RESEARCH ARTICLE

Uremic Retention Solute Indoxyl Sulfate Level Is Associated with Prolonged QTc Interval in Early CKD Patients

Wei-Hua Tang[1,2], Chao-Ping Wang[3], Fu-Mei Chung[3], Lynn L. H. Huang[4], Teng-Hung Yu[3], Wei-Chin Hung[3], Li-Fen Lu[5], Po-Yuan Chen[6], Ching-Hsing Luo[6], Kun-Tai Lee[1,2], Yau-Jiunn Lee[7], Wen-Ter Lai[1,2]*

1 Graduate Institute of Medicine, Collage of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, 2 Division of Cardiology, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan, 3 Division of Cardiology, Department of Internal Medicine E-Da Hospital, I-Shou University, Kaohsiung, Taiwan, 4 Institute of Biotechnology, National Cheng Kung University, Tainan, Taiwan, 5 Division of Cardiac Surgery, Department of Surgery, E-Da Hospital, I-Shou University, Kaohsiung, Taiwan, 6 Institute of Electric Engineering, National Cheng-Kung University, Tainan, Taiwan, 7 Lee's Endocrinologic Clinic, Pingtung, Taiwan

* kmuhep@yahoo.com.tw

Abstract

Total mortality and sudden cardiac death is highly prevalent in patients with chronic kidney disease (CKD). In CKD patients, the protein-bound uremic retention solute indoxyl sulfate (IS) is independently associated with cardiovascular disease. However, the underlying mechanisms of this association have yet to be elucidated. The relationship between IS and cardiac electrocardiographic parameters was investigated in a prospective observational study among early CKD patients. IS arrhythmogenic effect was evaluated by in vitro cardio-myocyte electrophysiological study and mathematical computer simulation. In a cohort of 100 early CKD patients, patients with corrected QT (QTc) prolongation had higher IS levels. Furthermore, serum IS level was independently associated with prolonged QTc interval. In vitro, the delay rectifier potassium current (IK) was found to be significantly decreased after the treatment of IS in a dose-dependent manner. The modulation of IS to the IK was through the regulation of the major potassium ion channel protein Kv 2.1 phosphorylation. In a computer simulation, the decrease of IK by IS could prolong the action potential duration (APD) and induce early afterdepolarization, which is known to be a trigger mechanism of lethal ventricular arrhythmias. In conclusion, serum IS level is independently associated with the prolonged QTc interval in early CKD patients. IS down-regulated I_K channel protein phosphorylation and the I_K current activity that in turn increased the cardiomyocyte APD and QTc interval in vitro and in the computer ORd model. These findings suggest that IS may play a role in the development of arrhythmogenesis in CKD patients.

Introduction

The importance of cardiovascular diseases (CVD) and cardiac arrhythmias in chronic kidney disease (CKD) is currently well established. Abnormal corrected QT (QTc) prolongation on the electrocardiogram, which is an independent risk factor for sudden cardiac death (SCD), is frequently found in patients with CKD and end-stage renal disease (ESRD) [1,2]. However, traditional cardiovascular risk factors are insufficient to explain and accurately predict QTc prolongation and SCD in this population. As the renal function deteriorates, levels of uremic retention toxins and proinflammatory cytokines increase [3]. Various inflammatory mediators, such as C-reactive protein (CRP), IL-6 and platelet-activating factor, have been associated with arrhythmias through the modulation of ion channel function [4,5]. In CKD patients, the protein bound uremic retention solutes, such as Indoxyl sulfate (IS), is associated with the elevation of inflammatory mediators [6,7], elevation of serum IL-6 [8], anti-oxidant modulation [6,7] and monocyte-mediated inflammation [9]. Our previous studies have also demonstrated that increased serum IS may be a possible risk factor in the pathogenesis of coronary atherosclerosis in CKD patients [10,11]. However, the association between IS levels and the cardiac arrhythmias risk indicator remains was still unknown. In the present study, we thus examined the relationship of serum IS levels with electrocardiographic parameters in a cohort of early CKD patients.

The delayed rectifier potassium current (I_K) plays an important role in the repolarization of cardiac action potentials and is one of the major currents that determines the action potentials duration and QT interval [12,13]. The potassium channel trafficking and regulation are involved in certain inherited or acquired cardiac channelopathies, such as long QT syndrome and heart failure [14]. For these reasons, the IS effect on cardiomyocyte electrophysiology was explored in vitro and in a mathematical computer simulation model in the present report.

Materials and Methods

Participants

The study investigated 100 consecutive CKD patients who underwent cardiac multi-slice CT or coronary angiography for exclusion of CAD due to typical and atypical chest pain, with intermediate pretest indications for CAD, from June 2006 to June 2008 at the E-Da Hospital. Before the examination, all of the each patients received a detailed interview covering their medical and personal histories. Patients with previous diagnoses of cerebrovascular diseases, heart failure, cardiomyopathy, coronary heart diseases, congenital heart disease, cardiac arrhythmias or taking medication that had a QT prolongation effect [15] were excluded from this study. Type 2 diabetes (T2DM) was defined as a past or current diagnosis of T2DM and/or the need for medical therapy. Hypertension was defined as a systolic blood pressure (SBP) $\geq$ 140 mmHg, a diastolic blood pressure (DBP) $\geq$ 90 mmHg, or if the patient was under antihypertensive treatment. Dyslipidemia was defined according to the criteria of Adult Treatment Panel III, or if the patient was under lipid lowering treatment. Former and current smokers were analyzed as one group, and compared with those who had never smoked.

Estimated glomerular filtration rate (eGFR) had been calculated and followed up according to the extended Modification of Diet in Renal Disease Study formula 3–6 months before the study [16]. Written informed consent was obtained before the enrollment. This study was approved by the Human Research Ethics Committee of I-Shou University E-Da Hospital.

Laboratory measurement

Peripheral blood samples were taken after fasting for 8 hours overnight and before the examination. Complete blood counts and serum creatinine, sodium, potassium, calcium, uric acid,

albumin, glucose and lipid profiles (included plasma triglycerides, total cholesterol, LDL-C, HDL-C) were determined as our previous reports [17,18].

