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Free serum concentrations of the protein-bound retention solute *p*-cresol predict mortality in hemodialysis patients

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Based on *in vitro* data, protein-bound uremic retention solutes have increasingly been recognized to play a pathophysiological role in the uremic syndrome. *p*-Cresol, a representative of this group of molecules, has been shown to be implicated in uremic immunodeficiency and endothelial dysfunction, potentially linking its serum levels to mortality. Thus far, however, no clinical information on this issue is available. To determine the relationship between *p*-cresol and all-cause mortality, 175 prevalent hemodialysis (HD) patients were enrolled in a prospective study. At baseline, serum levels of the water-soluble solutes urea, creatinine, and phosphate, the middle molecule β_2-microglobulin, total and free concentrations of the protein-bound solute *p*-cresol, and several risk factors for mortality were evaluated. During a median follow-up of 34 months, 60 patients died. Baseline comorbidity (Davies score) (hazard ratio (HR), 1.49; 95% confidence interval (95% CI), 1.19–1.86), impaired nutritional status (HR, 4.22; 95% CI, 2.15–8.29), time since initiation of dialysis (HR, 0.98; 95% CI, 0.97–1.00), and higher free concentrations of the protein-bound solute *p*-cresol (HR, 2.28; 95% CI, 1.12–4.64) were independently associated with mortality (multivariate Cox proportional hazards analysis). Our data suggest that free serum levels of *p*-cresol, a representative of the protein-bound uremic retention solutes, are associated with mortality in HD patients. These findings may encourage nephrologists to widen their field of interest beyond the scope of small water-soluble uremic solutes and middle molecules.

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When in the 1960s dialysis strategies as a maintenance therapy for stage 5 chronic kidney disease were introduced in clinical practice, the quality of life and survival of patients improved substantially. Efficiency of dialysis was estimated based on the kinetics of small water-soluble uremic retention solutes, notably urea nitrogen.[1] The findings of early studies were supportive for this approach, since they indicated a benefit of higher removal of urea nitrogen with regard to morbidity and mortality.[2]

However, despite less efficient removal of small water-soluble molecules by peritoneal dialysis as compared to hemodialysis (HD), peritoneal dialysis patients were seen to have a similar clinical condition as their HD counterparts.[3] This observation questioned the importance of small water-soluble molecules in the uremic syndrome. It gave rise to the middle-molecule hypothesis,[4] which suggests a pathophysiological role for molecules with a higher molecular mass than urea nitrogen in the development of the uremic syndrome. Nevertheless, since it was impossible at that time to identify these solutes, urea kinetic modeling became established as the most valuable tool for assessment of dialysis adequacy.[5] During the previous decades, the middle-molecule hypothesis has regained interest by the characterization of several representatives of this molecular mass class.[6] Moreover, recent research work has directed attention towards another distinct group of uremic retention molecules: the protein-bound solutes.[7] Although most of them have a low molecular mass, binding to serum proteins hampers their dialytic removal. *p*-Cresol, a 108-Da colonic fermentation metabolite of the amino acid tyrosine, is considered a prototype of this group of uremic solutes. The protein binding of this molecule was shown to be about 90% in stage 5 chronic kidney disease patients.[8] Among different *in vitro* studies, several have suggested that *p*-cresol plays a role in the immunodeficiency of uremia.[9–11] Recently, a link between *p*-cresol and endothelial dysfunction has been demonstrated.[12,13] In spite of these *in vitro* findings, only few data on the clinical importance of the solute have been reported to date. Free serum concentrations of *p*-cresol were shown to be higher in HD patients hospitalized for infectious disease.[14]

Furthermore, a positive relationship was found between total serum levels of *p*-cresol and a uremic symptom score in patients treated with peritoneal dialysis, whereas a correlation with small water-soluble solutes and the middle molecule β_2-microglobulin was absent.[15] Based on the above-mentioned *in vitro* relationships of *p*-cresol with immune and endothelial cell dysfunction, it is tempting to speculate on this solute as a contributor to the high mortality of dialysis patients. Thus far, however, no information on this issue is available. The principal aim of this prospective observational cohort study was to investigate the association between total and free serum levels of *p*-cresol and survival in prevalent HD patients.

