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Original Article

Protein-bound uraemic toxins, dicarbonyl stress and advanced glycation end products in conventional and extended haemodialysis and haemodiafiltration

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ABSTRACT

Background. Protein-bound uraemic toxins (PBUT), dicarbonyl stress and advanced glycation end products (AGEs) associate with cardiovascular disease in dialysis. Intensive haemodialysis (HD) may have significant clinical benefits. The aim of this study was to evaluate the acute effects of conventional and extended HD and haemodiafiltration (HDF) on reduction ratio (RR) and total solute removal (TSR) of PBUT, dicarbonyl stress compounds and AGEs.

Methods. Thirteen stable conventional HD patients randomly completed a single study of 4-h HD (HD4), 4-h HDF (HDF4), 8-h HD (HD8) and 8-h HDF (HDF8) with a 2-week interval between the study sessions. RR and TSR of PBUT [indoxyl sulphate (IS), *p*-cresyl sulphate (PCS), *p*-cresyl glucuronide, 3-carboxyl-4-methyl-5-propyl-2-furanpropionic acid (CMPF), indole-3-acetic acid (IAA) and hippuric acid] of free and protein-bound AGEs [N^{ϵ}-(carboxymethyl)lysine (CML), N^{ϵ}-(carboxyethyl)lysine (CEL), N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine, pentosidine], as well as of dicarbonyl compounds [glyoxal, methylglyoxal, 3-deoxyglucosone], were determined.

Results. Compared with HD4, HDF4 resulted in increased RR of total and/or free fractions of IAA and IS as well as increased RR of free CML and CEL. HD8 and HDF8 showed a further increase in TSR and RR of PBUT (except CMPF), as well as of dicarbonyl stress and free AGEs compared with HD4 and HDF4. Compared with HD8, HDF8 only significantly increased RR of total and free IAA and free PCS, as well as RR of free CEL.

Conclusions. Dialysis time extension (HD8 and HDF8) optimized TSR and RR of PBUT, dicarbonyl stress and AGEs, whereas HDF8 was superior to HD8 for only a few compounds.

Keywords: advanced glycation end products, dicarbonyl stress, haemodiafiltration, haemodialysis, intensive, protein-bound uraemic toxins

INTRODUCTION

Accumulation of uraemic toxins is associated with morbidity and mortality in patients with end-stage renal disease (ESRD). Whereas conventional haemodialysis (HD) is effective in removing low-molecular-weight (MW) uraemic toxins, it is less efficient in removing middle-MW and protein-bound toxins [1–3]. Evidence regarding toxicity of protein-bound uraemic toxins (PBUT) is predominantly available for the indole and phenol-type toxins [2–11]. The addition of convection and the use of protein-leaking membranes have resulted in improvement in reduction ratio (RR) and/or a reduction in plasma levels of PBUT [12, 13], although data are conflicting [14]. Extended slow-flow dialysis techniques showed no or limited additive effect on the removal of PBUT [15, 16]. However, no

studies have compared the removal between short and long dialysis timeframes while maintaining the same blood and dialysis flow rates, and there are no data on the effect of extended haemodiafiltration (HDF).

Other types of toxins are dicarbonyl compounds, such as methylglyoxal (MG), glyoxal (GO) and 3-deoxyglucosone (3-DG). Dicarbonyl stress may be involved in vascular damage, either directly or through the formation of advanced glycation end products (AGEs) [17–19]. The formation of N^{ϵ}-(carboxymethyl)lysine (CML) may be mediated by GO and 3-DG [20, 21]. N^{ϵ}-(Carboxyethyl)lysine (CEL) can be formed by a non-enzymatic reaction between MG and lysine, whereas the formation of pentosidine is likely mediated through the Amadori pathway [20, 22]. N_{δ}-(5-Hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1) is a major AGE and is formed by the reaction of MG with arginine. Tissue damage by AGEs results from protein cross-linking and from activation of AGE-specific receptors (RAGEs) [20, 23]. Plasma levels of CML are related to mortality in dialysis patients [24]. Few studies have assessed the effect of dialysis on dicarbonyl stress compounds and AGEs [25–28].

Intensive (short daily and nocturnal) HD is associated with significant improvement of several clinical and laboratory markers [29]. This could be partially explained by the optimized removal of uraemic solutes [29–31]. Given the scarcity of comparative studies differentiating between the respective effects of time and convection on non-conventional uraemic toxins, the aim of the present study was to compare the reduction in concentration, removal and clearance of PBUT, dicarbonyl stress compounds and AGEs between conventional and extended HD and HDF sessions.

