Reduced protein bound uraemic toxins in vegetarian kidney failure patients treated by haemodiafiltration

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Abstract

Introduction Indoxyl sulfate (IS) and p cresyl sulfate (PCS) are protein bound toxins which accumulate with chronic kidney disease. Haemodiafiltration (HDF) increases middle molecule clearances and has been suggested to increase IS and PCS clearance. We therefore wished to establish whether higher convective clearances with HDF would reduce IS and PCS concentrations.

Methods We measured total plasma IS and PCS in a cohort of 138 CKD5d patients treated by On-line HDF (Ol-HDF), by HPLC.

Findings Mean patient age was 64.6±16.5 years, 60.1% male, 57.3% diabetic, median dialysis vintage 25.9 months (12.4-62.0). The mean ICS concentration was 79.8±56.4 umol/l and PCS 140.3±101.8 umol/l. On multivariate analysis, IS was associated with serum albumin (β 4.31,p<0.001), and negatively with residual renal function (β-4.1,p=0.02) and vegetarian diet(β -28.3,p=0.048) and PCS negatively with log CRP (β-75.8, p<0.001) and vegetarian diet (β -109, p=0.001). Vegetarian patients had lower IS and PCS levels (median 41.5 (24.2-71.9) vs 78.1 (49.5-107.5) and PCS (41.6 (14.2-178.3) vs 127.3 (77.4-205.6) umol/l respectively, p<0.05. Vegetarian patients had lower preOl-HDF serum urea, and phosphate (13.8 ±3.8vs 18.4 ±5.2 mmol/l, and 1.33 ±0.21 vs1.58 ±0.45 mmol/l), and estimated urea nitrogen intake (1.25±0.28 vs 1.62 ±0.5 g/kg/day) respectively, all p<0.05

Conclusions Plasma IS and PCS concentrations were not lower with Ol-HDF compared to previous studies in haemodialysis patients. However those eating a

vegetarian diet had reduced IS and PCS concentrations. Although this could be due to differences in dietary protein intake, a vegetarian diet may also potentially reduce IS and PCS production by the intestinal microbiome.

Introduction

Chronic kidney disease (CKD) leads to the accumulation of nitrogenous waste products of metabolism. The mortality of CKD patients treated by dialysis (CKD5d) remains high, and intuitively a greater amount of dialysis designed to remove these toxins would be expected to increase patient survival. The National Co-operative Dialysis Study (NCDS), demonstrated that short term patient outcomes were determined by the time averaged urea concentration. The results from the NCDS were used to define a critical threshold for haemodialysis urea clearance as a sessional Kt/Vurea [12]. However a subsequent randomised prospective haemodialysis study (HEMO study) [3], did not show that higher dialyzer urea clearances improved patient survival.

Although urea accumulation can lead to carbamylation of haemoglobin and other proteins [4,5], urea is only one of a number of potential toxins that accumulate in CKD5d patients [6]. Serum beta 2 microglobulin, a middle sized azotaemic toxin, has been linked to an increased cardiovascular mortality and secondary analysis of the HEMO study also reported an association between increasing serum beta 2 microglobulin concentrations and mortality [7]. Although this effect of increasing beta 2 microglobulin may be due to loss of residual renal

function, or inflammatory states, rather than reduced dialyzer beta 2 microglobulin clearance.

As the prospective randomised Membrane Permeability Outcome (MPO) trial was designed to compare high and low flux dialyzers did not observe any overall survival advantage with high flux dialyzers [8]. Although haemodiafiltration, which increases middle sized solute clearances [9], was described more than 50 years ago [10], it is only in recent times that haemodiafiltration has become an established treatment for many patients in Europe . Studies delivering the highest convective clearances reported a survival benefit for on-line haemodiafiltration (Ol-HDF) [11], whereas trials delivering lower convective doses did not [12]. Although these studies differed in patient characteristics including age, dialysis vintage and residual renal function ,there was a suggestion that Ol-HDF, by improving the spectrum of azotaemic toxin removal may increase dialysis patient survival [13]. There has been debate as to whether HDF increases clearance of indoxyl sulfate and p-cresyl sulfate, with some studies reporting increased clearance [14].