RESULTS

Baseline characteristics of the patients are shown in the first column of Table 1. After a follow-up of 30.1±0.4 months (median 34.0, minimum–maximum 22.0–34.0), 60 of the 175 patients (34%) had died. Causes of death were cardiovascular in 30, infectious in 12, and malignancy in four. Of the remaining 14, one patient died due to a hemorrhagic complication after abdominal surgery and one of noninfectious respiratory insufficiency due to end-stage pulmonary emphysema. In six patients cause of death was defined as cachexia without evidence for malignancy or infection. Six patients died at home from a nonspecified cause. In all, 28 patients were transplanted and six were transferred to another dialysis centre during follow-up.

Table 2 shows the relative risks of death estimated by univariate Cox proportional hazards analysis. Age at baseline, serum albumin, C-reactive protein (CRP), creatinine, phosphate, residual renal function, impaired nutritional status according to Subjective Global Assessment (SGA), Malnutrition–Inflammation Score (MIS), Davies score and grade, atherosclerotic disease, and diabetes were significantly associated with mortality. Total serum levels of *p*-cresol were not associated with outcome. Free concentrations of the solute, however, were significantly related to mortality. Figure 1 shows the Kaplan–Meier survival curves of patients with high ($\geqslant$1.97 mg/l, $n=88$) and with low ($<$1.97 mg/l, $n=87$) free serum levels of *p*-cresol. The survival difference between the two groups was statistically significant (log-rank *P*-value 0.041).

The second and third columns of Table 1 show baseline data of patients with high and low free concentrations of *p*-cresol separately. As compared to the latter, those with high levels had lower serum albumin, higher total *p*-cresol concentrations, and lower renal Kt/*V* of urea nitrogen (rKt/*V*). Moreover, they were older, had been treated with dialysis for a longer time, and were more likely to be treated

Table 1 | Baseline characteristics of the patients

Variable	All (*n*=175)	Free *p*-cresol $\geqslant$1.97 mg/l (*n*=88)	Free *p*-cresol $<$1.97 mg/l (*n*=87)	*P*
Age (years)	64.7±1.1 (67, 26–89)	68.2±1.6 (72, 26–89)	61.2±1.6 (62, 31–86)	0.001
Sex (male/female)	108/67	50/38	58/29	0.180
Time since dialysis initiation (months)	29.8±2.5 (18.1, 2.3–158.1)	37.8±4.2 (24.6, 2.3–158.1)	21.7±2.2 (14.9, 2.5–128.0)	0.021