MATERIALS AND METHODS

Design of the study

Details of the study and of the cohort have been described previously [31]. FX80® dialysers were used for HD, and FX800® dialysers were used for HDF. Blood flow was 300 mL/min and dialysate flow 600 mL/min in all study sessions. Total substitution volume was 15 L for HDF4 and 30 L for HDF8. Online HDF was performed in post-dilution mode. The study was approved by the local ethics committee at the Maastricht University Medical Centre under number NL34908.068.10/MEC10-2-098 and was registered under clinicaltrials.gov number NCT01328119. Included patients gave written informed consent.

Blood and dialysate sampling and measurements

Serum samples were taken from the inlet bloodlines immediately before the onset of dialysis and at the end of the study session. A mixture of spent dialysate and ultrafiltrate was continuously collected in a fractionated fashion in a bag. At the end of the treatment and after thorough mixing, a 10 mL of sample was drawn from the collection bag in order to quantify solute concentration. All samples were stored at −80°C until analysis.

To establish the total concentration of hippuric acid (HA) [79 Da, protein binding (PB) ∼40–50%], indole-3-acetic acid (IAA) (175 Da, PB ∼75–95%), indoxyl sulphate (IS) (212 Da, PB ∼90–95%), *p*-cresyl sulphate (PCS) (187 Da, PB ∼95%), *p*-cresyl glucuronide (PCG) (284 Da, PB ∼10–30%) and 3-carboxyl-4-methyl-5-propyl-2-furanpropionic acid (CMPF) (240 Da, PB ∼100%) [1, 3], serum and dialysate samples were deproteinized by heat denaturation and were analysed by reversed-phase high-performance liquid chromatography (RP-HPLC) [32]. IS and IAA (excitation λ_{ex}: 280 nm; emission λ_{em}: 340 nm), as well as PCS and PCG (λ_{ex}: 265 nm; λ_{em}: 290 nm), were determined by fluorescence analysis. HA and CMPF were analysed by UV detection at 254 nm [33]. Due to technical problems, dialysate concentrations of PCS could not be measured. Serum total protein (TP) was analysed according to standard methods.

The following dicarbonyl compounds (α-oxaldehydes) were measured: GO (58 Da), MG (72 Da, PB 99%) and 3-DG (162 Da). As representatives of AGEs, CEL (±200 Da), CML (204 Da, PB > 95%), MG-H1 and pentosidine (342 Da, PB > 95%) [20, 32] were measured in its free form as well as in protein-bound form. Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS) was used to determine protein-bound and free CML, CEL and MG-H1, as well as to analyse the dicarbonyl compounds GO, MG and 3-DG [34, 35]. Pentosidine was detected in plasma protein hydrolysates by one-column RP-HPLC method [36].

Calculations

Total solute removal (TSR) was calculated by multiplying the dialysate solute concentration of the 10 mL representative spent dialysate sample by the sum of dialysate volume, the ultrafiltration volume and (in the HDF sessions) the substitution volume. To calculate TSR of dicarbonyl stress compounds, we subtracted the concentration of fresh dialysate from the concentration of dialysate collected at the end of the study session. Dialytic clearances (Cls) were calculated as TSR divided by the dialysis duration and by the log mean of the average of pre- and post-dialysis solute blood concentrations. RR of solutes was defined as a function of pre-dialysis (C_{pre}) and post-dialysis (C_{post}) concentrations ($RR = [1 - (C_{post}/C_{pre})] \times 100$). For all protein-bound compounds, concentration at the dialysis end (C_{post}) was corrected for haemoconcentration based on TP concentration at start versus end of the dialysis session.

Statistical analysis

Data are expressed as mean ± SD. The Kolmogorov–Smirnov test was used to assess the normal distribution of the data. Comparison of mean values among groups was done using repeated measures ANOVA, and, if significant, followed by Fisher's least significant difference (LSD) test to assess differences between individual time points. The tests were considered significant if the P-value was <0.05. P-values are presented in the Results section if the value was between 0.01 and 0.05. The IBM SPSS Statistics 20 program was used for statistical analyses.

For paired comparison of two groups, 11 patients would be needed to observe significant differences in RR of 10% between two groups with a power of 0.8 and an alpha of 0.05. The RR of PBUT was used as the primary outcome parameter.

In a recent study by Meert *et al.* [13], RR of PCS was 38 ± 10% for conventional HD as compared with 50 ± 8% for HDF.

