21]. The role of the ABCG2 transporter in the intestinal excretion of IS, and whether it inhibits intestinal UA excretion in CDK patients, is unclear [17, 18].

It must be mentioned that another ABC transporter, ABCC2, could also be involved in IS excretion by the gut [22]. Like ABCG2, ABCC2 is also expressed in major physiological barriers, i.e., the apical membrane of hepatocytes, epithelial cells in the renal tubules, placental syncytiotrophoblast, and the intestinal epithelium [22–24]. Therefore, ABCC2 also promotes the excretion of organic anions conjugated with glutathione, glucuronate, or sulfate; therefore, it would not be farfetched if ABCC2 could also be involved in the excretion of IS [22, 23].

A human colorectal adenocarcinoma cell line, which is morphologically and biochemically very similar to the epithelium of the small intestine, has been widely used

in substance absorption and excretion studies, especially by ABC transporters. Recent in vitro studies [19, 21] on these cells suggest that the SNPs rs72552713 (376C>T), rs2231142 (421C>A) in ABCG2 gene, and rs2273697 (1249G>A) in the ABCC2 gene affect the transporter's activity, ultimately compromising the intestinal excretion of different substances, e.g., IS and UA.

Therefore, unraveling the role of these transporters in the excretion of IS could contribute toward the treatment of dialysis patients by improving its elimination or that of other uremic toxins from the body. This pilot study addresses the effect of polymorphisms ABCG2 and ABCC2 on IS level and whether UA concentration is associated with high IS concentration in patients under dialysis treatment.

Materials and Methods

Patients and Study Design

A monocentric, prospective, open cohort pilot study was performed. The study group included 108 patients undergoing dialysis treatment in the Nephrology Clinic of the University Hospital in Hradec Kralove (Czech Republic). This group consists of two subgroups. To the first one, 26 anuric patients with diuresis equal to or less than 200 mL/24 h were selected. To the second one, 82 oliguric patients with diuresis between 400 and 200 mL/24 h were selected. These patients did not have any digestive tract pathologies or did not use allopurinol.

Blood samples were collected from these patients to determine IS, urea, and UA levels in serum. The amount of urea formed between dialysis sessions was used as a reflection of dietary protein intake and thus to calculate PCRn [13]. Reference values for measured and calculated analytes are shown in Table 1. The blood samples were collected during the patients' monthly checks from an inserted hemodialysis needle before heparin administration. In those patients with a central venous catheter, blood collection was performed after the prior aspiration of 10 mL of blood, i.e., after the complete removal of the catheter anticoagulant plug.

Lastly, the presence of single nucleotide polymorphisms (SNPs) in the *ABCG2* and *ABCC2* genes was also evaluated in the patients as they may, among other things, act as transporters of IS into the gut. These genetic variants could determine the secretion efficacy of IS and thus have an impact upon its concentration in serum. The evaluated SNPs were rs72552713 (376C>T) and rs2231142 (421C>A) in the *ABCG2* gene, and rs2273697 (1249G>A) in the *ABCC2* gene.

Analytical Methods

Biochemical Methods

The predialysis concentration of IS in the patients' serum was determined through ion pair chromatography with electrochemical detection (UltiMate 3000 Series; Thermo Fisher Scientific, Waltham, MA, USA). The blood samples were drawn into tubes containing a gel separator and allowed to coagulate for 30 min, later centrifuging at 2,000 *g* for 10 min [10]. The obtained serum aliquots were stored at −75°C until analysis. Before evaluation, 350 µL of the serum sample were mixed with 350 µL of ultrapure water and 700 µL of 7% perchloric acid to deproteinize the samples and thus release all bound IS into its free form. The samples were mixed in a vortex, incubated at room temperature for 5 min, and centrifuged for 10 min at 10,000 *g* at 4°C [25]. The supernatant was transferred into a glass vial, from which 10 µL were taken and injected into the chromatograph. The analysis was performed on a Kinetex XB-C18 column (100 × 4.6 mm, 5 µL) at a flow rate of 0.3 mL/min. The mobile phase consisted of 85% phosphate buffer (0.025 mol/L, pH 4.2, containing sodium dihydrogen phosphate, sodium 1-octanesulfonate, and ethylenediaminetetraacetic acid) and 15% methanol. The voltage on the detector was set to +400 mV. IS was eluted at 5.5 min in isocratic mode [26]. Urea and UA were determined using an automated analytical system Cobas 8000 (Roche AG, Basel, Switzerland). All samples were processed according to institutional standards.