Treatment modality (HD/HDF)	107/68	63/25	44/43	0.004
Bicarbonate (mEq/l)	22.9±0.2 (22.6, 17.2–40.0)	22.7±0.2 (22.5, 17.7–26.7)	23.1±0.3 (22.8, 17.2–40.0)	0.642
Hemoglobin (g/dl)	11.6±0.1 (11.6, 7.3–15.0)	11.5±0.1 (11.5, 7.3–14.8)	11.6±0.2 (11.8, 8.0–15.0)	0.285
Albumin (g/dl)	3.64±0.03 (3.66, 2.69–4.48)	3.58±0.03 (3.61, 4.37–4.48)	3.70±0.04 (3.72, 2.85–4.48)	0.017
CRP (mg/l)	21.8±2.6 (8.0, 3.0–211.8)	20.8±3.4 (8.3, 3.0–211.8)	22.8±3.9 (7.5, 3.0–184.0)	0.564
Urea (mg/dl)	142.4±3.0 (138, 34–266)	155.9±4.4 (152, 63–266)	128.8±3.6 (129, 34–244)	$<$0.0001
Creatinine (mg/dl)	8.6±0.2 (8.6, 2.5–14.5)	8.9±0.3 (8.9, 2.8–14.0)	8.3±0.3 (8.2, 2.5–14.5)	0.097
Phosphate (mg/dl)	4.8±0.1 (4.6, 1.6–9.3)	4.7±0.2 (4.6, 1.9–9.3)	5.0±0.2 (4.8, 1.6–9.3)	0.309
β_2-Microglobulin (mg/l)	27.7±0.8 (26.5, 8.2–84.9)	30.4±0.9 (29.5, 15.0–63.1)	24.9±1.1 (23.6, 8.2–84.9)	$<$0.0001
Total *p*-cresol (mg/l)	19.0±0.9 (18.9, 0.3–60.5)	26.5±1.0 (23.4, 12.3–60.5)	11.4±0.8 (13.4, 0.3–25.0)	$<$0.0001
Free *p*-cresol (mg/l)	2.59±0.17 (1.97, 0.30–12.74)	4.12±0.24 (3.39, 1.97–12.74)	1.06±0.05 (1.08, 0.30–1.94)	$<$0.0001
% free *p*-cresol of total	13.03±0.44 (11.61, 4.61–39.59)	15.49±0.58 (14.25, 7.61–39.59)	9.70±0.42 (8.89, 4.61–23.58)	$<$0.0001
spKt/*V*	1.64±0.04 (1.50, 0.04–3.80)	1.66±0.06 (1.56, 0.04–3.62)	1.61±0.05 (1.49, 0.76–3.80)	0.276
rKt/*V*	0.06±0.01 (0, 0–0.79)	0.04±0.01 (0, 0–0.79)	0.07±0.01 (0.02, 0–0.43)	0.019
nPCR (g/kg/day)	0.93±0.02 (0.92, 0.15–1.71)	1.02±0.03 (1.03, 0.15–1.71)	0.85±0.02 (0.83, 0.45–1.51)	$<$0.0001
Body weight (kg)	66.6±1.1 (64.6, 42.5–112.0)	65.9±1.6 (63.2, 45.4–112.0)	67.2±1.5 (66.1, 42.5–106.5)	0.336
BMI (kg/m^2)	23.9±0.4 (23.0, 16.0–48.6)	24.2±0.6 (23.1, 16.9–48.6)	23.7±0.4 (22.7, 16.0–35.2)	0.929
Fat mass (kg)	18.4±0.9 (15.3, 3.4–56.3)	19.1±1.4 (15.4, 3.4–56.3)	17.4±1.2 (15.1, 5.8–40.0)	0.631
FFM (kg)	48.6±0.9 (49.3, 30.3–71.1)	47.9±1.2 (49.0, 32.9–64.9)	49.5±1.4 (51.1, 30.3–71.1)	0.421
MAMC (cm)	23.4±0.3 (23.2, 15.0–31.4)	23.3±0.4 (22.9, 17.0–31.4)	23.6±0.5 (23.8, 15.0–30.8)	0.362
SGA (% impaired nutritional status)	32.9%	44.0%	21.1%	0.003
MIS	6.17±0.33 (5, 0–22)	6.95±0.47 (6, 0–22)	5.37±0.45 (4, 0–22)	0.011
Davies score	1.84±0.11 (2, 0–6)	1.95±0.14 (2, 0–6)	1.72±0.16 (2, 0–5)	0.246
Davies grade (low/medium/high)	38/81/56	13/47/28	25/34/28	0.053
Atherosclerotic disease (%)	46.6%	54.0%	39.0%	0.048
Diabetes (%)	29.7%	33.0%	26.4%	0.346