RESULTS

Patient and treatment characteristics

Thirteen prevalent conventional HD patients underwent, in random order, at midweek, a 4-h HD (HD4) session, a 4-h post-dilution online HDF (HDF4) session, an 8-h HD (HD8) session and an 8-h post-dilution online HDF (HDF8) session, with a 2-week interval between the study sessions. Ten of the 13 patients were men. Mean age was 53.6 ± 20.4 years. Patients were on conventional HD therapy for 49 ± 29 months. Three patients had mean residual diuresis of 420 ± 96 mL per 24 h. Treatment characteristics of the different dialysis modalities were described previously [31]. Baseline pre-dialysis serum levels of PBUT, AGEs and dicarbonyl stress were comparable among the four strategies, except for higher baseline protein-bound CEL levels in HD8 compared with HDF4 and higher baseline protein-bound MG-H1 levels in HDF8 compared with HD8 (Table 1). Since the patients underwent only one respective study session followed by a 2-week interval of conventional routine HD (for wash-out reasons), we did not see any differences in pre-dialysis uraemic toxin levels at the end of each study period (Table 2).

Reduction ratio, total solute removal and dialytic clearance of protein-bound uraemic toxins

RRs of total and free fractions of PCG, HA, IAA, IS and PCS for the four different modalities are displayed in Figures 1 and 2, respectively. No free fraction of CMPF could be measured. Also, no significant reduction in CMPF was observed for any of the four modalities (Figure 1). For the total PBUT concentrations, HDF4 resulted in higher RR of IAA (P = 0.014) and IS (P = 0.035) compared with HD4. HD8 increased RR of total HA, IAA, IS and PCS compared with HD4 and HDF4 (Figure 1). HDF8 also resulted in significantly increased RR of total HA, IS and PCS compared with HD4 and HDF4, as well as in higher RR of total IAA compared with HD4, HDF4 and HD8 (P = 0.043). For the free PBUT fractions, HDF4 showed a significant increase in RR of IAA compared with HD4 (Figure 2). HD8 showed higher RR of free IAA and IS compared with HD4 and HDF4. HDF8 resulted in higher RR of free PCG, HA, IS and PCS (P = 0.046) compared with HD4 and HDF4, as well as higher RR of free IAA and free PCS (P = 0.045) compared with HD4, HDF4 and HD8.

TSR and Cl of PBUT (except for PCS, which could not be traced as such in the dialysate) are shown in Table 3. HDF4 did not result in a significant increase in TSR of PBUT compared with HD4. HD8 and HDF8 showed increased TSR of all PBUT compared with HD4 (PCG HDF8–HD4, P = 0.017; HA HD8–HD4, P = 0.023; HA HDF8–HD4, P = 0.040) except for TSR of IAA in HDF8 (HDF8–HD4, P = 0.058). HD8 and HDF8 increased TSR of HA compared with HDF4. No significant differences in TSR of PBUT between HD and HDF were found for the same length of dialysis, either conventional or extended. Cl of the different PBUT was similar among groups.

Reduction ratio, total solute removal and dialytic clearance of dicarbonyl stress and RR of AGEs

HDF4 did not increase RR of dicarbonyl stress compounds compared with HD4 (Figure 3). HD8 increased RR of MG and GO compared with HD4 and HDF4 (GO HD8–HDF4, P =

Table 1. Pre-treatment serum levels of PBUT, AGEs and dicarbonyl stress compounds

	HD4	HDF4	HD8	HDF8
PCG_{total}	0.60 ± 0.73	0.76 ± 1.04	0.63 ± 0.67	0.88 ± 1.15
PCG_{free}	0.54 ± 0.66	0.68 ± 0.96	0.58 ± 0.63	0.80 ± 1.08
HA_{total}	3.81 ± 2.20	4.12 ± 2.82	4.19 ± 2.53	4.37 ± 2.67
HA_{free}	2.15 ± 1.41	2.26 ± 1.66	2.43 ± 1.81	2.58 ± 1.87
IAA_{total}	0.21 ± 0.23	0.21 ± 0.16	0.19 ± 0.17	0.20 ± 0.17
IAA_{free}	0.063 ± 0.082	0.061 ± 0.057	0.055 ± 0.056	0.057 ± 0.052
IS_{total}	2.95 ± 1.37	3.08 ± 1.73	3.02 ± 1.28	2.99 ± 1.47
IS_{free}	0.18 ± 0.11	0.18 ± 0.12	0.18 ± 0.10	0.19 ± 0.13
PCS_{total}	2.79 ± 1.53	2.64 ± 2.04	2.79 ± 1.37	2.85 ± 1.80
PCS_{free}	0.34 ± .37	0.16 ± 0.15	0.24 ± 0.28	0.19 ± .15
CMPF	0.22 ± 0.26	0.22 ± 0.30	0.21 ± 0.25	0.22 ± 0.27
$CML_{protein\text{-}bound}$	10 493 ± 3283	10 553 ± 3050	10 690 ± 3331	10 743 ± 3194
CML_{free}	1733 ± 604	1854 ± 492	1839 ± 626	1783 ± 430
$CEL_{protein\text{-}bound}$	1897 ± 465	1869 ± 261	2065 ± 403*	2061 ± 468
CEL_{free}	986 ± 376	1061 ± 299	987 ± 358	1082 ± 423
$MG\text{-}H1_{protein\text{-}bound}$	3758 ± 77	3724 ± 883	3662 ± 996	4091 ± 841**
$MG\text{-}H1_{free}$	4786 ± 2416	5215 ± 1994	4981 ± 2330	6119 ± 5082
$Pentosidine_{protein\text{-}bound}$	272 ± 149	280 ± 160	277 ± 157	279 ± 149
MG	1839 ± 852	1815 ± 481	2079 ± 821	1794 ± 489
GO	1878 ± 439	1991 ± 371	1939 ± 462	1918 ± 289
3-DG	1740 ± 666	1675 ± 392	1829 ± 580	1528 ± 226