Table 1. Reference values

	Reference values
IS, µmol/L	<5.4
PCRn,[a] g/kg/day	1.0–1.2
Urea, mmol/L	2.8–8.1
UA – male, µmol/L	202.0–417.0
UA – female, µmol/L	143.0–339.0

[a] Reference values for patients undergoing dialysis treatment.

Molecular Biological Methods

Genomic DNA was isolated from whole non-coagulated blood using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Individual SNPs were analyzed by real-time polymerase chain reaction (qPCR) using allele-specific TaqMan probes (TaqMan Drug Metabolism Genotyping Assay). The qPCR reaction contained 5 µL of 2x TaqMan Genotyping Master mix, 0.5 µL of 20x TaqMan Drug Metabolism Genotyping Assay, 3.5 µL of DNase-free water, and 1 µL of genomic DNA. The qPCRs were performed in a Rotor Gene Q (Qiagen) as follows: 95°C for 10 min, followed by 40 cycles of 95°C/15 s and 60°C/60 s.

Statistical Analysis

Most of the statistical analyses were performed with the SigmaStat software version 3.1 (Systat Software Inc., US). The obtained data are presented as a median (first quartile; third quartile) or as a mean ± standard deviation. The normality of distribution was tested with a Shapiro-Wilk test, and the statistical difference between the groups was evaluated with one-way analysis of variance or with a Mann-Whitney rank-sum test. A p value of ≤0.05 was considered statistically significant. The correlation between the evaluated parameters was evaluated with Pearson's correlation coefficient. IS, PCRn, and UA data are normally distributed. The post hoc power of the study was performed with the post hoc power calculator-ClinCalc (www.https://clincalc.com/stats/Power.aspx) (see Table 2).

Table 2. Measured analytes and calculated values

	N	Median (first quartile; third quartile)	*p* values	Power
IS, µmol/L	108	128.4 (103.0; 184.7)		
PCRn, g/kg/day	108	1,07 (0.93; 1.20)		
IS anuric patients, µmol/L	26	160.4 (122.6; 200.5)	**0.005**	86.9%
IS oliguric patients, µmol/L	82	124.6 (98.7; 168.1)		
Urea before dialysis procedure, mmol/L	108	21.0 (17.1; 23.5)	N/A	N/A
Urea after dialysis procedure, mmol/L	108	5.1 (4.1; 6.5)		
UA, µmol/L	108	367.5 (325.0; 405.5)		
UA, wild type 421C>A (ABCG2)[a], µmol/L	89	360.5 (324.8; 397.0)	**0.003**[a,c]	100.0%
UA, heterozygous g. 421C>A (ABCG2)[b], µmol/L	17	400 (336.8; 454.5)	**0.031**[b,c]	100.0%
UA, µmol/L homozygous g. 421C>A (ABCG2)[c], µmol/L	2	505.3 (493.5; 517.0)	**0.033**[a,b]	49.0%
UA, wild type 1249G>A (ABCC2), µmol/L	60	360.5 (324.7; 418.0)	>0.05	3.7%
UA, heterozygous g. 1249G>A (ABCC2), µmol/L	46	372.0 (329.5; 400.0)		
UA, homozygous g. 1249G>A (ABCC2), µmol/L	2	342.5 (286.0; 399.0)		
IS, wild type 421C>A (ABCG2), µmol/L	89	130.0 (103.0; 187.8)	>0.05	11.4%
IS, µmol/L heterozygous g. 421C>A (ABCG2)	17	123.4 (77.25; 186.25)		
IS, homozygous g. 421C>A (ABCG2), µmol/L	2	142.4 (122.8; 162.0)		
IS, wild type 1249G>A (ABCC2), µmol/L	60	130.0 (101.0; 187.8)	>0.05	2.7%
IS, heterozygous g. 1249G>A (ABCC2), µmol/L	46	126.7 (103.6; 186.8)		
IS, homozygous g. 1249G>A (ABCC2), µmol/L	2	114.8 (90.7; 138.9)		
PCRn, wild type 421C>A (ABCG2), g/kg/day	89	1.07 (0.92; 1.21)	>0.05	5.2%
PCRn, heterozygous g. 421C>A (ABCG2), g/kg/day	17	1.02 (0.91; 1.13)		
PCRn, g/kg/day homozygous g. 421C>A (ABCG2)	2	1.3 (1.10; 1.58)		
PCRn, wild type 1249G>A (ABCC2), g/kg/day	60	0.90 (0.85; 0.94)	>0.05	11.5%
PCRn, heterozygous g. 1249G>A (ABCC2), g/kg/day	46	1.06 (0.89; 1.20)		
PCRn, homozygous g. 1249G>A (ABCC2), g/kg/day	2	1.1 (0.96; 1.21)		