HD, hemodialysis; HDF, hemodiafiltration; CRP, C-reactive protein; spKt/*V*, single-pool Kt/*V* of urea nitrogen; rKt/*V*, renal Kt/*V* of urea nitrogen; nPCR, normalized protein catabolic rate; BMI, body mass index; FFM, fat-free mass; MAMC, mid-arm muscle circumference; SGA, Subjective Global Assessment; MIS, Malnutrition–Inflammation Score. Data are expressed as mean±s.e.m. (median, minimum–maximum). Differences between patients with high ($\geqslant$1.97 mg/l) and low ($<$1.97 mg/l) free *p*-cresol were evaluated using Mann–Whitney *U*-test or the χ^2 test for association where appropriate. Two-sided *P*-values are indicated in the last column.

Table 2 | Univariate Cox proportional hazards analysis for association of baseline variables with all-cause mortality (*n*=175)

Variable	Unit of increase	HR	*P*
Age	1 year	1.04 (1.02–1.07)	0.0002
Sex	Male vs female	0.65 (0.39–1.08)	0.096
Time since dialysis initiation	1 month	1.00 (0.99–1.01)	0.717
Treatment modality	HDF vs HD	0.58 (0.33–1.04)	0.065
Bicarbonate	1 mEq/l	0.98 (0.89–1.08)	0.685
Hemoglobin	1 g/dl	0.86 (0.72–1.04)	0.123
Albumin	0.1 g/dl	0.89 (0.82–0.96)	0.004
	<3.5 g/dl vs ⩾3.5 g/dl	1.76 (1.06–2.94)	0.030
CRP	10 mg/l	1.07 (1.01–1.13)	0.024
	⩾8.0 mg/l vs <8.0 mg/l	2.00 (1.17–3.40)	0.011
Urea	1 mg/dl	1.00 (0.99–1.01)	0.667
Creatinine	1 mg/dl	0.79 (0.70–0.88)	<0.0001
Phosphate	1 mg/dl	0.83 (0.70–0.99)	0.036
β_2-microglobulin	10 mg/l	1.05 (0.83–1.33)	0.678
Total *p*-cresol	10 mg/l	1.17 (0.95–1.46)	0.142
Free *p*-cresol	1 mg/l	1.10 (1.00–1.21)	0.058
	⩾1.97 vs <1.97 mg/l	1.73 (1.02–2.94)	0.043
% Free *p*-cresol of total	1%	1.03 (0.98–1.07)	0.270
spKt/*V*	1 unit	0.71 (0.38–1.33)	0.287
rKt/*V*	1 unit	0.01 (0.00–1.19)	0.060
	Present vs absent	0.51 (0.30–0.88)	0.016
nPCR	1 g/kg/day	0.84 (0.28–2.58)	0.762
Body weight	1 kg	1.00 (0.98–1.02)	0.852
BMI	1 kg/m^2	1.02 (0.96–1.08)	0.465
Fat mass	1 kg	1.02 (0.99–1.05)	0.287
FFM	1 kg	0.98 (0.94–1.01)	0.127
MAMC	1 cm	0.90 (0.81–1.01)	0.068
SGA	'Impaired' vs 'normal'	3.29 (1.87–5.80)	<0.0001
MIS	1 unit	1.11 (1.05–1.19)	0.001
Davies score	1 unit	1.63 (1.36–1.96)	<0.0001
Davies grade	1 grade	2.65 (1.74–4.04)	<0.0001
Atherosclerotic disease	Present vs absent	2.19 (1.29–3.74)	0.004
Diabetes	Present vs absent	2.37 (1.43–3.94)	0.001

HD, hemodialysis; HDF, hemodiafiltration; CRP, C-reactive protein; spKt/*V*, single pool Kt/*V* of urea nitrogen; rKt/*V*, renal Kt/*V* of urea nitrogen; nPCR, normalized protein catabolic rate; BMI, body mass index; FFM, fat-free mass; MAMC, mid-arm muscle circumference; SGA, Subjective Global Assessment; MIS, Malnutrition–Inflammation Score.
Relative risks of death for each variable are given as HR (95% CI) and *P*-value for the univariate Cox proportional hazards analysis.

with HD instead of hemodiafiltration (HDF). While their daily protein intake per kg body weight (normalized protein catabolic rate) was higher, they showed more signs of impaired nutritional status based on SGA and had a higher MIS. Finally, a significantly larger proportion of patients with high free levels of *p*-cresol at baseline had atherosclerotic disease as compared to those with low levels. To identify independent determinants of free *p*-cresol concentrations, a multivariate linear regression analysis was performed as outlined in Materials and Methods. Since MIS is mathematically dependent on other relevant variables (albumin and SGA), it was not introduced into the model. Also, Davies score, Davies grade, diabetes, and atherosclerotic disease are by definition covarying variables. For that reason, only Davies score was used. In the final regression model, the following variables were retained as independent predictors

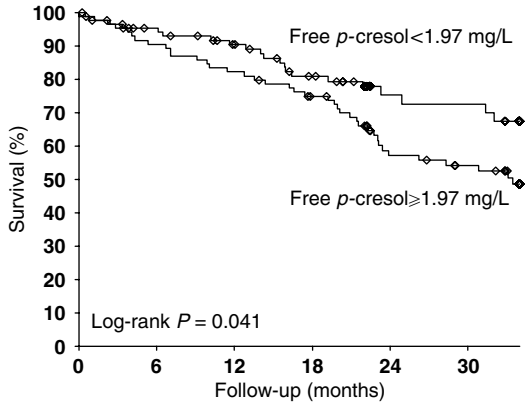

Figure 1 | Kaplan–Meier survival curves of patients with high free serum levels of *p*-cresol (⩾1.97 mg/l, *n* = 88) and patients with low free serum levels of *p*-cresol (<1.97 mg/l, *n* = 87). The *P*-value of the log-rank test is indicated in the figure (◇, censored value).

of the free serum level of *p*-cresol: serum albumin ($P<0.0001$), age ($P=0.013$), total *p*-cresol ($P<0.0001$), and impaired nutritional status based on SGA ($P=0.005$). The respective regression coefficients allowed formulating the following equation, which explained 77.6% (adjusted R^2) of the variability of free *p*-cresol (mg/l): 4.92–1.30 × (serum albumin (g/dl))–0.02 × (age (years)) + 0.17 × (total *p*-cresol (mg/l)) + 0.60 (in case of impaired nutritional status according to SGA).