Data in mean ± SD.
IS (indoxyl sulphate), PCS (p-cresyl sulphate), PCG (p-cresyl glucuronide), IAA (indole-3-acetic acid), CMPF (3-carboxyl-4-methyl-5-propyl-2-furanpropionic acid) and HA (hippuric acid) in mg/dL.
CML (N-carboxymethyl-lysine), CEL (N-carboxyethyl-lysine), MG-H1 (N-hydromethyl-imidazolon-ornithine), pentosidine, MG (methylglyoxal), GO (glyoxal), 3-DG (3-deoxyglucosone) in nmol/L.
*P < 0.05 versus HDF4, **P < 0.05 versus HD8.

Table 2. Pre-dialysis concentrations of studied uraemic toxins 2 weeks after one single study session of the respective dialysis modalities (single study sessions were separated by a 2-week interval of routine conventional HD)

	2 weeks post-HD4	2 weeks post-HDF4	2 weeks post-HD8	2 weeks post-HDF8
PCG_{total}	1.03 ± 1.09	0.42 ± 0.41	0.97 ± 1.30	0.63 ± 0.85
PCG_{free}	0.94 ± 1.02	0.37 ± 0.37	0.89 ± 1.21	0.57 ± 0.77
HA_{total}	4.81 ± 2.67	3.33 ± 2.30	4.12 ± 2.57	3.97 ± 2.56
HA_{free}	2.84 ± 2.03	1.87 ± 1.47	2.38 ± 1.67	2.31 ± 1.79
IAA_{total}	0.17 ± 0.10	0.18 ± 0.18	0.22 ± 0.16	0.22 ± 0.26
IAA_{free}	0.054 ± 0.042	0.048 ± 0.050	0.065 ± 0.059	0.066 ± 0.089
IS_{total}	3.16 ± 1.12	3.00 ± 1.61	3.03 ± 1.53	2.72 ± 1.56
IS_{free}	0.20 ± 0.12	0.18 ± 0.13	0.20 ± 0.12	0.17 ± 0.12
PCS_{total}	3.10 ± 1.90	2.31 ± 1.19	3.02 ± 1.93	2.88 ± 1.88
PCS_{free}	0.21 ± 0.16	0.26 ± 0.34	0.20 ± 0.16	0.28 ± 0.39
CMPF	0.26 ± 0.37	0.23 ± 0.29	0.23 ± 0.29	0.16 ± 0.12
$CML_{protein\text{-}bound}$	9965 ± 2292	10 273 ± 3784	10 388 ± 2987	10 751 ± 3630
CML_{free}	1910 ± 419	1747 ± 526	1843 ± 489	1649 ± 801
$CEL_{protein\text{-}bound}$	2036 ± 229	1878 ± 593	1886 ± 289	2028 ± 463
CEL_{free}	1130 ± 272	1023 ± 377	1028 ± 420	909 ± 449
$MG\text{-}H1_{protein\text{-}bound}$	3878 ± 879	3960 ± 787	3670 ± 808*	3790 ± 1024
$MG\text{-}H1_{free}$	5394 ± 1975	5802 ± 5377	5211 ± 2869	4884 ± 3111
$Pentosidine_{protein\text{-}bound}$	263 ± 116	254 ± 149	267 ± 161	291 ± 172
MG	1803 ± 644	1859 ± 786	1750 ± 539	2046 ± 955
GO	2029 ± 271	1788 ± 488	1893 ± 311	1849 ± 418
3-DG	1787 ± 427	1630 ± 541	1606 ± 496	1537 ± 392

Data in mean ± SD.