To determine the blood total IS, A UPLC assay was used as our previously reported [10,17,18]. In brief, the blood for total IS determination was drawn, centrifuged, and stored at-80°C for subsequent assay immediately after blood sampling. The serum samples were deproteinized by the addition of 3 parts methanol to 1 part serum for determination of IS. A UPLC assay, using detection at the 280 nm of the PDA detector, was performed at room temperature on an ACQULITY UPLC BEH phenyl column of 2.1×100 mm. Quantitative results were obtained and calculated as concentrations (μmol/L). The sensitivity of this assay was 1.061 μmol/L for IS.

ECG, QT and QTc interval measurement

Twelve-lead ECGs were collected at the baseline examination by standardized protocol. Standard interval (heart rate, PR, QRS, QT intervals), amplitudes (R, S, and T waves and J and ST segment) were analyzed by standard protocol as described elsewhere [19]. The QT and QTc interval were calculated by the post-processing of ECG signals using the superimposed median beat method and Bazett's formula ($QTc = QT/\sqrt{RR}$). QRS interval with a bundle branch block pattern, duration longer than 120 ms, extremely rapid (> 150 beats per minute) or extremely slow heart rate (< 40 beats per minute) ECG were excluded from this study [20,21]. The definition of prolonged QTc interval in our study was according to the 2009 AHA/ACCF/HRS recommendations. The adjusted QT of 460 ms or longer in women and 450 ms or longer in men was considered a prolonged QT interval [22].

H9c2 cell culture and IS treatment

Embryonic rat heart-derived cardiac H9c2 cells (BCRC 60096, Bioresource Collection and Research Center, Taiwan) were cultured in DMEM supplemented with 10% fetal bovine serum under an atmosphere of 95% air 5% CO_2 at 37°C. Experiments were carried out using mononucleated myoblasts culture for 2–5 days [23]. All culture media were controlled to pH 7.4 before use. The cells were incubated 48 hours with IS (purchased from Sigma) at a concentration of 0.1μM, 1μM and 300μM, as previously described [24], before the experiment. In addition, we did not investigate the effect of albumin and potassium in the present study for the following reasons. Meijers et al. revealed that the presence of albumin, even in abnormal high concentration, still did not significantly affect the protein-bound uremic toxin biological effect [25]. Further, in this study because p-cresyl sulfate was synthesized as a potassium salt, cells exposed to culture medium supplemented with equimolar concentrations of potassium chloride were used as controls. We found that the addition of 1.0 mmol/L of potassium chloride did not alter the number of EMPs compared with growth medium alone [25].

Western blot analysis

The protein level of Kv2.1 and phosphorylated Kv2.1 in H9c2 cells was analyzed by Western blot [26,27]. In brief, after the total protein content was extracted, separated and transferred to Immobilon PVDF membranes (Millipore Corp., California, USA), rabbit polyclonal antibodies for Kv 2.1 (Millipore Corp., California, USA) or rabbit polyclonal antibodies for phosphorylated-Ser805 Kv2.1 (Sigma, USA) were added and incubated at room temperature. A secondary antibody (anti-rabbit, Millipore Corp., California, USA) conjugated with horseradish peroxidase was added, and the antigen-antibody complexes were detected by enhanced chemiluminescence (Millipore Corp., California, USA). Densitometric analysis was conducted using LabWorks 4.5 Image Acquisition and Analysis software (Ultra-Violet Products Ltd., UK).

Patch-clamp cell electrophysiological studies

The whole-cell potassium outward currents were recorded using an Axopatch 700A amplifier (Axon Instruments, Union City, CA, USA). The details of the methods has been described in previous reports [23,28,29]. Briefly, H9c2 cells were placed in a recording dish and perfused with a bath solution and the cells were voltage clamped. Step-pulse protocols and data acquisition were performed using pCLAMP software (Axon Instruments). Membrane capacitance was calculated from the peak amplitudes and time constant decay of capacity transients elicited by 10 mV, hyperpolarizing voltage pulses from holding potential of -50 mV. All electrical recordings were performed at room temperature.

Mathematical computer model for cardiomyocyte action potential and pseudo-ECG

The latest mathematical model of the O'Hara-Rudy dynamic human ventricular model (ORd model) was used in our experiment [30]. Cardiomyocyte action potential was mathematically constructed to include ionic currents, ionic pumps, ionic exchangers, and intracellular ionic regulation processes of Na^+, K^+ and Ca^{2+}.

A Markov model for I_K is derived from previously published K channel models [31]. The current traces figures and data of I_K in H9c2 cells with and without IS treatment was digitized and formulated into a new Markov model computer equation and inserted into the ORd model to evaluate the IS effect on human cardiomyocyte action potential.

A pseudo-ECG was constructed and simulated by the transmural wedge model [32]. The numerical method of forward Euler with the Rush and Larsen method was used to compute pseudo-ECG with an integration time-step size (0.005ms) [33].

Statistical analysis

Data normality was analyzed using the Kolmogorov-Smirnov test. Continuous, normally distributed variables are presented as mean ± SD, and non-normally distributed variables as median (interquartile range). Statistical differences in variables were compared using the Wilcoxon rank-sum test. Categorical variables were recorded as frequencies and/or percentages, and inter-group comparisons were analyzed with the Fisher's exact test. The general linear modeling function analysis was used to control for potential confounders other than age and sex. Simple linear regression analysis was used to examine the association and independence between serum IS and the values of other parameters. Using multiple logistic regression, variables were assessed for independent associations with the prolonged QTc interval. Multivariate adjusted ORs are presented with 95% confidence interval (CI). All the tests were two-tailed, and a p value of < 0.05 was considered statistically significant. All of the statistical analyses were performed using SAS statistical software, version 8.2 (SAS Institute Inc., Cary, NC, USA).

Results

Clinical characteristics

The general demographics of the participants are summarized in Table 1. All the participants were with stage 2 or 3 CKD. The average eGFR was 63.0 ± 14.6 ml/min/1.73m^2 (Table 1). All the patients in our studies were found with normal coronary angiogram over the cardiac multislice CT or coronary angiography. Fifty-six percent of our patients were male. The mean age of our patients is 61 ± 10 years and the average body mass index of our patients was 25.2 ± 3.6 kg/m^2. Sixty-five percent of the patients had hypertension, 22% had diabetes, and 53% were diagnosed with hyperlipidemia, and 32% admitted to the use of tobacco.

Table 1. Patient demographics of 100 chronic kidney disease patients.