Based on the findings of the univariate Cox proportional hazards analyses (Table 2) and taking the variables related to the free concentration of *p*-cresol into account (Table 1), the following parameters were entered in the multivariate survival analysis: age, sex, time since initiation of dialysis, treatment modality, hemoglobin, albumin, CRP, urea, creatinine, phosphate, β_2-microglobulin, free *p*-cresol, rKt/*V*, normalized protein catabolic rate, fat-free mass, mid-arm muscle circumference, SGA, and Davies score. Free *p*-cresol was entered as a binary variable (⩾1.97 mg/l or <1.97 mg/l) (Model A) or as a continuous variable (Model B). The proportionality assumption was fulfilled for all of the variables studied (proportionality test overall *P*-value = 0.536). In Table 3 the final results of Models A and B are given. Time since initiation of dialysis, Davies score, and impaired nutritional status based on SGA were consistently retained as independent factors influencing survival. Depending on the definition of the variable, the free serum level of *p*-cresol remained in the final model (Model A) or lost significance in the final step of the initial screening (Model B).

DISCUSSION

The results of this prospective observational cohort study suggest that, besides other well-known predictors of survival, the free serum level of the protein-bound uremic retention solute *p*-cresol is related to mortality in patients treated with dialysis. Several *in vitro* data have suggested a pathophysiological role of the solute in some important aspects of the uremic syndrome. *p*-Cresol depressed the respiratory burst

Table 3 | Multivariate Cox proportional hazards analysis of baseline variables associated with all-cause mortality (*n*=175)[a]

Variable	Unit of increase	HR (95% CI)	*P*
Model A ('free p-cresol' entered as a binary variable)			
Months on dialysis	1 month	0.98 (0.97–1.00]	0.005
Free *p*-cresol	high vs low[b]	2.28 (1.12–4.64)	0.023
SGA	'impaired' vs 'normal'	4.22 (2.15–8.29)	<0.0001
Davies score	1 unit	1.49 (1.19–1.86)	0.001
Model B ('free p-cresol' entered as a continuous variable)			
Age	1 year	1.03 (1.00–1.06)	0.047
Months on dialysis	1 month	0.98 (0.97–1.00)	0.007
SGA	'impaired' vs 'normal'	4.42 (2.25–8.71)	<0.0001
Davies score	1 unit	1.45 (1.17–1.80)	0.001

SGA, Subjective Global Assessment.
Relative risk of death for each variable is given as HR (95% CI) and *P*-value for Cox proportional hazards analysis.
[a]See Materials and methods for detailed description of the statistical methods used.
[b]'High' means $\geqslant$1.97 mg/l, 'low' means <1.97 mg/l.

reactivity of granulocytes and monocytes at concentrations encountered in stage 5 chronic kidney disease patients.[9] Later it was shown that the solute inhibits the synthesis of platelet-activating factor, another aspect of leukocyte function.[10] Further evidence for a role of *p*-cresol in the immunodeficiency of uremia was provided by the recent finding of its inhibitory effect on cytokine-induced expression of the endothelial adhesion molecules intercellular adhesion molecule-1 and vascular cellular adhesion molecule-1.[11] Endothelial dysfunction is another characteristic of the uremic syndrome.[16] It is considered to be an important contributor to the burden of cardiovascular atherosclerotic complications in renal disease patients. That *p*-cresol might be implicated in this disorder was suggested recently by two *in vitro* studies that described an inhibition of the regenerative properties and an increase in the permeability of endothelial cells by uremic concentrations of the solute.[12,13] In accordance with the data linking *p*-cresol to immunodeficiency, a study by De Smet *et al.*[14] described higher free serum levels of the solute in a group of HD patients hospitalized for infectious disease as compared to those hospitalized for other reasons. From the absence of a difference in total *p*-cresol between the two groups and results of *in vitro* experiments described in the same paper, the authors concluded that, similar to protein-bound drugs, the free, rather than the total, concentration of *p*-cresol is responsible for its biological activities. Our findings are in agreement with that thesis. Indeed, although survival of patients with total serum levels of *p*-cresol $\geqslant$18.9 mg/l was worse than in those with lower levels (50 vs 66%, log-rank *P*-value = 0.456), only the free serum concentrations of the solute conferred significantly to outcome.