As such we wished to determine whether there was an effect of convection on the removal of these protein bound azotaemic toxins.

Patients and methods

We measured total plasma indoxyl sulfate and p-cresylsulfate in 138 established chronic kidney dialysis patients attending for routine outpatient Ol-HDF treatments under the care of the Royal Free Hospital, using Fresenius

114 4008H, 5008 (Fresenius AG, Bad Homburg, Germany) or BBraun Dialogue+
115 (BBraun, Melsungen, Germany) dialysis machines, with high flux polysulphone
116 dialyzers (Nipro, Osaka, Japan) [15], and anticoagulated with tinzaparin (Leo
117 Laboratories, Market Harborough, UK) [16]. Ultrapure quality water was used
118 for all treatments.

119 Serum biochemistry samples were analysed with a standard multi-channel
120 biochemical analyzer (Roche Integra, Roche diagnostics, Lewes, UK), using the
121 bromocresol green method for albumin determination, and the same assay for C
122 reactive protein (CRP) as the UK National Amyloid Centre. Haemoglobin (Hb) was
123 measured by the sodium lauryl sulphate-Hb method (XE-2100 Sysmex
124 Corporation, Kobe, Japan) [17]. Serum beta-2-microglobulin was measured by
125 rate nephelometry (www.Dako.com, Image 800 analyser, Beckman Coulter, High
126 Wycombe, UK). Dialysate sodium delivery was checked by flame photometry and
127 ion electrophoresis [18]. Patients were weighed pre and post haemodiafiltration
128 using regularly calibrated scales and extracellular water to total body water
129 measured with multifrequency bioelectrical impedance (Biospace 720, Biospace,
130 Seoul, Korea) [19]. Protein nitrogen accumulation was estimated from pre and
131 post dialysis urea concentrations and body water, and then normalised for body
132 weight (nPNA) [20].

133 Patients were reviewed by renally qualified dieticians, and dietary
134 histories obtained by patient dietary recall. Residual renal function was
135 measured from timed urine collections obtained between the 1st and 2nd OI-HDF

treatments of the week with corresponding blood tests, and expressed as the average of urea and creatinine clearance.

Indoxyl sulfate (IS) and p cresyl sulfate (p-CS) were measured by high pressure liquid chromatography (HPLC). In brief 200 ul plasma samples were incubated for 5 minutes at room temperature with 20uL of competitive inhibitor sodium caprylate (0.24 umol/l). 20uL of Internal standard naphthalene sulphonic acid (0.5 mmol/l) was added. Samples were deproteinated by addition of 2 ml ice cold acetone, centrifugation at 4000 rpm for 10 minutes followed by the addition of 2 ml ice cold dichloromethane to the resulting supernatant and further centrifugation at 4000 rpm for 10 minutes. The top aqueous phase was removed, stabilised with 20uL of 1M HCl and placed in a Waters 717 plus auto-sampler (Waters Corporation, Milford, Massachusetts, USA) set at 15°C, 10 μl sample injection, 20 minutes run time, 10 minutes delay. Samples were injected onto a Fortis C18 column (150 x 4.6 OD, 3 mM) (Fortis Technologies Ltd, Neston, Cheshire, UK) for peak separation using a Perkin Elmer Series 200 quaternary gradient pump (Perkin Elmer Life and Analytical Sciences, Shelton, Connecticut, USA) to control a mobile phase of A (0.2% trifluoroacetic acid in water) and B(0.2% trifluoroacetic acid in acetonitrile) with a gradient flow of %A/B 85/15 for 5 minutes, 80/20 for 5 minutes, 0/100 for 2 minutes at 0.6 ml/minute flow rate, 0/100 for 3 minutes at 0.8 ml/min flow rate and 86/15 for 2 minutes at 1 ml/minute flow rate. A Waters 2475 multi wavelength fluorescence detector was used to detect IS and internal standard at 280/360 nm and p-CS at 260/296 nm excitation/emission wavelength. TotalChrom Navigator-900