IS (indoxyl sulphate), PCS (p-cresyl sulphate), PCG (p-cresyl glucuronide), IAA (indole-3-acetic acid), CMPF (3-carboxyl-4-methyl-5-propyl-2-furanpropionic acid) and HA (hippuric acid) in mg/dL.

CML (N-carboxymethyl-lysine), CEL (N-carboxyethyl-lysine), MG-H1 (N-hydromethyl-imidazolon-ornithine), pentosidine, MG (methylglyoxal), GO (glyoxal), 3-DG (3-deoxyglucosone) in nmol/L.

*P < 0.05 versus HDF4 (paired *t*-tests were performed; LSD was not valid because of the missing data for every patient, since no blood samples were taken 2 weeks after the last study session).

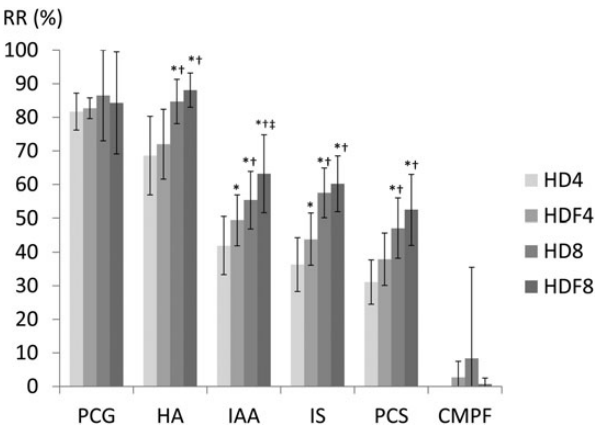

FIGURE 1: RRs of total PBUT. Abbreviations: PCG: p-cresyl glucuronide; HA: hippuric acid; IAA: indole-3-acetic acid; IS: indoxyl sulphate; PCS: p-cresyl sulphate; CMPF: 3-carboxyl-4-methyl-5-propyl-2-furanpropionic acid; HD4: 4-h haemodialysis; HDF4: 4-h haemodiafiltration; HD8: 8-h haemodialysis; HDF8: 8-h haemodiafiltration. *P < 0.05 versus HD4; †P < 0.05 versus HDF4; ‡P < 0.05 versus HD8.

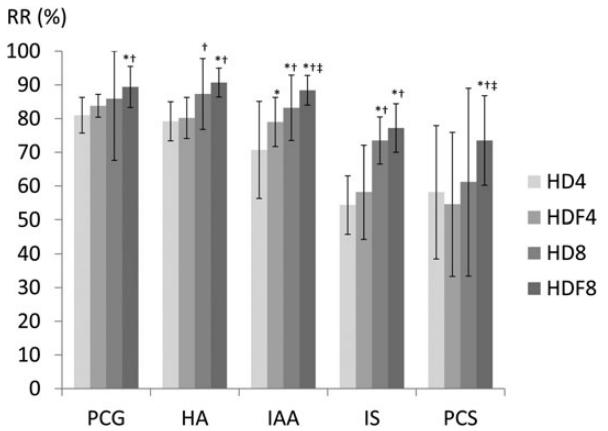

FIGURE 2: RRs of free PBUT. Abbreviations: PCG: p-cresyl glucuronide; HA: hippuric acid; IAA: indole-3-acetic acid; IS: indoxyl sulphate; PCS: p-cresyl sulphate; HD4: 4-h haemodialysis; HDF4: 4-h haemodiafiltration; HD8: 8-h haemodialysis; HDF8: 8-h haemodiafiltration. *P < 0.05 versus HD4; †P < 0.05 versus HDF4; ‡P < 0.05 versus HD8.

0.036), and HDF8 increased RR of MG and GO compared with HD4. RR of 3-DG was not increased by HD8 and HDF8 compared with HD4 and HDF4 (RR 19.1 ± 27.2% in HD4, 12.7 ± 17.4% in HDF4, 11.1 ± 19.0% in HD8, and 12.0 ± 17.3% in HDF8 sessions) (Figure 3).