Instead of attributing our findings to the sole influence of *p*-cresol, it seems more reasonable to consider the molecule as a representative of the group of protein-bound retention solutes. Indeed, also other members of this group have been found to be associated with immune dysfunction, endothelial cell dysfunction, and, closely related to the latter, oxidative stress. Like *p*-cresol, indoxyl sulfate was found to inhibit endothelial proliferation and wound repair.[12] Moreover, the molecule was shown to induce free radical production by renal tubular cells and activate nuclear factor κB, a central mediator of atherosclerosis.[17] Recently, another protein-bound uremic compound, phenylacetic acid, was identified to have an inhibitory impact on inducible nitric oxide synthase, an enzyme with a protective potential on endothelial function.[18]

In accordance with earlier observations, our data confirm that the relative risk of death in dialysis patients increases with higher age, the presence of atherosclerotic disease, diabetes, and higher overall comorbidity (Table 2).[19] Residual renal function seems to be a protective factor. Although the latter has been shown before in several studies including peritoneal dialysis patients, only few other studies with HD patients report on this issue.[20,21] Finally, parameters reflecting impaired nutritional status and/or inflammation, such as low serum albumin, low creatinine, high CRP, unfavorable SGA, and high MIS, are strong predictors of mortality. These observations are in agreement with literature data reporting on the association between malnutrition, inflammation, atherosclerosis, and mortality in renal disease patients.[22] In each of the multivariate models, comorbidity and nutritional status were retained as independent factors influencing survival (Table 3). Depending on the definition of the variable, the free serum level of *p*-cresol remained in the model or lost its significance in one of the final elimination steps. This discrepancy between Models A and B may seem confusing at first sight. In our opinion, however, it reflects the complex interplay between (free) *p*-cresol and other important baseline variables as far as their relationship with outcome is concerned. As can be appreciated from Table 1 and from the multivariate regression analysis, baseline free serum concentrations of *p*-cresol were indeed significantly related to age, serum albumin, impaired nutritional status according to SGA, MIS, and the presence of atherosclerotic disease, all associated with mortality themselves. The present study, however, does not allow to define the exact role of (free) *p*-cresol within this group of variables and further investigation will be needed to resolve this intriguing question. Nevertheless, whatever the strength and causality of the relationship between *p*-cresol and the other variables, it is clear from our data that a high free serum level of the solute (or related solutes) constitutes an additive risk factor for mortality in a dialysis population. Furthermore, it should be noted that only higher free levels of *p*-cresol were related to mortality, whereas no such relationship was seen for any of the other uremic retention molecules studied. This is of particular interest in view of the results of the recent hemodialysis (HEMO) and adequacy of dialysis Mexico (ADEMEX) studies.[19,23] These randomized trials failed to show an improvement of patient outcome by increasing the removal of low-molecular-mass water-soluble solutes and even middle molecules above current standards. Besides

several other hypotheses that have been put forward to explain these findings,[24,25] one should consider the limited removal of protein-bound solutes by the dialysis strategies evaluated in these trials. In view of the results of the present study, there seems to be an incentive to search for strategies that improve protein-bound solute removal and to investigate their impact on patient outcome.

Finally, from a methodological point of view, one might question the validity of survival statistics based on a single baseline measurement of the *p*-cresol level. Indeed, considerable bias could be expected if intraindividual variability over time were large. However, by measurement of three pre-dialysis *p*-cresol serum levels in seven patients (a) during 1 week and (b) 12 weeks apart, the pre-dialysis *p*-cresol was found to be acceptably stable (coefficient of variation (a) 12.1 ± 2.3% (median 10.5, minimum–maximum 2.0–19.4); (b) 12.6 ± 2.3% (median 9.6, minimum–maximum 6.1–21.3)). Another issue worth addressing is the fact that the median free *p*-cresol concentrations used to divide the study population in a high and low free *p*-cresol cohort should not be regarded as a clinically applicable cutoff value. We used this approach to enable reliable statistics on groups of equal and sufficient size. Again, larger trials with longer follow-up times will help to define relevant cutoff points that can be used in clinical algorithms.

In conclusion, our data suggest for the first time that free serum levels of the protein-bound uremic retention solute *p*-cresol are associated with mortality in patients treated with HD. Besides the molecule's own toxic effects, this finding probably reflects the toxicity of an entire group of physicochemically related retention solutes. Taking the results of the recent HEMO and ADEMEX studies into consideration, we believe that these findings may encourage scientists and clinicians to widen their field of interest beyond the scope of the small water-soluble uremic solutes and middle molecules.[19,23]