159 software (Perkin Elmer Life and Analytical Sciences, Shelton, Connecticut, USA)
160 was used to collate the peak data via a PE Nelson 900 Series interface [21].
161
162 Statistical analysis
163 All categorical data were reported as number (percentage) and numeric
164 data as mean ± standard deviation (SD). Comparison between two groups was
165 performed with t test or Mann-Whitney U test for non-normally and non-
166 parametric distributed variables, or Chi square test with Yates' correction.
167 Nonparametric variables were log transformed for correlation analysis. Simple
168 correlation was performed, and variables with $p<0.1$ were then included in a step
169 backward multiple linear regression analysis to determine which variables were
170 associated with indoxyl sulfate and p-cresyl sulfate concentrations. Variables
171 which were not statistically significant were excluded from analysis unless they
172 improved model fit. Data were analysis by SPSS statistic software (version 22,
173 Chicago, USA). The level of significance was defined as a p value <0.05.
174 Study approval was granted by the UK NHS national ethics committee
175 IRAS project number 129559, and the study was undertaken in keeping the
176 Helsinki accord with informed patient consent and trial registration
177 ISRCTN70556765.
178
179 Results
180 Total plasma IS and P-CS concentrations were measured in 138 adult
181 patients receiving Ol-HDF, mean age 64.6±16.5 years, 60.1% male, 57.3%

diabetic, median dialysis (Ol-HDF) vintage 25.9 months (12.4-62.0). Stoke-Davies co-morbidity score 1.0 (1-1) and grade 1 (0-1) [22]. 51 patients were Caucasoid, 42 South Asian, 37 African-Afro-Caribbean with the remainder being Asian and one from North Africa. On dietary recall renally trained dieticians recorded that 16 patients were strict vegetarians and the remainder of the cohort were omnivores.

Pretreatment haemoglobin was 110.1±17.4 g/l, serum albumin 39.3±4.2 g/l, urea 17.9±5.3 mmol/l, creatinine 677±254 umol/l, calcium 2.32± 0.13 mmol/l, phosphate 1.55±0.43mmol/l, glucose7.5±2.7 mmol/l , CRP 6mg/l (2.0-12.3 , β2 microglobulin 28.9±10.6mg/l and median 24 hour urine volume 367ml (210-865). Median dialysis session time 3.75 hours (3.5-4.0), dialyzer surface area 2.1 m^2 (1.7-2.1), blood flow 300 ml/min (300-350), litres of convection 15.4 ±2.4 l, tinzaparin dose 2500 IU (1500-3500), dialysate sodium 136mmol/l (136-137). Sessional urea reduction ratio was 73.4±7.4%, and single pool Kt/V_{urea} 1.55±0.32, with a nPNA 1.58±0.48g/kg/day. Pre-dialysis weight 74.3± 17.3 kg, and 72.4±17.0 kg post dialysis. Extracellular water/total body water ratio pre-dialysis was 0.403±0.013 and post dialysis 0.395±0.021. 55 patients had some residual renal function, median creatinine clearance 0 (0-2.2) ml/min/1.73m^2.

54.4% of patients were prescribed antihypertensive medications, median number of classes of anti-hypertensive medications prescribed per patient 1 (0-1). 43.9% were prescribed calcium containing phosphate binders, 25.2% sevelamer hydrochloride 25.2% and 4.3% lanthanum carbonate.