N/A, not applicable; N, number of individuals.

Results

Patient Group

The study group consisted of 108 patients on dialysis treatment (26 anuric patients, 82 oliguric patients), 41 females and 67 males. The mean age of the group was of 67.3 ± 12.3 years. The median of the renal replacement therapy was of 9.8 years (5.2; 13.4), maximum is 33.3 years, 68 patients underwent hemodiafiltration therapy, and 40 were treated by hemodialysis. The mean ideal body weight of these patients was of 78.9 ± 16.5 kg, body mass index 28.8 ± 5.4 kg/m^2, and body surface area 1.92 ± 0.2 m^2. A 34% (n = 37) of the patients in the group were diagnosed with diabetes.

Glycemia 6.3 mmol/L (4.9; 8.3), natremia 138.0 mmol/L (137.0; 140.0), potassium in serum 5.5 mmol/L (5.1; 6.0), kalcemia 2.19 mmol/L (2.12; 2.27), fosfatemia 1.72 mmol/L (1.38; 2.13), and creatininemia 796.0 µmol/L (652.0; 940.0) were also determined in all studied patients. A subgroup of anuric patients included 26 members, 9 females and 17 males. The mean age of the group was of 66.7 ± 10.5 years. Hemodiafiltration therapy underwent 24 patients and 2 patients were treated by hemodialysis. The mean ideal body weight of these patients was of 85.0 ± 18.7 kg, body mass index 30.6 ± 5.2 kg/m^2, and body surface area 1.99 ± 0.2 m^2. A 44% (n = 25) of the patients in this subgroup were diagnosed with diabetes.

A subgroup of oliguric patients consisted of 82 members, 32 females and 50 males. The mean age of the group was of 67.4 ± 13.2 years. Hemodiafiltration therapy underwent 44 patients, and 38 patients were treated by hemodialysis. The mean ideal body weight of these patients was of 77.0 ± 15.4 kg, body mass index 28.2 ± 5.3 kg/m^2, and body surface area 1.90 ± 0.2 m^2. A 31% (n = 12) of the patients in this subgroup were diagnosed with diabetes.

Measured Analytes and Calculated Values

IS Concentration

The IS concentration was 128.4 µmol/L (103.0; 184.7) in the study group. Concentration of IS was 160.4 µmol/L (122.6; 200.5) in anuric subgroup of patients and 124.6