MATERIALS AND METHODS

Patients

In total, 175 patients with stage 5 chronic kidney disease (108 males, 67 females) who had undergone regular dialysis treatment for at least 2 months (29.8 ± 2.5 months (median 18.1, minimum–maximum 2.3–158.1)) in two Belgian HD centers (University Hospital, Leuven and Virga Jesse Hospital, Hasselt) were enrolled at two inclusion dates (January 1, 2002 and January 1, 2003). Causes of renal disease were diabetic nephropathy ($n = 34$), polycystic kidney disease ($n = 18$), glomerular disease ($n = 40$), tubulointerstitial disease ($n = 25$), vascular disease ($n = 8$), and unknown etiologies ($n = 50$). At inclusion, none of the patients had malignant disease, which could be expected to influence prognosis. All patients were being treated three times 4 h a week with HD or hemodiafiltration (post-dilution mode with 20 l of substitution fluid) using synthetic polysulfone or polyamide dialysis membranes with a surface area of 1.4–1.8 m^2. Dialyzers were not reused. The dialysate and/or substitution fluid contained 138.0 mEq/l sodium, 1.0–3.0 mEq/l potassium, 1.22 mg/dl magnesium, 6.0 mg/dl calcium, 32.0 mEq/l bicarbonate, 18.0 mg/dl acetate, and 100 mg/dl glucose. Purity of the fluids was monitored according to internationally accepted guidelines.[26] Dialysis efficiency was targeted based on single-pool Kt/*V* of urea nitrogen according to the National Kidney Foundation-Kidney Disease Outcomes Quality Initiative guidelines.[5] Dry weight was defined in every patient to achieve a normotensive edema-free state. The study was performed according to the principles of the Declaration of Helsinki Principles and was approved by the ethical committees of the University Hospital, Leuven and the Virga Jesse Hospital, Hasselt. Informed consent was obtained from all patients.

Procedures

Baseline evaluation. At inclusion, the following analyses were performed on a blood sample taken immediately before the mid-week dialysis treatment: bicarbonate (mEq/l), hemoglobin (g/dl), albumin (g/dl), urea (mg/dl), creatinine (mg/dl), phosphate (mg/dl), β_2-microglobulin (mg/l), and total and free *p*-cresol (mg/l). As a baseline measure of inflammation, the mean of all CRP (mg/l) levels, measured from 45 days before to 45 days after the inclusion date, was calculated.

Dialysis efficiency was evaluated according to the National Kidney Foundation-Kidney Disease Outcomes Quality Initiative guidelines.[5] Single-pool Kt/*V* of urea nitrogen was calculated using the second-generation logarithmic formula of Daugirdas.[27] Residual renal function was estimated from an interdialytic urine collection and expressed as weekly rKt/*V*.

As a measure of daily protein intake, normalized protein catabolic rate (g/kg/day) was calculated.[28] Anthropometrical parameters measured were body weight (kg) and body mass index, calculated from height and body weight (kg/m^2). Furthermore, fat mass, fat-free mass, and mid-arm muscle circumference were calculated from skinfold measurements and mid-arm circumference according to the appropriate formulas.[29] All measurements were performed by a skilled dietician within the first hour after the end of the dialysis session, when the patient was considered to be at dry weight.

Overall assessment of nutritional status was performed by SGA according to Detsky *et al.* and was reported as 'impaired nutritional status' (SGA scores B or C) or 'normal nutritional status' (SGA score A).[30] As a parameter reflecting both nutritional status and inflammation, the MIS was calculated. This comprehensive scoring system with a possible range from 0 to 30 combines components of the SGA with three additional elements (serum albumin, total iron-binding capacity, and body mass index).[31]

Based on a review of the medical records, comorbidity at baseline was assessed using the method described by Davies *et al.*[32] In brief, seven categories of comorbid conditions were scored as '1' (present) or '0' (absent). The total sum of scores was used to determine the grade of comorbidity. Grade 0 (low risk) is a zero score, grade 1 (medium risk) is a score of 1–2 and grade 2 (high risk) a cumulative score of $\geqslant 3$. Patients scoring 1 in the categories 'ischemic heart disease' and/or 'peripheral vascular disease' were considered to have atherosclerotic disease. The presence or absence of diabetes was also considered as a separate variable.