Simple univariate analysis was performed for both plasma IS and PCS (Table 1), and IS was most strongly associated with serum albumin and creatinine and negatively with residual renal function, whereas PCS the associations were weaker but positive for serum urea, and prescription of HMG CoA 3 reductase inhibitors (statins),and co-morbidity. There was no association with convection volumes delivered by Ol-HDF and PCS concentrations, and only a weak correlation between convection volume and IS (r^2=0.04, p=0.049). However both IS and PCS were negatively associated with a vegetarian diet. We then performed a backward linear regression analysis (table 2), and IS was independently associated with serum albumin, and negatively with residual renal function and vegetarian diet. Whereas, PCS was associated with statin prescription and negatively with CRP and vegetarian diet.

We then compared those eating a vegetarian diet to those consuming meat (table 3). Vegetarians had lower pre-dialysis serum urea and creatinine concentrations , and dietary nitrogen intake, but did not differ in residual renal function, or dialysis urea based clearances or convective volume exchange achieved. Both IS and PCS were lower in the vegetarian cohort (Figure 1). Serum phosphate was also lower (table3), despite more patients taking phosphate binders in the meat eaters (77.9% vs 43.8%, Chi square 8.97, p=0.03), with no difference in calcium containing phosphate binders prescribed compared to sevelamer or lanthanum (45.9 vs 31.3; 27.0 vs 12.5; and 4.9 vs 0%, respectively). Although there were no differences in sex, age or diabetes, more vegetarians were South Asians (meat eaters 40.9% Caucasoid, 21.3% South Asians and

30.3% African-Afro-Caribbeans, and vegetarians 6.3% Caucasoids; 93.7% South Asians, and 0% African-Afro-Caribbeans respectively, Chi square 35.6,p<0.001). Correcting muscle mass and body fat for height, to derive muscle and fat indices, then the vegetarian cohort had lower muscle mass, but fat mass was similar (p=0.052) (Figure 2).

Discussion

Indoxyl sulfate and P cresyl sulfate are protein bound toxins which accumulate in patients with chronic kidney disease treated by dialysis. Plasma total concentrations in our cohort are similar to those reported from previous studies in dialysis patients [23]. Although earlier reports suggested that convective dialysis therapies could lead to a reduction in IS and PCS [24], we only observed a weak association between the Ol-HDF convective dose and IS concentrations, and other reports showed no difference in IS or PCS concentrations and different convective therapies [25]. Comparing our cross sectional cohort data with previous studies, then treatment with Ol-HDF does not appear to lead to a substantial reduction in protein bound solutes. We only found a modest association and dialysis session duration, in keeping with previous reports of marginal differences in IS and PCS plasma concentrations and dialysis session time. Increasing dialyzer surface area has previously been suggested to increase clearance [26], we only found a weak univariate association between plasma IS and PCS and dialyzer surface area, that was lost on multiple linear regression. As we had standardised dialysate flow rates we did

not examine the effect of different dialysate flow rates [26], but there was no effect of convection volume and PCS concentrations and only a very modest univariate association with IS concentrations.

There was no association between dialytic urea clearance or convective volume exchange and PCS concentrations, but there were weak univariate associations for IS, which were lost on multivariate analysis. In keeping with previous observations IS concentrations were lower in those dialysis patients who had retained residual renal function [27].

IS and indoxyl glucuronide are produced from tryptophan derived indoles following intestinal bacterial metabolism, whereas p-cresyl sulfate and p-cresyl glucuronide are derived from p-cresol following bacterial metabolism of tyrosine. Both are then predominantly bound by albumin. Generally on univariate analysis we found an association between those variables associated with increased protein intake, protein turnover, whereas there was a negative association with residual renal function and those variables associated with reduced dietary intake. On multivariate analysis, IS was associated with albumin, which is generally a marker of health and nutritional status, and negatively with residual renal function and vegetarian diet. Similarly PCS was associated with statin prescription, a potential surrogate for cardiovascular disease, and also dietary intake, and these protein bound toxins have been linked to increased cardiovascular risk for dialysis patients [28]. We suspect that the negative association with CRP could be secondary to the negative effects of low grade inflammation on dietary intake. Although it is recognised that acute illnesses can

affect the gastrointestinal biome, and so by altering bacterial numbers and populations could also have an effect in reducing production of these gut derived uraemic toxins [29].