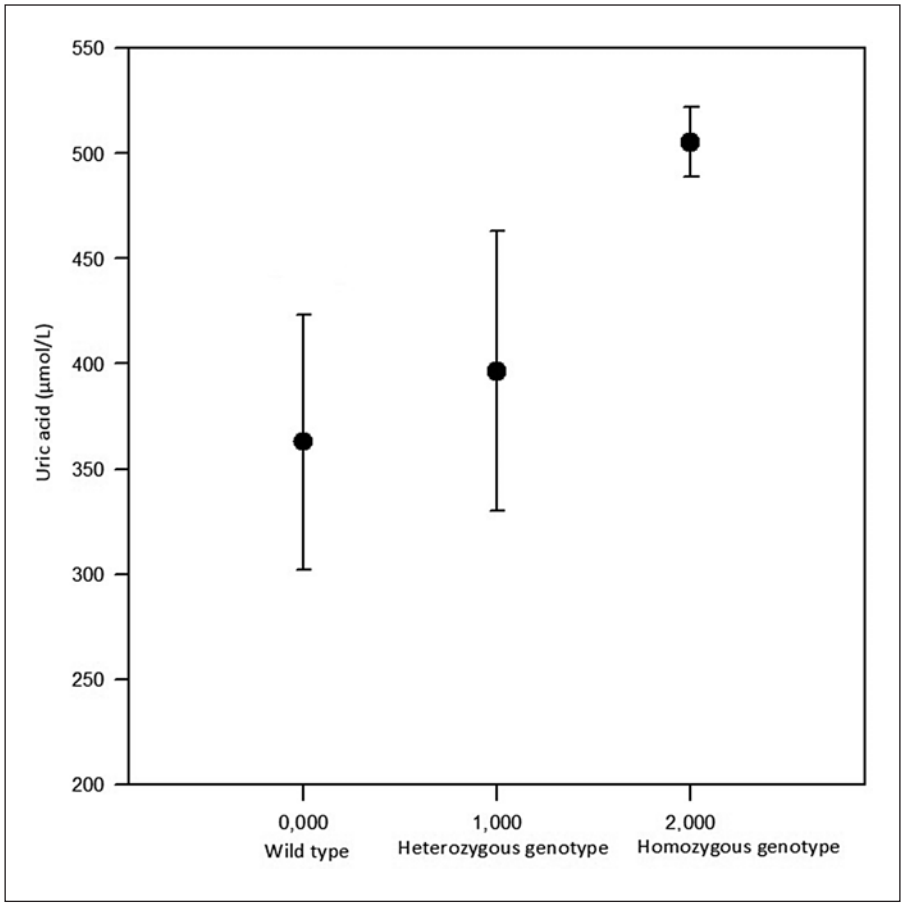

Fig. 1. Comparison of uric acid concentration between ABCG2 421C>A gene polymorphism ($p = 0.003$, $p = 0.031$, $p = 0.033$).

µmol/L (98.7; 168.1) in oliguric subgroup. A statistical difference in IS concentration was demonstrated between anuric and oliguric patients ($p = 0.005$).

Urea Concentration and PCRn

The urea concentration was 5.1 mmol/L (4.1; 6.5) after dialysis, increasing to 21.0 mmol/L (17.1; 23.5) prior to the following dialysis session (shown in Table 1) in the study group. These data were used to determine PCRn 1.07 g/kg/day (0.93; 1.20), finding a significant correlation ($p = 0.040$) between PCRn and IS concentration in the patients' serum. There was no significant difference in urea concentration between anuric and oliguric patients. No statistically significant difference was found for PCRn in the two subgroups either.

ABC Transporter Polymorphisms

The *ABCG2* 421C>A (rs2231142) gene polymorphism was detected in 17% ($n = 19$) of patients, with only two having a homozygous genotype. The rare *ABCG2* 376 C>T (rs72552713) variant was not detected in any of the patients. The 1249 G>A (rs2273697) polymorphism in the *ABCC2* gene was found in 45% ($n = 48$) of the patients, 46 patients having heterozygous genotype, only two having a homozygous genotype. However, no significant correlation was observed between the tested polymorphisms and IS concentration in the whole patient group and in the subgroups too. There is no statistical difference between PCRn patients carrying the above ABCG2 and ABCC2 polymorphisms and standard carriers (see Table 2).

UA Comparison

The amount of UA in patients in ABCG2 was compared. The UA concentration was 360.5 µmol/L (324.8; 397.0) in the patients with wild-type genotype, 400 µmol/L (336.8; 454.5) in the patients with heterozygous genotype, and 505.3 µmol/L (493.5; 517.0) in the patients with homozygous genotype (shown in Fig. 1). There was a significant difference in UA between the patients with a wild-type genotype, heterozygous genotype, and homozygous genotype of ABCG2 ($p = 0.003$, $p = 0.031$, $p = 0.033$). No significant difference could be observed between UA

and IS values in neither ABCG2 gene polymorphism nor in the patients regardless of the genotype. Also, there was no significant difference in UA concentration between anuric and oliguric patients.

Discussion

The present study evaluates the clarification of effects involved in decreasing toxic concentrations of IS in patients treated with renal replacement therapy. Although some mechanisms were described using in vitro studies, they were not confirmed in the group of dialysis patients. A total of 108 patients from the Nephrology Clinic of the University Hospital in Hradec Kralove were included in the study.