Age (years)	61 (54–69)
Men/Women (n, %)	56/44 (56/44)
Hypertension (% yes/no)	65/35
Diabetes mellitus (% yes/no)	22/78
Hyperlipidemia (% yes/no)	53/47
Current smoking (% yes/no)	32/68
Body mass index (kg/m^2)	25.2 ± 3.6
Systolic blood pressure (mmHg)	129 ± 19
Diastolic blood pressure (mmHg)	75 ± 11
Fasting sugar (mg/dl)	111.2 ± 37.3
Total cholesterol (mg/dl)	181.0 ± 41.6
Triglyceride (mg/dl)	154.7 (77.5–173.5)
HDL-cholesterol (mg/dl)	44.8 ± 12.9
LDL-cholesterol (mg/dl)	106.1 ± 37.2
Hematocrit (%)	40.3 ± 4.6
Creatinine (mg/dl)	1.4 ± 0.9
GFR-MDRDGFR-E (ml/min/1.73m^2)	63.0 ± 14.6
Albumin (mg/dl)	4.1 ± 0.3
Indoxyl sulfate (µmol/L)	6.1 (0.9–6.1)
Hs-CRP (mg/L)	4.8 (0.8–4.0)
Electrocardiographic parameters	
Rate (bpm)	73.0 ± 16.3
PR interval (ms)	161.5 ± 24.2
QRS duration (ms)	94.0 ± 17.3
QT interval (ms)	401.8 ± 44.6
QTc interval (ms)	436.7 ± 40.4

Values expressed as number (percent), mean ±SD, or median (25th to 75th percentile), as appropriate. Bpm: beats per minute, HDL: high-density lipoprotein, LDL: low-density lipoprotein, Hs-CRP: high-sensitivity C-reactive protein.

doi:10.1371/journal.pone.0119545.t001

The median serum IS level was 6.1 µmol/L and the median of high sensitive CRP was 4.8 mg/L. The average heart rate was 73.0 ± 16.3 bpm, PR interval 161.5 ± 24.2ms, QRS duration 94.0 ± 17.3ms, QT interval 401.8 ± 44.6ms and QTc interval 436.7 ± 40.4ms.

Association between serum IS and patients' clinical characteristics

A univariate analysis was performed to test the association between the clinical and biochemical variables with log-transformed serum IS levels (Table 2). Serum IS was found positively associated with the age, creatinine, QRS duration, QT and QTc interval. In contrast, serum IS concentration was inversely associated to hematocrit, albumin, and eGFR.

Patient clinical laboratory data stratified by QTc status

The patients were divided into the normal QTc and prolonged QTc groups according to the criteria of AHA/ACCF/HRS [22] to investigate whether biological factors affect QTc (Table 3). Of the 100 patients in our study, 26 patients had prolonged QTc and had significantly higher serum IS levels but lower HDL levels compared to those of the normal QTc patients even after adjusting for age and sex.

Table 2. Clinical and biochemical variables associated in univariate analysis with log indoxyl sulfate.

Variable	Unit	β Coefficient (confidence interval)	p value
Age	year	0.329 (0.006 to 0.023)	0.001
Sex	Men v women	0.115 (-0.076 to 0.286)	0.253
Hypertension	Yes (65) v no (35)	0.057 (-0.135 to 0.243)	0.573
Diabetes mellitus	Yes (22) v no (78)	0.182 (-0.017 to 0.412)	0.070
Hyperlipidemia	Yes (53) v no (47)	0.017 (-0.166 to 0.196)	0.868
Current smoking	Yes (32) v no (67)	0.059 (-0.136 to 0.249)	0.561
Body mass index	kg/m^2	-0.124 (-0.041 to 0.010)	0.218
Systolic blood pressure	mmHg	0.048 (-0.004 to 0.006)	0.633
Diastolic blood pressure	mmHg	-0.171 (-0.016 to 0.001)	0.088
Fasting sugar	mg/dl	0.031 (-0.002 to 0.003)	0.770
Total cholesterol	mg/dl	-0.160 (-0.004 to 0.000)	0.116
Triglyceride	mg/dl	0.037 (-0.269 to 0.391)	0.714
HDL-cholesterol	mg/dl	-0.070 (-0.009 to 0.005)	0.500
LDL-cholesterol	mg/dl	-0.201 (-0.005 to 0.000)	0.051
Hematocrit	%	-0.237 (-0.045 to -0.003)	0.025
Creatinine	mg/dl	0.524 (0.954 to 1.878)	<0.001
Estimated GFR	ml/min/1.73m^2	-0.521 (-0.021 to -0.009)	<0.001
Albumin	mg/dl	-0.273 (-0.629 to -0.050)	0.022
Hs-CRP	mg/L	-0.067 (-0.018 to 0.010)	0.580
ECG rate	bpm	0.102 (-0.003 to 0.008)	0.310
PR interval	ms	0.047 (-0.003 to 0.005)	0.656
QRS duration	ms	0.351 (0.004 to 0.014)	<0.001
QT interval	ms	0.237 (0.000 to 0.004)	0.018
QTc interval	ms	0.336 (0.002–0.006)	0.001

HDL: high-density lipoprotein, LDL: low-density lipoprotein.

doi:10.1371/journal.pone.0119545.t002

Association between IS and prolonged QTc interval

Multivariate logistic regression analysis was performed to estimate the effects of serum IS level together with several other parameters in the presence of prolonged QTc interval. The presence of prolonged QTc interval was associated with age, sex, and serum IS level (Table 4).

The effect of IS on H9c2 ventricular cardiomyocyte potassium outward current

To evaluate the effect of IS on H9c2 cell, in the beginning, we tested the acute effect of IS on the delayed rectifier potassium current in cardiac H9c2 cells with an IS treatment of 16 hours over night. However, there was no significant change in the potassium current in the treated group compared to that of the controls. As a result, we prolong the duration of treatment to 48 hours and founded that the potassium current was significantly decreased in the IS treated group compared to that of the controls. To our knowledge, there are few reports investigating IS effect on cardiomyocytes, though Lekawanvijit et al. found that IS induced cardiomyocyte hypertrophic change after 48 hours of IS treatment [24]. For this reason, we designed the condition of cell incubation for 48 hours.

The results of the patch-clamp cell electrophysiological study revealed that the I_K was significantly decreased after treatment with IS for 48 hours (Fig 1A). The average relationships between I_K and membrane potential calculated from the measured peak current amplitudes

Table 3. Patient clinical laboratory data according to QTc classification.