In our dialysis cohort we had a group of predominantly South Asian vegetarians, who had lower plasma IS and PCS levels than their meat eating counterparts. Residual renal function and dialysis treatments were not different. However these vegetarian patients did have a lower estimated dietary protein intake, and lower predialysis serum urea and creatinine concentrations, and lower muscle mass, but similar fat mass. Our study is the first to report that a vegetarian diet leads to lower IS and PCS levels in kidney dialysis patients. Our results are supported by previous studies which have reported that low protein diets reduce serum concentrations of protein bound azotaemic toxins [30], and a small study which reported that the production rate of IS and PCS were lower in healthy vegetarians compared to healthy omnivores [31]. In addition, serum phosphate concentrations were also lower, despite fewer patients prescribed phosphate binders, and studies in vegetarian chronic kidney disease patients have also reported lower phosphate levels thought to be due to differences in plant phytate intake [32].

The bacterial population in the colon changes in patients with chronic kidney failure [29], with a switch to more bacteria capable of producing IS and PCS. It is equally recognised that not only sudden changes in health, but abrupt changes in dietary intake can also lead to changes in the gastrointestinal microbiome [29]. There are differences between animal and vegetable proteins,

and as such, the lower IS and PCS concentrations we report in our vegetarian patients could potentially lead to differences in gut bacterial populations and reduced IS and PCS production. Other studies have shown that a deliberate intervention to increase in dietary fibre for 6 weeks can also reduce IS and PCS concentrations [33] We do not think that this would have a major effect in our dialysis population due to the dietary advice given to restrict higher phosphate containing foodstuffs, such as bran and other high fibre foodstuffs. The majority of our vegetarian patients came for the South Asian community, and this group of patients have better 5 year survival on dialysis than Northern Europeans. Although our study is cross sectional, we report for the first time that vegetarian dialysis patients have lower IS and PCS levels , and as such further prospective interventional studies are required to investigate whether changes in diet can reduce protein bound and other uraemic toxins [34].

The authors have no conflicts of interest
The data contained in this paper have not been previously published
Authorship

Sakina Kandouz, Ali Shendi Mohamed Ali, Yishan Zheng and Susan Sandeman collected, processed and analysed samples. Andrew Davenport organized ethical approvals. All authors contributed to drafting the paper and approved the final version.

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Figure 1. Plasma concentrations of indoxyl sulfate and p cresyl sulfate in non-vegetarian and vegetarian patients. Results expressed as median (interquartile range).* $p<0.05$ vs non-vegetarian

Figure 2. Muscle and Fat mass measured post haemodiafiltration by multifrequency bioimpedance indexed to height. Results expressed as median (interquartile range) * $p<0.05$ vs non-vegetarian.

Table1. Pearson correlation analysis for indoxylsulfate (IS) and p-cresyl sulfate (PCS).Nonparametric data were transformed prior to analysis. Serum variables pre midweek dialysis session. Residual renal function (RRF -combined 24 hour urinary urea and creatinine clearance),dialyzer surface area (dialyzer) Number of classes of antihypertensive medications (No BPmeds), sessional Kt/V (Kt/V),dialysate potassium (Potassium D, sodium (Dialysate Na),β2 microglobulin (β2M), phosphate binders (binders), total body water(TBW), litres ofconvective clearance (litres), prescription HMG CoA 3 reductase inhibitor (statin),Stoke-Davies Co-morbidity grade (comorbidity),dialysis session time (time), C reactive protein (CRP),g urea nitrogen/kg/day .G N/day/kg