The concentration of IS was determined in the studied group of patients and a statistically difference was observed in IS between oliguric and anuric patients. Residual diuresis in oliguric patients is probably the reason for the significantly lower concentration of IS sulfate in oliguric subgroup of patients. Therefore, the strategy of dialysis centers is also to maintain residual diuresis as long as possible so that IS and similar substances can be at least partially removed by tubular secretion.

The amount of urea accumulated between dialysis sessions, as a reflection of dietary protein intake, was determined and used to calculate PCRn. The comparison between PCRn and IS concentration showed a statistically significant correlation between these factors. Based on these results, we could consider that an increased dietary protein intake in these patients is a "double-edged sword," not only because it compensates for protein and amino acid loss during dialysis sessions but also because it contributes to higher IS concentration. The positive correlation between IS and protein intake can be influenced by eating habits, dietary composition, and the microbial flora in the colon [12].

So far from now, the only available method to control IS concentration in dialysis patients is the tight control of dietary protein intake to prevent both its deficit and excess. Other potential way to decrease the IS concentration in plasma of dialysis patients can be the influence on *ABCG2* and *ABCC2* transporters activity. Unfortunately, despite the encouraging observations made in in vitro studies and in animal experiments, no significant differences in IS level in serum were observed between *ABCG2* and *ABCC2* SNPs (shown in Table 1). The ABCG2 transporter allegedly eliminates IS via enteral excretory function [17, 18]. The *ABCG2* 421C>A polymorphism results in decreased intestinal transport activity (previously known as breast cancer resistance protein, BCRP) [18]. However, in spite of significantly higher UA concentration in serum (shown in Table 1) in the patients with that SNP, we could not detect any effect of ABCG2 polymorphism on IS level. The significant difference of UA serum concentration confirms functioning bowel elimination pathway in dialysis patients. The presence of a less functioning allele containing SNP 421C>A results in significantly increased UA concentration and the effect is more profound in homozygosity. The lack of influence of this SNP on IS serum concentration excludes any significant effect of this elimination pathway in renal replacement therapy patients. Similarly, no influence of SNP 1249G>A in ABCC2 allele in IS concentration was found. From a therapeutic standpoint, this is an unfortunate finding as the expression of ABCG2 and ABCC2 can be easily stimulated by several compounds, e.g., glycyrrhizin, a principal compound of licorice extracts [27]. To the best of our knowledge, no similar study evaluating the influence of ABCC2 and ABCG2 polymorphisms on IS concentration has been published yet.

Conclusion

A high IS level in serum has a detrimental effect on the whole organism, often causing increased oxidative stress, vascular toxicity, and risk of cardiovascular complications. Unfortunately, the current dialysis strategies are not able to reduce the concentration of IS in a significant manner. Residual renal function (residual diuresis) is an important factor in reducing IS levels. Therefore, the strategy of dialysis centers is also to maintain residual diuresis as long as possible. The concentration of IS increases proportionally with dietary protein intake as estimated by PCRn analysis. ABCC2 and ABCG2 gene polymorphisms did not affect the IS level in serum, thus other molecular targets of decreasing the toxin concentration in circulation should be discovered. Thus, diet seems to be still the most important way to diminish IS concentration.

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Statement of Ethics

All procedures and examinations were approved by the University Hospital Hradec Kralove Ethics Committee (Ref. Numbers: 201811 S16P, 201910 I71P) according to the Declaration

of Helsinki (June 1964 and later amendments). The patients in the study were properly informed of the methods and aims of the study in verbal and written form and provided written informed consent. This trial is registered with EU PAS Register of Studies EU-PAS38785.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Sylvie Dusilová Sulková, Roman Šafránek, and Radomír Hyšpler conceived and designed the study; Adéla Tomášová drafted the manuscript; Radomír Hyšpler did the critical revision of the manuscript; Adéla Tomášová, Marcela Chmelařová, Ivana Baranová, Helena Párová, Petr Moučka, Zora Nývltová, and Karolína Štochlová performed samples analysis; Radomír Hyšpler, Alena Tichá, and Adéla Tomášová performed statistical analysis; Ladislava Pavlíková provided technical and material support; Vladimír Palička and Zdeněk Zadák provided study supervision.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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