	QTc <450 ms in women / <460 ms in men	QTc ≥450 ms in women / ≥460 ms in men	p-value
No	74	26	
Age (years)	59.0 ± 9.0	66.4 ± 11.4	0.002
Sex (male/female)	44/30	12/14	0.260
Current smoking (n, %)	23 (31.1)	9 (34.6)	0.805
BMI (kg/m^2)	25.0 ± 3.2	25.7 ± 4.3	0.437
Systolic BP (mmHg)	128 ± 14	130 ± 31	0.620
Diastolic BP (mmHg)	76 ± 10	73 ± 13	0.341
Fasting glucose (mg/dl)	105.8 ± 23.9	126.8 ± 59.4	0.300
Total cholesterol (mg/dl)	184.3 ± 36.5	170.8 ± 54.0	0.071
Triglyceride (mg/dl)	103.5 (74.5–173.5)	112.0 (81.8–176.5)	0.506
HDL-cholesterol (mg/dl)	46.1 ± 12.1	40.5 ± 14.6	0.007
LDL-cholesterol (mg/dl)	107.6 ± 31.5	101.3 ± 51.8	0.216
NA (mEq/L)	139.9 ± 2.5	139.0 ± 4.0	0.190
K (mEq/L)	4.0 ± 0.8	4.0 ± 0.6	0.682
Calcium (mg/dl)	8.5 ± 1.1	8.8 ± 0.7	0.184
Hematocrit (%)	40.6 ± 4.6	39.5 ± 4.5	0.293
Creatinine (mg/dl)	1.1 (1.0–1.2)	1.1 (1.0–1.7)	0.157
Albumin (mg/dl)	4.1 ± 0.3	4.0 ± 0.4	0.218
Estimated GFR (ml/min/1.73m^2)	64.6 ± 12.2	57.8 ± 20.1	0.343
Indoxyl sulfate (μmol/L)	2.8 (0.9–5.2)	6.1 (0.9–11.3)	0.019

Data are expressed as mean ± SD, number (%), or median (interquartile range). BMI, body mass index; BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

doi:10.1371/journal.pone.0119545.t003

Table 4. Multiple logistic regression analysis with presence of prolonged QTc interval as the dependent variable.

	exp(B)	95% Confidence Interval	p-value
Age	4.94	1.04–2.44	0.045
Sex	0.05	0.01–0.59	0.017
BMI	1.12	0.91–1.38	0.285
Systolic BP	1.02	0.98–1.06	0.424
Diastolic BP	0.97	0.91–1.05	0.458
Fasting glucose	1.02	0.99–1.04	0.087
Total cholesterol	1.02	0.95–1.09	0.547
Triglyceride	0.99	0.98–1.01	0.312
HDL-cholesterol	0.95	0.86–1.04	0.270
LDL-cholesterol	0.98	0.92–1.05	0.548
Smoking	9.19	0.91–10.16	0.061
Na	0.89	0.66–1.19	0.428
K	0.55	0.18–1.66	0.285
Calcium	1.50	0.63–3.56	0.358
Indoxyl sulfate	7.35	1.12–4.48	0.037

BMI, body mass index; BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
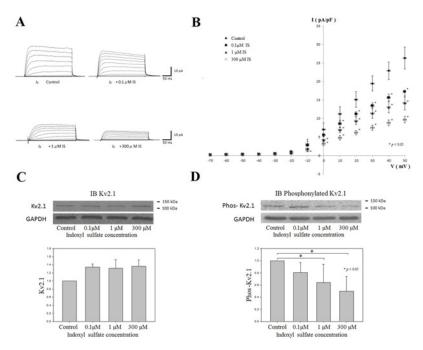
doi:10.1371/journal.pone.0119545.t004

Fig 1. The effect of IS on H9c2 cardiomyocyte I_K and potassium channel protein Kv2.1 expression. (A) The representative current traces for delayed rectifier potassium outward currents (I_k) in H9c2 cells with different indoxyl sulfate (IS) concentration treatment. I_k were elicited by 300 ms depolarizing step pulses from -70 to 50 mV at a holding potential of -60 mV. (B) The average relationships between I_k (pA/pF) and membrane potential in the control, 0.1μM IS, 1 μM IS and 300μM IS groups (n = 6 for each groups) comparing the IS treated group with the control group, I_k was significantly decreased at membrane potentials from 0 mV to 50 mV in a dose-dependent manner. (C and D) The expression of Kv2.1 and phosphorylated Kv2.1 by Western blot in the H9c2 cells treated with different concentration of IS (0.1μM, 1 μM and 300μM). The expressions of Kv2.1 were not significantly different among the control and IS-treated groups (C). However, the phosphorylated Kv2.1 was significantly decreased in the 1 μM IS-and 300 μM-IS treated groups (D). (n = 6 for each groups) *: $p<0.05$ as compared with the control group.

doi:10.1371/journal.pone.0119545.g001

showed that I_K was significantly decreased at the membrane potentials from 0 mV to 50 mV in a dose-dependent manner (Fig 1B).

Kv2.1 Western Blot Analysis with and without IS treatment in H9c2 cardiomyocyte

Potassium ion channel protein Kv2.1 is the major subunit protein comprised of ion channels that generate I_K in H9c2 cell [23]. Western Blot analysis revealed that there was an increase in Kv2.1 level after the treatment of IS. However, there were no significant differences noted between the groups (Fig 1C). In contrast, the expression of phosphorylated Kv2.1 was found to be significantly decreased in the IS-treated groups (Fig 1D).