TSR and Cl of dicarbonyl stress compounds are shown in Table 4. HDF4 did not increase TSR of GO, MG or 3-DG compared with HD4. HD8 increased TSR of GO compared with HD4 (P = 0.035) as well as TSR of 3-DG compared with HD4 and HDF4. Similarly to HD8, HDF8 resulted in increased TSR of GO compared with HD4 (P = 0.041) as well as increased TSR of 3-DG compared with HD4 and HDF4. There was also a trend to higher TSR for MG in HD8 and HDF8 compared with HD4 and HDF4. Cl of the different dicarbonyl stress compounds was similar among groups except for lower Cl of GO in HDF8 compared with HD4 (P = 0.017).

Table 3. TSRs and dialytic clearances (Cls) of PBUT

	PCG	HA	IAA	IS
TSR_{HD4}	108.0 ± 139.0	622.7 ± 411.4	14.6 ± 10.9	148.2 ± 80.3
TSR_{HDF4}	133.5 ± 183.5	657.7 ± 450.3	16.5 ± 10.0	176.5 ± 91.2
TSR_{HD8}	169.0 ± 182.5*	965.0 ± 802.2**	22.4 ± 13.9*	239.5 ± 128.4*
TSR_{HDF8}	180.7 ± 235.1*	945.5 ± 615.7**	19.6 ± 11.9	249.1 ± 130.0*
Cl_{HD4}	158.8 ± 42.3	118.7 ± 35.6	41.5 ± 18.0	25.5 ± 8.1
Cl_{HDF4}	151.1 ± 35.2	123.0 ± 20.1	40.4 ± 15.8	27.6 ± 7.2
Cl_{HD8}	143.3 ± 40.7	114.0 ± 26.9	38.6 ± 14.2	25.0 ± 6.5
Cl_{HDF8}	133.5 ± 41.6	114.7 ± 26.9	38.2 ± 17.6	28.0 ± 9.9

Data in mean ± SD.
TSR for PCG (p-cresyl glucuronide), HA (hippuric acid), IAA (indole-3-acetic acid) and IS (indoxyl sulphate) in mg.
Cl in mL/min.
*P < 0.05 versus HD4, **P < 0.05 versus HDF4.

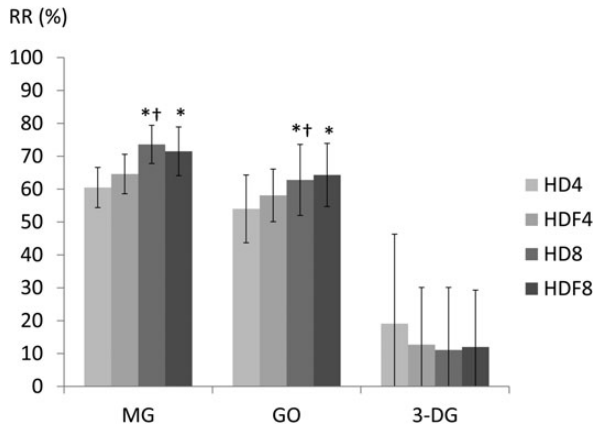

FIGURE 3: RRs of dicarbonyl stress. Abbreviations: MG: methylglyoxal; GO: glyoxal; 3-DG: 3-deoxyglucosone; HD4: 4-h haemodialysis; HDF4: 4-h haemodiafiltration; HD8: 8-h haemodialysis; HDF8: 8-h haemodiafiltration. *P < 0.05 versus HD4; †P < 0.05 versus HDF4.

Table 4. TSRs and dialytic clearances (Cls) of dicarbonyl stress compounds

	GO	MG	3-DG
TSR_{HD4}	14.9 ± 4.5	39.5 ± 13.2	51.6 ± 23.5
TSR_{HDF4}	15.9 ± 5.7	46.2 ± 15.9	48.8 ± 21.8
TSR_{HD8}	21.2 ± 10.0*	62.0 ± 29.7	94.8 ± 25.3**
TSR_{HDF8}	21.9 ± 7.7*	57.9 ± 24.0	102.7 ± 34.8**
Cl_{HD4}	47.8 ± 12.1	163.2 ± 46.7	122.1 ± 55.9
Cl_{HDF4}	54.0 ± 24.6	184.7 ± 63.8	124.2 ± 22.8
Cl_{HD8}	37.9 ± 15.4	124.4 ± 45.9	107.5 ± 37.4
Cl_{HDF8}	39.0 ± 13.7*	128.0 ± 51.1	116.5 ± 29.4

Data in mean ± SD.
TSR for GO (glyoxal), MG (methylglyoxal) and 3-DG (3-deoxyglucosone) in µmol.
Cl in mL/min.
*P < 0.05 versus HD4, **P < 0.05 versus HDF4.