IS	r	p	PCS	r	p
PCS	0.362	<0.001	IS	0.362	<0.001
albumin	0.328	<0.001	statin	0.267	0.002
creatinine	0.324	<0.001	urea	0.206	0.015
RRF	-0.266	0.002	comorbidity	0.209	0.016
dialyzer	0.233	0.006	vegetarian	-0.197	0.020
No BP meds	0.225	0.008	CRP	-0.18	0.035
Kt/V	-0.204	0.014	G N/day/kg	0.172	0.044
Potassium D	-0.192	0.024	time	0.170	0.046
vegetarian	-0.192	0.024			
Sodium D	-0.182	0.033			
β2 M	0.184	0.031			
binders	0.174	0.041			
TBW	-0.182	0.045			
litres	0.199	0.049			

Table2;multiple linear regression analysis for indoxylsulfate (IS) and p-cresyl sulfate (PCS). Residual renal function (RRF -combined 24 hour urinary urea and creatinine clearance). C reactive protein (CRP), prescription HMG CoA 3 reductase inhibitor (statin). Standard error of β (SE),confidence limits (CL).ICS model corrected r^2= 0.179,and PCS,r^2=0.235

ICS	β	SE	t	95% CL	F	p
albumin	4.31	1.1	4.02	2.2 to6.4	16.1	<0.001

RRF	-4.1	1.3	-3.1	-6.7 to-1.5	0.87	0.02
vegetarian	-28.3	14.2	-1.99	-56.4to-0.24	0.98	0.048
PCS						
CRP	-75.8	19.4	-3.9	-114 to -37	15.4	<0.001
vegetarian	-109	31.8	-3.4	-172to -46	11.8	0.001
statin	49.1	19.1	2.4	11.1to 87.0	6.6	0.012

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532 Table3: Patient demographics of those who were vegetarian and those who were
533 non-vegetarian. Serum values pre mid- week dialysis session, and body
534 composition postdialysis. Extra cellularwater (ECW) total body water (TBW),
535 Residual renal function (RRF -combined 24 hour urinary urea and creatinine
536 clearance), C reactive protein (CRP). Chi square (X2)
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variable	Non vegetarian	vegetarian	P value
Age yr	63.7 ±16.9	71.7 ±11.6	0.07
Male %	59.0	68.8	X2=0.5,p>0.5

Diabetic %	55.7	68.9	X2=0.27,p>0.5
Vintage months	25.7 (13.5-62)	29.8 (10.9-69.2)	0.97
RRF ml/min1.73m^2	0 (0-2.2)	0 (0-1.2)	0.23
Weight kg	71.7 ±18.1	65.6 ±12.1	0.20
BMI kg/m2	26.5 ± 6.0	25.4 ± 5.2	0.50
ECW/TBW	0.394 ±0.022	0.399 ±0.016	0.39
Haemoglobin g/l	109.3 ±17.8	116.1 ±12.9	0.15
Albumin g/l	39.4 ±4.3	38.7 ±3.5	0.53
Urea mmol/l	18.4 ±5.2	13.8 ±3.8	<0.01
Creatinine umol/l	699 ±258	503 ±115	<0.01
Phosphate mmol/l	1.58 ±0.45	1.33 ±0.21	0.02
Cholesterol mmol/l	3.96 ±1.25	3.56 ±0.96	0.22
LDL cholesterol mmol/l	2.06 ±0.93	1.60 ±0.79	0.06
CRP mg/l	6.5 (2-13)	4 (1.3-8.3)	0.17
B2 microglobumin mg/l	28.2 (22.5-34.5)	25.1 (17.1-32.3)	0.09
Urea reduction ratio	73.3 ±7.4	73.8 ±7.8	0.81
Sessional Kt/V_{urea}	1.55 ±0.32	1.58 ±0.35	0.74
Session time h	3.64 ±0.53	3.59 ±0.52	0.73
Convection volume L	15.2 ±2.3	16.5±3.7	0.13
G N/kg/day	1.62 ±0.49	1.25 ±0.28	<0.01

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