Mathematical computer simulation of IS effect on human cardiomyocyte electrophysiology

From the computer calculations and simulation results, the decrease of I_K caused by the increase of treated IS concentration will gradually prolong the constructed action potential duration (APD) and pseudo ECG QT interval (Fig 2). In addition, early afterdepolarization (EAD) was noted in the higher suppression of I_K simulation, which mimicked the high IS effect on the
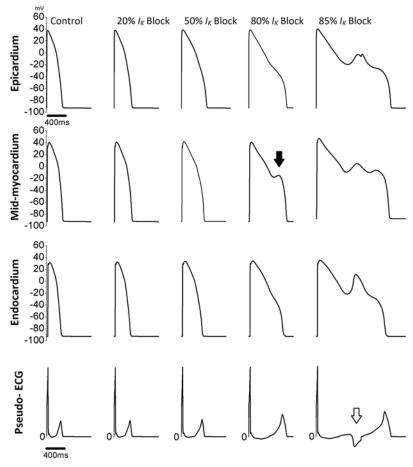
Fig 2. Ventricular cardiomyocyte action potential (AP) and pseudo-ECG constructed by the O'Hara-Rudy dynamic human ventricular model. The suppression of inward rectifier potassium current (I_K) mimics the effect of indoxyl sulfate toxicity to ventricular cardiomyocyte AP. The AP duration was gradually increased and the QT interval was also prolonged with the increment of I_K suppression. The early afterdepolarization was noted in the higher suppression of I_K especially in the mid-myocardial cardiomyocyte (arrow). The ventricular arrhythmias like ECG was also noted when the I_K was severely suppressed (empty arrow).

doi:10.1371/journal.pone.0119545.g002

cell electrophysiological studies (Fig 2, arrow). The constructed pseudo ECG also showed the ventricular arrhythmias like ECG when the I_K was severely suppressed (Fig 2, empty arrow).

Discussion

In the present study, we demonstrated that serum IS concentrations are correlated with age, hematocrit, creatinine, estimated GFR, albumin, QRS duration, and QTc interval. The serum IS concentration was increased in QTc prolongation patients in contrast with the normal QTc controls; in addition, multiple logistic regression analysis also confirmed this independent association, even in a fully adjusted model. Furthermore, in cell electrophysiological study, IS decreased the I_K in rat ventricular cardiomyocyte through the regulation of the major potassium ion channel protein Kv 2.1 phosphorylation. To our knowledge, this is the first report to observe that the serum IS level is associated with QTc prolongation and that the possible mechanism of this phenomenon is through the down-regulation of I_K channel protein phosphorylation and the I_K current activity that in turn increases the APD and QTc interval.

QT prolongation is usually found in patients with chronic renal diseases and multiple mechanisms have been suggested, such as electrolyte imbalance, autonomic nerve dysfunction, rapid changes in electrolyte plasma concentrations during hemodialysis and cardiac hypertrophy [34]. However, there have been few reports demonstrating the relationship between early stages of CKD and QT interval. Cardiorenal syndrome (CRS), which indicates that in the heart and kidney, acute or chronic dysfunction of one organ may induce acute or chronic dysfunction in the other, is caused by multiple factors, including non-dialyzable uremic toxins, such as IS [35].

Recently, IS has been reported to have the profibrotic and prohypertropic effects on cardiomyocytes [24], free radical production [7], endothelial microparticle release [36], vascular smooth muscle cell proliferation [37], and adherens junctions disruption of vascular endothelial cells [38]. Clinically, all of these pathogenetic states can contribute to vascular damage [39], progression of renal diseases [40], coronary artery disease [10], and even mortality [39] in CKD patients. In the present study, we found that there is a strong association between QTc interval and serum IS level in early CKD patients. Importantly, an increase in circulating IS levels can occur in the early stages (2 and 3) of CKD [41], and the combined prevalence of stages 2 and 3 accounts for 67% of all CKD stages [42].

The biological mechanisms involving IS level in the pathogenesis of QTc prolongation is not well understood. According to Ronco et al., IS is the strongest evidence-based uremic toxin involved in type 2 and type 4 CRS pathophysiology, which is mainly attributable to the profibrotic effects [35]. Recently, IS has also been proven to have a direct effect on cardiac fibroblasts and induced cardiac fibrosis in an animal study [24]. As cardiac fibrosis is known as one of the mechanisms of cardiac dysfunction, QT prolongation and cardiac arrhythmia [43], as well as high serum IS levels may be associated with QTc prolongation. In addition, disturbances in gap junctional intercellular communication affect the electrical coupling between heart muscle cells and the underlie prolongation of QRS and QT intervals [44]. Several uremic retention toxins, such as homocysteine [45] and p-cresol [46] have been shown to be involved in the disassembly of connexin and the disruption of the adherens junction of cardiomyocyte and QT prolongation in an animal model [45]. Therefore, whether IS also involved in the modulation of cardiomyocyte connexin needs further investigation.

I_K is one of the core current determinants of the cardiomyocyte APD, with the increase of APD prolonging the ECG QT interval [13]. The Kv2.1 protein is the major subunit protein comprising the ion channel in H9c2 cell, which generates the I_K [23], and previous studies have also shown the Kv2.1 channel activity is regulated by phosphorylation [47]. Here, we demonstrated that IS decreased the expression of phosphorylated Kv2.1 in the H9c2 cell, down-regulated the Kv2.1 channel activity and decreased I_K, which suggests that IS may play a role in the development of QTc prolongation. This finding provides evidence that uremic toxins are related to arrhythmogenesis, and beyond the present traditional risk factors. From a literature review, we uncovered no documented mechanism describing IS as down-regulates phopho-Kv2.1. However, Park et al. showed that the Kv2.1 potassium channel is regulated by variable phosphorylation [48], in which protein kinase C (PKC) is usually involved [47,49]. Previous studies have demonstrated that PKC was associated with inflammation, oxidative stress, and many other pathological states [50,51]. IS has also been proven to be involved with free radical production and the elevation of inflammatory mediators [6,7,9]. In addition, the induction of nephrotoxicity by IS is mediated by organic anion transporters (OATs), such as OAT1 and OAT3, which are localized in the basolateral membrane of renal proximal tubular cells [52]; OAT1 activity/function is modulated via phosphorylation mediated by the PKC pathway [53]. Hence, it is possible that IS might down regulates phopho-Kv2.1 via the PKC pathway, but further experiments and studies will need to verify this.

It is hard to demonstrate any channelopathies in human cardiomyocyte and their consequent effects on the human heart surface ECG. Recently, both experimental and theoretical models have been used in procedures studying the biological factors that induce arrhythmias [30]. In our computer simulation experiment, the prolonged APD, EAD phenomenon, prolonged QT interval and ventricular arrhythmias-like ECG appearance were noted (Fig 2). The EAD induction and trigger activity were the major arrhythmogenesis in ventricular arrhythmias. The induction of EAD in the experiment suggests an IS arrhythmogenic effect and its possible role in the arrhythmias and SCD among CKD patients.