HDF4 showed a significant increase in RR of free CML (P = 0.016) and CEL (P = 0.034) compared with HD4 (Figure 4). HD8 resulted in higher RR of free CML, CEL and MG-H1 compared with HD4 and HDF4. HDF8 increased RR of free MG-H1 compared with HD4 and HDF4, as well as increased RR of free CEL compared with HD4, HDF4 and HD8 (free CEL HDF8–HD8, P = 0.016). No reduction in the protein-bound AGEs (CML, CEL, MG-H1 and pentosidine) was found in the four different dialysis modalities (Figure 4).

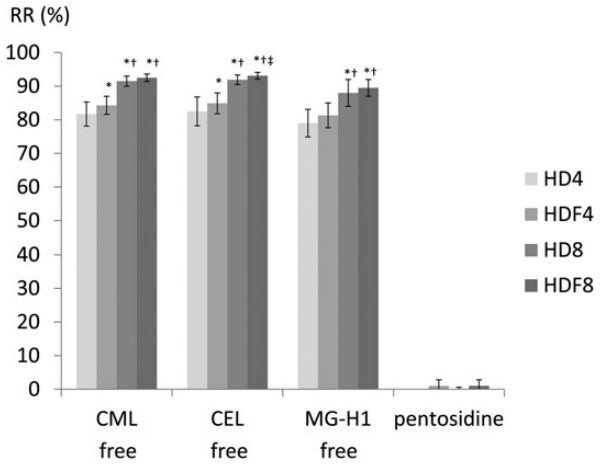

FIGURE 4: RRs of AGEs. Abbreviations: CML: N^{ϵ}-(carboxymethyl)lysine; CEL: N^{ϵ}-(carboxyethyl)lysine; MG-H1: N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine; HD4: 4-h haemodialysis; HDF4: 4-h haemodiafiltration; HD8: 8-h haemodialysis; HDF8: 8-h haemodiafiltration. *P < 0.05 versus HD4; †P < 0.05 versus HDF4; ‡P < 0.05 versus HD8.

DISCUSSION

This randomized crossover study shows, for the first time, markedly increased TSR and RR of PBUT, dicarbonyl stress compounds and AGEs by prolonging dialysis treatment time. HD8 and HDF8 increased RR and TSR of most PBUT, dicarbonyl stress and free AGEs compared with HD4 and HDF4. HDF8 resulted in the highest RR of total and free IAA, free PCS and free CEL compared with HD4, HDF4 and HD8. The effects of time and convection on the reduction and removal of uraemic toxins could be optimally disentangled by comparing conventional and extended sessions of HD and HDF using identical prescriptions of blood and dialysate flow among the four groups.

The most important finding of this study is an increased removal of PBUT during long dialysis, if blood and dialysate flows are kept at 300 and 500 mL/min, respectively. This is in contrast with findings from Basile *et al.*, who observed no increased removal of IAA, IS, HA and homocysteine during 8 h of slow-flow dialysis while using blood and dialysate flows of only 190 mL/min [15]. Since time added more than convection to their removal, it seems that the clearance of hippuric, indolic and phenolic PBUT is predominantly diffusive, and thus dependent upon blood and dialysate flows. It also suggests that their clearance is mainly achieved by removal of their free fraction. Increasing dialysis time will progressively lower free PBUT levels, facilitating dissociation of bound toxins from albumin resulting in continued availability of these toxins for diffusion to the dialysate, but with PB as a refraining factor. Moreover, a multi-compartmental behaviour of PBUT, by which increasing dialysis time could allow refilling of PBUT from secondary compartments, was also suggested by Meijers *et al.* [16], who found lower RR than in our study with nocturnal HD but with lower blood and dialysate flows. The importance of diffusion on removal of PBUT

during extended dialysis was also highlighted by Sirich *et al.* [37], who showed better clearance if a high dialyser surface was combined to high dialysate flow. Luo *et al.* [38] demonstrated an increase in dialytic clearance of PBUT by increasing dialysate flow through the use of two high-flux dialysers in series. Also, in a study of short daily HD, in which blood flow of 250 mL/min and dialysate flow of 500 mL/min were applied, a reduction in pre-dialytic levels of *p*-cresol, IS and IAA was observed [33].

The limited additive effect of convection on PBUT could be explained by the fact that HDF mainly contributes to optimized removal of middle molecules, whereas the additive effect on small molecules (such as the free fraction of PBUT) is limited. RR of total IS and RR of total and free IAA in our study was higher with HDF4 compared with HD4, without a significant increase in TSR. Interestingly, whereas no effect of HDF4 on the RR of PCS was observed compared with HD4, RR of free PCS was higher for HDF8 compared with HD4 and HDF4. In the literature, divergent results of the effects of convective techniques on RR and/or TSR of PBUT are reported, but even if a positive effect was found, the difference was never spectacular [12–14, 39, 40].