Biochemical analyses. Hemoglobin, total iron-binding capacity, CRP, bicarbonate, creatinine, urea, phosphate, and β_2-microglobulin were measured using standard laboratory techniques. The bromocresol green method was used for the determination of albumin. Total *p*-cresol (i.e. combined free and protein-bound fraction) was analyzed by the gas chromatography mass spectrometry technology as described earlier.[15,33] In summary, after deproteinization (acid and heat), addition of the internal

standard (2,6-dimethylphenol), and extraction (ethyl acetate), the samples were transferred to the gas chromatography mass spectrometry (Trace GC-MS, Thermofinnigan, San José, CA, USA) for injection and separation on a column, followed by identification of *p*-cresol by mass spectrometry. For the determination of free *p*-cresol, serum was first ultrafiltered at room temperature with an ultrafiltration membrane (Centrifree and MPS Micropartition UF Devices, Amicon, Beverly, MA, USA) with a molecular cutoff of 30 000 Da, followed by the same sample preparation and gas chromatography mass spectrometry analysis. Quantitative results were obtained by the internal standard method and calculated as concentrations (mg/l). The method has low intra- and inter-assay variabilities (coefficient of variation 3.33 and 5.30%, respectively). The detection limit for measurement of *p*-cresol is 0.15 mg/l and the limit of quantification is 0.30 mg/l. Values below the limit of quantification were treated as 0.30 mg/l in the statistical evaluation.

Follow-up and study end. Patients were followed up until November 30, 2004. During follow-up, deaths were accurately recorded and cause of death was specified. Causes of death were categorized as cardiovascular, infectious, malignancy or other.

Statistical analysis

Data are expressed as mean $\pm$ s.e.m. The median and range of each variable are given between brackets. For some analyses, the total group of patients was dichotomized based on the serum level of albumin (<3.5 g/dl or $\geqslant 3.5$ g/dl; HEMO study's standard of care as cutoff[19]), the free concentration of *p*-cresol ($\geqslant 1.97$ mg/l or <1.97 mg/l; median as cutoff), the serum level of CRP ($\geqslant 8.0$ mg/l or <8.0 mg/l; median as cutoff), residual renal function (present ($rKt/V>0$) or absent ($rKt/V=0$)) or Davies score ($\geqslant 2$ or <2; median as cutoff). The baseline characteristics of patients with high ($\geqslant 1.97$ mg/l) and low (<1.97 mg/l) free *p*-cresol were compared using the Mann–Whitney *U*-test. Categorical data were compared by the χ^2 test for association. To identify independent determinants of free concentrations of *p*-cresol, multivariate linear regression analysis was performed, including all univariately associated variables ($P<0.2$). After excluding colinearity, the best subset of variables was selected by backward elimination on $P<0.2$. This subset was then subjected to a final backward elimination procedure on $P<0.05$. Inspection of residual plots assured that the *a priori* assumptions for linear regression were justified.

Survival analysis was performed using two different statistical approaches. First, the univariate Cox proportional hazards model was used and the relative risk of death was expressed as a HR. Second, the cumulative incidence of death during follow-up was estimated by the Kaplan–Meier method, and the log-rank test was used to compare the differences of variables affecting survival. In each approach, data were censored at transplantation, transfer to another dialysis center or at the end of follow-up (November 30, 2004). Variables that affected all-cause mortality and/or were related to the free serum level of *p*-cresol ($P<0.2$) were included in a multivariate Cox proportional hazards analysis. First, the most relevant of them were selected by a backward elimination on $P<0.2$. The selected subset of variables was then entered in a backward elimination procedure on $P<0.05$ to define the best model explaining the outcome variable. The proportionality assumption was tested by using the 'proportionality test' statement within the PHREG procedure and by inspection of log(−log(survival)) curves. HRs are given with their 95% CIs. *P*-values less than 0.05 were considered significant. The SAS version 8.02 (SAS Institute, Cary, NC, USA) software program was used for the statistical analysis.

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Note added in proof

Between acceptance and publication of the present paper, two interesting studies on the molecule *p*-cresol have been published. Indeed, Martinez *et al*. (Martinez AW, Recht NS, Hostetter TH *et al*. Removal of *p*-cresol sulfate by hemodialysis. *J Am Soc Nephrol* 2005; **16**: 3430–3436) and we (de Loor H, Bammens B, Evenepoel P *et al*. Gas chromatographic-mass spectrometric analysis for measurement of *p*-cresol and its conjugated metabolites in uremic and normal serum. *Clin Chem* 2005; **51**: 1535–1538), independently of each other, found that *p*-cresol in human serum is almost entirely sulfated (*p*-cresylsulfate). The fact that *p*-cresylsulfate in body fluids is detected as *p*-cresol is due to the deconjugating effect of the analytical methodology used.

Although the findings of the above-mentioned papers may ask for a critical reappraisal of the *in vitro* toxicity data on *p*-cresol, they do not abrogate clinical associations such as the ones described in the present paper. Furthermore, in both studies the important protein binding of *p*-cresylsulfate was confirmed, supporting its use as a representative of the specific group of protein-bound uremic retention solutes originating from colonic protein fermentation.

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