Among these non-dialyzable uremic toxins, many biological and pathological effects have been previously discussed [54,55]. However, there are lacks of reports showing the arrhythmogenic effect of these non-dailyzable uremic toxins in human heart or animal models. IS is the representative molecule of uremic toxin and already known to be associated with the pathogenesis of many uremic syndrome. Previous studies have shown that IS has profibrotic and prohypertropic effects on cardiomyocytes [24,56], which are also known to be related to free radical production and the elevation of inflammatory mediators, which are in turn proven to affect cardiac ion channel function [24,48,49,56]. It is therefore reasonable to select IS over other toxins for investigation into its arrhythmogenic effect. Further work is required to confirm these findings in other uremic toxins.

Some limitations of this study need to be considered. First, our study population was relatively small. Further, the cross-sectional design limits our ability to infer any causal relationship between total IS levels and QTc prolongation. Second, we did not determined the plasma magnesium and bicarbonate levels in our patients due to hospital standard cardiac multi-slice CT or coronary angiography examination preparation protocols; as such, it is unfortunate that we could not demonstrate whether these electrolytes also affect QTc prolongation. Third, in our study, the IS affected I_K in vitro at very low concentrations, even below the normal population serum IS level [57]. Moreover, the potassium concentration in the added IS potassium salt (0.1uM to 0.3mM) was relatively low compared to the potassium concentration in the DMEM culture medium (5.3mM) used in the experiment. Hence, the additional potassium effect on the action potentials in the experiment could have been eliminated. As the cardiac electrical activity is a complex system, it is no doubt plausible that there exists another modulation system in vivo to contend with the IS biological effect, such as the equilibrium state of IS protein binding capacity [24]. Finally, it is still unclear whether IS affects the expression of other cardiac ion channels, ion currents and cardiomyocyte electrophysiology. Further investigation is warranted.

In conclusion, our study demonstrated that the QTc interval was prolonged in early CKD patients with a higher serum IS level. The arrhythmogenic effect of IS was shown through the inhibition of I_K. By the progress of renal disease and the interaction of CRS, the effect and role of IS on the arrhythmogenesis among CKD patients might be enhanced in conjunction with the advance of renal function impairment. As a result, the arrhythmogenic effect of IS should be taken seriously.

Supporting Information

S1 Code. Mathematical computer model for cardiomyocyte action potential and pseudo-ECG. In our experiment, cardiomyocyte action potential was mathematically constructed by the latest mathematical model of the O'Hara-Rudy dynamic human ventricular model. The codes and equations we used in our experiment were downloaded and modified from the Open access and supplemental material journal PLoS computational Biology.
(DOC)

Acknowledgments

Author Contributions

Conceived and designed the experiments: WHT YJL WTL. Performed the experiments: WHT CPW WCH KTL PYC. Analyzed the data: FMC LLHH. Contributed reagents/materials/analysis tools: WHT LFL CHL. Wrote the paper: WHT FMC THY CPW.

References

1. Genovesi S, Rossi E, Nava M, Riva H, De Franceschi S, Fabbrini P, et al. A case series of chronic haemodialysis patients: mortality, sudden death, and QT interval. Europace. 2013; 15: 1025–1033. doi: 10.1093/europace/eus412 PMID: 23284142

2. Straus SM, Kors JA, De Bruin ML, van der Hooft CS, Hofman A, Heeringa J, et al. Prolonged QTc interval and risk of sudden cardiac death in a population of older adults. J Am Coll Cardiol. 2006; 47: 362–367. PMID: 16412861

3. Shamseddin MK, Parfrey PS. Sudden cardiac death in chronic kidney disease: epidemiology and prevention. Nat Rev Nephrol. 2011; 7: 145–154. doi: 10.1038/nrneph.2010.191 PMID: 21283136

4. Hoffman BF, Guo SD, Feinmark SJ. Arrhythmias caused by platelet activating factor. J Cardiovasc Electrophysiol. 1996; 7: 120–133. PMID: 8853022

5. Hoffman BF, Feinmark SJ, Guo SD. Electrophysiologic effects of interactions between activated canine neutrophils and cardiac myocytes. J Cardiovasc Electrophysiol. 1997; 8: 679–687. PMID: 9209969

6. Aoyama I, Shimokata K, Niwa T. An oral adsorbent downregulates renal expression of genes that promote interstitial inflammation and fibrosis in diabetic rats. Nephron. 2002; 92: 635–651. PMID: 12372949

7. Motojima M, Hosokawa A, Yamato H, Muraki T, Yoshioka T. Uremic toxins of organic anions upregulate PAI-1 expression by induction of NF-kappaB and free radical in proximal tubular cells. Kidney Int. 2003; 63: 1671–1680. PMID: 12675842

8. Lee CT, Kuo CC, Chen YM, Hsu CY, Lee WC, Tsai YC, et al. Factors associated with blood concentrations of indoxyl sulfate and p-cresol in patients undergoing peritoneal dialysis. Perit Dial Int. 2010; 30: 456–463. doi: 10.3747/pdi.2009.00092 PMID: 20338972

9. Ito S, Higuchi Y, Yagi Y, Nishijima F, Yamato H, Ishii H, et al. Reduction of indoxyl sulfate by AST-120 attenuates monocyte inflammation related to chronic kidney disease. J Leukoc Biol. 2013; 93: 837–845. doi: 10.1189/jlb.0112023 PMID: 23362306

10. Chiu CA, Lu LF, Yu TH, Hung WC, Chung FM, Tsai IT, et al. Increased levels of total P-Cresylsulphate and indoxyl sulphate are associated with coronary artery disease in patients with diabetic nephropathy. Rev Diabet Stud. 2010; 7: 275–284. doi: 10.1900/RDS.2010.7.275 PMID: 21713315

11. Hsu CC, Lu YC, Chiu CA, Yu TH, Hung WC, Wang CP, et al. Levels of indoxyl sulfate are associated with severity of coronary atherosclerosis. Clin Invest Med. 2013; 36: E42–49. PMID: 23374599

12. Noble D, Tsien RW. Outward membrane currents activated in the plateau range of potentials in cardiac Purkinje fibres. J Physiol. 1969; 200: 205–231. PMID: 5761944

13. Carmeliet E. K+ channels and control of ventricular repolarization in the heart. Fundam Clin Pharmacol. 1993; 7: 19–28. PMID: 8458599