RR and TSR of dicarbonyl products and free AGEs were also significantly enhanced by increasing treatment time. Convection had an additional effect on RR of free CML and CEL. MG, GO and 3-DG are relatively small substances with high PB reversibility [41]. PB reversibility, however, does not appear to apply for the AGEs CML, CEL and pentosidine, whose total fractions actually increased during dialysis, likely as a result of haemoconcentration. A recent meta-analysis showed a significant reduction in pre-dialytic plasma levels of pentosidine but not in the RR of pentosidine and AGEs with high-flux HD or HDF as compared with low-flux HD [40], suggesting that other mechanisms (e.g. a decrease in inflammation) might lead to beneficial effects of these treatments on AGE levels. Lin *et al.* observed increased RR and clearance of AGEs with online HDF as compared with high-flux HD [28]. Beck *et al.* also found a reduction in plasma AGEs after a session with online HDF [42]. Among the scanty studies addressing the removal of dicarbonyl compounds, Agalou *et al.* found that a single 4-h HD session with a polysulfone dialyser reduced levels of free CML, CEL, pentosidine but also of free GO and MG, in a way fairly similar to the findings in our study with standard 4-h HD [25]. However, our results were markedly more impressive during the extended sessions.

Of note, we observed for a number of molecules divergent effects on RR versus TSR in the comparison of HD versus HDF as well as of extended versus short treatment time sessions. In general, significant differences in RR without differences in TSR between HD and HDF are to be explained by a more efficient removal during HDF from the plasmatic compartment by highly efficient dialysis, which is not matched by a corresponding solute shift from the extraplasmatic to the intraplasmatic compartment. With an increase in treatment time, the dissociation of intercompartmental transport becomes less pronounced as there is more opportunity for equilibration between compartments. An additional explanation for divergent effects on RR and TSR may be the differential generation and reactivity of studied molecules, which may especially apply for the dicarbonyl stress compounds.

Our study has some limitations. First, patient sample size was small. Also, long-term effects of the different treatments on these non-traditional uraemic toxin levels could not be assessed; in order to study long-term differences in pre-dialysis concentrations of the different treatment modalities, we should have treated these patients with these respective dialysis modalities for a prolonged period of time instead of during only one study session. Convective volumes during HDF were relatively low, especially for 4-h HDF [43, 44]. Dialysate concentrations of free AGEs were not measured. No post-dialysis blood samples were taken to assess rebound of uraemic toxins, but this bias was obviated by also assessing TSR.

This study has several strengths. First, it is a randomized crossover study of multiple dialysis modalities analysing a multitude of non-traditional uraemic toxins that have been related to outcome as well as end-organ complications in clinical and experimental studies. Direct dialysate quantification was performed to assess uraemic toxin removal. Lastly, blood flow and dialysate flow were identical in HD and HDF treatments allowing the strict separation of the time and convection component.

In conclusion, in this randomized crossover study, extended HD and HDF, using comparable blood flows as in standard dialysis sessions, enhanced RR and removal of PBUT, dicarbonyl stress and free AGEs. Conventional HDF had an additive effect on the RR of some PBUT and free AGEs but not on dicarbonyl compounds. The highest RR was obtained with extended HDF, especially for RR of total and free IAA and free PCS, as well as RR of free CEL. The clinical relevance of these findings needs to be addressed in controlled prospective trials.

AUTHORS' CONTRIBUTIONS

Research idea and study design: T.C., S.E., G.G., R.V., F.V.D.S., K.M.L., J.P.K. and C.G.S.; data acquisition: T.C., S.E., G.G., J.S., M.V.L., M.A.W. and C.G.S.; data analysis/interpretation: T.C., S.E., R.V., G.G., J.P.K. and C.G.S.; statistical analysis: T.C.; supervision or mentorship: J.P.K. and C.G.S. Each author contributed important intellectual content during manuscript drafting or revision and accepts accountability for the overall work by ensuring that questions pertaining to the accuracy or integrity of any portion of the work are appropriately investigated and resolved. C.G.S. takes responsibility that this study has been reported honestly, accurately and transparently; that no important aspects of the study have been omitted; and that discrepancies from the study as planned (and, if relevant, registered) have been explained.

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CONFLICT OF INTEREST STATEMENT

The results presented in this paper have not been published previously in whole or part, except in abstract format.

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