14. Nattel S, Maguy A, Le Bouter S, Yeh YH. Arrhythmogenic ion-channel remodeling in the heart: heart failure, myocardial infarction, and atrial fibrillation. Physiol Rev. 2007; 87: 425–456. PMID: 17429037

15. Kannankeril P, Roden DM, Darbar D. Drug-induced long QT syndrome. Pharmacol Rev. 2010; 62: 760–781. doi: 10.1124/pr.110.003723 PMID: 21079043

16. Levey AS, Coresh J, Greene T, Stevens LA, Zhang YL, Hendriksen S, et al. Using standardized serum creatinine values in the modification of diet in renal disease study equation for estimating glomerular filtration rate. Ann Intern Med. 2006; 145: 247–254. PMID: 16908915

17. Wang CP, Lu LF, Yu TH, Hung WC, Chiu CA, Chung FM, et al. Associations among chronic kidney disease, high total p-cresylsulfate and major adverse cardiac events. J Nephrol. 2013; 26: 111–118. doi: 10.5301/jn.5000111 PMID: 22460182

18. Wang CP, Lu LF, Yu TH, Hung WC, Chiu CA, Chung FM, et al. Serum levels of total *p*-cresylsulphate are associated with angiographic coronary atherosclerosis severity in stable angina patients with early stage of renal failure. Atherosclerosis. 2010; 211: 579–583. doi: 10.1016/j.atherosclerosis.2010.03.036 PMID: 20427046

19. Magnani JW, Wang N, Nelson KP, Connelly S, Deo R, Rodondi N, et al. Electrocardiographic PR interval and adverse outcomes in older adults: the Health, Aging, and Body Composition study. Circ Arrhythm Electrophysiol. 2013; 6: 84–90. doi: 10.1161/CIRCEP.112.975342 PMID: 23243193

20. Malik M, Hnatkova K, Batchvarov V, Gang Y, Smetana P, Camm AJ. Sample size, power calculations, and their implications for the cost of thorough studies of drug induced QT interval prolongation. Pacing Clin Electrophysiol. 2004; 27: 1659–1669. PMID: 15613131

21. Salvi V, Karnad DR, Panicker GK, Natekar M, Hingorani P, Kerkar V, et al. Comparison of 5 methods of QT interval measurements on electrocardiograms from a thorough QT/QTc study: effect on assay sensitivity and categorical outliers. J Electrocardiol. 2011; 44: 96–104. doi: 10.1016/j.jelectrocard.2010.11.010 PMID: 21238976

22. Rautaharju PM, Surawicz B, Gettes LS, Bailey JJ, Childers R, Deal BJ, et al. AHA/ACCF/ HRS recommendations for the standardization and interpretation of the electrocardiogram: part IV: the ST segment, T and U waves, and the QT interval: a scientific statement from the American Heart Association Electrocardiography and Arrhythmias Committee, Council on Clinical Cardiology; the American College of Cardiology Foundation; and the Heart Rhythm Society. Endorsed by the International Society for Computerized Electrocardiology. J Am Coll Cardiol. 2009; 53: 982–991. doi: 10.1016/j.jacc.2008.12.014 PMID: 19281931

23. Wang W, Hino N, Yamasaki H, Aoki T, Ochi R. KV2.1 K+ channels underlie major voltage-gated K+ outward current in H9c2 myoblasts. Jpn J Physiol. 2002; 52: 507–514. PMID: 12617756

24. Lekawanvijit S, Adrahtas A, Kelly DJ, Kompa AR, Wang BH, Krum H. Does indoxyl sulfate, a uraemic toxin, have direct effects on cardiac fibroblasts and myocytes? Eur Heart J. 2010; 31: 1771–1779. doi: 10.1093/eurheartj/ehp574 PMID: 20047993

25. Meijers BK, Van Kerckhoven S, Verbeke K, Dehaen W, Vanrenterghem Y, Hoylaerts MF, et al. The uremic retention solute p-cresyl sulfate and markers of endothelial damage. Am J Kidney Dis. 2009; 54: 891–901. doi: 10.1053/j.ajkd.2009.04.022 PMID: 19615803

26. Murakoshi H, Shi G, Scannevin RH, Trimmer JS. Phosphorylation of the Kv2.1 K+ channel alters voltage-dependent activation. Mol Pharmacol. 1997; 52: 821–828. PMID: 9351973

27. Ito T, Nuriya M, Yasui M. Regulation of Kv2.1 phosphorylation in an animal model of anoxia. Neurobiol Dis. 2010; 38: 85–91. doi: 10.1016/j.nbd.2010.01.002 PMID: 20079839

28. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch. 1981; 391: 85–100. PMID: 6270629

29. Lee KT, Tang PW, Tsai WC, Liu IH, Yen HW, Voon WC, et al. Differential effects of central and peripheral fat tissues on the delayed rectifier k outward currents in cardiac myocytes. Cardiology. 2013; 125: 118–124. doi: 10.1159/000350360 PMID: 23711914

30. O'Hara T, Virag L, Varro A, Rudy Y. Simulation of the undiseased human cardiac ventricular action potential: model formulation and experimental validation. PLoS Comput Biol. 2011; 7: e1002061. doi: 10.1371/journal.pcbi.1002061 PMID: 21637795

31. Zagotta WN, Hoshi T, Aldrich RW. Shaker potassium channel gating. III: Evaluation of kinetic models for activation. J Gen Physiol. 1994; 103: 321–362. PMID: 8189208

32. Gima K, Rudy Y. Ionic current basis of electrocardiographic waveforms: a model study. Circ Res. 2002; 90: 889–896. PMID: 11988490

33. Rush S, Larsen H. A practical algorithm for solving dynamic membrane equations. IEEE Trans Biomed Eng. 1978; 25: 389–392. PMID: 689699

34. Covic A, Diaconita M, Gusbeth-Tatomir P, Covic M, Botezan A, Ungureanu G, et al. Haemodialysis increases QT(c) interval but not QT(c) dispersion in ESRD patients without manifest cardiac disease. Nephrol Dial Transplant. 2002; 17: 2170–2177. PMID: 12454229

35. Ronco C, Haapio M, House AA, Anavekar N, Bellomo R. Cardiorenal syndrome. J Am Coll Cardiol. 2008; 52: 1527–1539. doi: 10.1016/j.jacc.2008.07.051 PMID: 19007588

36. Faure V, Dou L, Sabatier F, Cerini C, Sampol J, Berland Y, et al. Elevation of circulating endothelial microparticles in patients with chronic renal failure. J Thromb Haemost. 2006; 4: 566–573. PMID: 16405517

37. Yamamoto H, Tsuruoka S, Ioka T, Ando H, Ito C, Akimoto T, et al. Indoxyl sulfate stimulates proliferation of rat vascular smooth muscle cells. Kidney Int. 2006; 69: 1780–1785. PMID: 16612331

38. Peng YS, Lin YT, Chen Y, Hung KY, Wang SM. Effects of indoxyl sulfate on adherens junctions of endothelial cells and the underlying signaling mechanism. J Cell Biochem. 2012; 113: 1034–1043. doi: 10.1002/jcb.23435 PMID: 22213462

39. Barreto FC, Barreto DV, Liabeuf S, Meert N, Glorieux G, Temmar M, et al. Serum indoxyl sulfate is associated with vascular disease and mortality in chronic kidney disease patients. Clin J Am Soc Nephrol. 2009; 4: 1551–1558. doi: 10.2215/CJN.03980609 PMID: 19696217

40. Wu IW, Hsu KH, Lee CC, Sun CY, Hsu HJ, Tsai CJ, et al. p-Cresyl sulphate and indoxyl sulphate predict progression of chronic kidney disease. Nephrol Dial Transplant. 2011; 26: 938–947. doi: 10.1093/ndt/gfq580 PMID: 20884620

41. Atoh K, Itoh H, Haneda M. Serum indoxyl sulfate levels in patients with diabetic nephropathy: relation to renal function. Diabetes Res Clin Pract. 2009; 83: 220–226. doi: 10.1016/j.diabres.2008.09.053 PMID: 19027976

42. National Kidney Foundation. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Am J Kidney Dis. 2002; 39: S1–266. PMID: 11904577

43. Zhang T, Yong SL, Drinko JK, Popović ZB, Shryock JC, Belardinelli L, et al. LQTS mutation N1325S in cardiac sodium channel gene SCN5A causes cardiomyocyte apoptosis, cardiac fibrosis and contractile dysfunction in mice. Int J Cardiol. 2011; 147: 239–245. doi: 10.1016/j.ijcard.2009.08.047 PMID: 19762097

44. Quan XQ, Bai R, Liu N, Chen BD, Zhang CT. Increasing gap junction coupling reduces transmural dispersion of repolarization and prevents torsade de pointes in rabbit LQT3 model. J Cardiovasc Electrophysiol. 2007; 18: 1184–1189. PMID: 17711442

45. Rosenberger D, Gargoum R, Tyagi N, Metreveli N, Sen U, Maldonado C, et al. Homocysteine enriched diet leads to prolonged QT interval and reduced left ventricular performance in telemetric monitored mice. Nutr Metab Cardiovasc Dis. 2011; 21: 492–498. doi: 10.1016/j.numecd.2009.11.014 PMID: 20227264

46. Peng YS, Lin YT, Wang SD, Hung KY, Chen Y, Wang SM. P-cresol induces disruption of cardiomyocyte adherens junctions. Toxicology. 2013; 306: 176–184. doi: 10.1016/j.tox.2013.02.015 PMID: 23466501

47. Song MY, Hong C, Bae SH, So I, Park KS. Dynamic modulation of the kv2.1 channel by SRC-dependent tyrosine phosphorylation. J Proteome Res. 2012; 11: 1018–1026. doi: 10.1021/pr200770v PMID: 22106938

48. Park KS, Mohapatra DP, Misonou H, Trimmer JS. Graded regulation of the Kv2.1 potassium channel by variable phosphorylation. Science. 2006; 313: 976–979. PMID: 16917065

49. Walsh KB, Zhang J. Neonatal rat cardiac fibroblasts express three types of voltage-gated K+ channels: regulation of a transient outward current by protein kinase C. Am J Physiol Heart Circ Physiol. 2008; 294: H1010–1017. PMID: 18156198

50. Inoguchi T, Sonta T, Tsubouchi H, Etoh T, Kakimoto M, Sonoda N, et al. Protein kinase C-dependent increase in reactive oxygen species (ROS) production in vascular tissues of diabetes: role of vascular NAD(P)H oxidase. J Am Soc Nephrol. 2003; 14: S227–232. PMID: 12874436

51. Cain BS, Meldrum DR, Harken AH. Protein kinase C in normal and pathologic myocardial states. J Surg Res. 1999; 81: 249–259. PMID: 9927548

52. Enomoto A, Takeda M, Tojo A, Sekine T, Cha SH, Khamdang S, et al. Role of organic anion transporters in the tubular transport of indoxyl sulfate and the induction of its nephrotoxicity. J Am Soc Nephrol. 2002; 13: 1711–1720. PMID: 12089366

53. Zhang Q, Hong M, Duan P, Pan Z, Ma J, You G. Organic anion transporter OAT1 undergoes constitutive and protein kinase C-regulated trafficking through a dynamin- and clathrin-dependent pathway. J Biol Chem. 2008; 283: 32570–32579. doi: 10.1074/jbc.M800298200 PMID: 18818201

54. Jourde-Chiche N, Dou L, Cerini C, Dignat-George F, Vanholder R, Brunet P. Protein-bound toxins-update 2009. Semin Dial. 2009; 22: 334–339. doi: 10.1111/j.1525-139X.2009.00576.x PMID: 19708977

55. Neirynck N, Vanholder R, Schepers E, Eloot S, Pletinck A, Glorieux G. An update on uremic toxins. Int Urol Nephrol. 2013; 45: 139–150. doi: 10.1007/s11255-012-0258-1 PMID: 22893494

56. Lekawanvijit S, Kompa AR, Manabe M, Wang BH, Langham RG, Nishijima F, et al. Chronic kidney disease-induced cardiac fibrosis is ameliorated by reducing circulating levels of a non-dialysable uremic toxin, indoxyl sulfate. PLoS One. 2012; 7: e41281. doi: 10.1371/journal.pone.0041281 PMID: 22829936

57. Vanholder R, De Smet R, Glorieux G, Argilés A, Baurmeister U, Brunet P, et al. Review on uremic toxins: classification, concentration, and interindividual variability. Kidney Int. 2003; 63: 1934–1943. PMID: 12675874