Title: Micronutrient and amino acid losses during renal replacement therapy for acute kidney injury.

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Running title:	Micronutrient loss during RRT for AKI
Abstract word count:	218 words, 1,498 characters.
Main text word count:	3937 words
Total word count:	3937

Author contributions

M.A.J.D. conceived the study; D.S.G. and M.A.J.D. designed research; W.C.O., J.C.A., D.S.G., B.M., M.R., A.S., D.H., & M.A.J.D. conducted the research; D.S.G., W.C.O. and M.A.J.D. co-wrote the manuscript. All authors critically evaluated the paper. D.S.G. conducted the statistical analyses. D.S.G. and M.A.J.D. have primary responsibility for its final content.

Abstract

Introduction

Malnutrition is common in patients with acute kidney injury (AKI), particularly in those requiring renal replacement therapy (RRT). RRT removes metabolic waste products and toxins but it will inevitably also remove useful molecules such as micronutrients, which might aggravate malnutrition. RRT modalities vary in mechanism of solute removal e.g. intermittent hemodialysis (IHD) uses diffusion, continuous veno-venous hemofiltration (CVVH) uses convection and sustained low-efficiency diafiltration (SLEDf) uses a combination of these.

Methods

We assessed micronutrient and amino acid losses in 3 different RRT modalities in patients with AKI (IHD, n=27; SLEDf, n=12; CVVH, n=21) after correction for dialysis dose and plasma concentrations.

Results

Total losses were affected by modality, generally CVVH >> SLEDf > IHD (e.g. amino acid loss was 18.69 ± 3.04, 8.21 ± 4.07 and 5.13 ± 3.1g, respectively; $P<0.001$). Loss of specific trace elements (e.g. copper and zinc) during RRT was marked, with considerable heterogeneity between RRT types (e.g. +849 and +2325 µg/L lost during SLEDf vs IHD, respectively), whereas effluent losses of copper and zinc decreased during CVVH (effect size relative to IHD, -3167 and -1442 µg/L, respectively). B-vitamins were undetectable in effluent, but experimental modelling estimated 40-60% loss within first 15 mins of RRT.

Conclusion

Micronutrient and amino acid losses are marked during RRT in patients with AKI, with variation between RRT modalities and micronutrients.

Key words: Acute Kidney Injury, malnutrition, trace elements, amino acids, B vitamins, renal replacement therapy

Introduction

Prevalence of disease related malnutrition (DRM) in AKI has been estimated at up to 42% and is an independent predictor of mortality [1]. Co-morbidities, prolonged hospital stay and RRT may exacerbate malnutrition. RRT may be required in AKI to remove metabolic waste products and toxins, and to regulate fluid and electrolyte balance. Inevitably, mechanisms by which RRT removes unwanted substances will also remove some essential solutes such as micronutrients and amino acids [2, 3].

Many RRT modalities are now available to support patients with severe AKI. These vary in duration of treatment (intermittent to continuous) and mechanism of solute clearance (diffusion, convection or combination). These differences are likely to affect micronutrient losses. Despite high risk of DRM in patients with AKI and the huge financial burden of AKI to health services [4], little research has been published on micronutrient losses in acute RRT. We studied micronutrient losses in three RRT modalities routinely used in our renal and intensive care units: intermittent haemodialysis (IHD), sustained low efficiency diafiltration (SLEDf) and continuous veno-venous haemofiltration (CVVH). These treatments vary in duration (intermittent, intermediate and continuous, respectively) and mechanism of solute removal (diffusion (IHD), convection (CVVH) or combination (SLEDf)). Specific micronutrients and amino acids were selected for investigation based on their physiological and nutritional importance, especially in critical illness, and if their physical properties suggested removal primarily by diffusion or convection. Those fulfilling both criteria were of particular interest, such as the amino acids glutamine, arginine and taurine. Micronutrients of interest included the trace elements zinc, selenium, iron and manganese and several B-vitamins. We also wished to quantify losses of water-soluble B-vitamins: thiamine (B1), pyridoxine (B6), folic acid (B9) and cobalamin (B12). All are relatively small water-soluble molecules (B12, 1355 g/mol) so extensive losses during RRT are likely. Patients requiring long term RRT for ESRD usually receive supplements containing thiamine and other water-soluble vitamins [5], but the extent of losses between different RRT modalities is not known.

To describe micronutrient and amino acid losses in patients with AKI receiving different types of RRT (IHD, SLEDf and CVVH) we conducted a prospective, observational study. We hypothesised firstly that water-soluble physiologically-free molecules in plasma (including trace elements, amino acids and B-vitamins) are lost in clinically significant amounts in acute RRT and secondly that nutrient losses differ qualitatively and quantitatively between RRT modalities because of different mechanisms of solute clearance.

Methods

Expanded materials and methods are available in Supplementary information.

Clinical study and sampling protocol: Regional ethics committee approval and sponsorship from Nottingham University Hospitals NHS Trust were obtained. Patients with AKI were eligible for recruitment once a plan for RRT was made. Inclusion and exclusion criteria and general study sampling protocol are illustrated in Figure 6. Blood sampling: a first blood sample was taken prior to RRT commencement and plasma obtained and stored appropriately until analysis ('base'). Further regular sampling occurred at intervals during each RRT session with the 'mid' or 'end' sample designated post-hoc as those samples taken at the mid-way or end of session (e.g. at 2 and 4h for a 4h IHD treatment; at 3h and 6h for a 6h SLEDf treatment; or at 12h and 24h for a 24h CVVH treatment). The first two RRT sessions were included in the study, but if only a single session was required, no further samples were taken. Effluent sampling: Spot-samples were obtained from the effluent port of each RRT machine on a schedule similar to blood sampling. 'Baseline' effluent was considered as the first sampled dialysate after machine priming had occurred, prior to patient attachment. For CVVH, a sample of the effluent was taken from each discarded 5L bag at 12 and 24h after commencing treatment.

Analysis of free amino acids: in plasma and effluent (490 µl) used an Amino Acid Analyser (Biochrom 20; Pharmacia LKB, Biochrom Ltd, Cambridge, UK) after deproteinization and derivatization, as described previously [6]. Values for 32 α–amino acids were determined as µmoles/L but were mass-corrected to µg/L for absolute quantification of loss.

Analysis of major and trace elements: Elemental analysis was conducted on 500 µl of plasma or effluent by inductively coupled plasma-mass spectrometry (ICP-MS; iCAP™ Q, ThermoFisher Scientific Inc., Waltham, MA, USA), as described previously [7, 8]. Certified reference materials (CRMs) were included in duplicate for each ICP-MS batch run (x60-120 samples) and were SeroNorm™ L-2 (REF203105, LOT0903107) and SeroNorm™ L-2 (REF210705, LOT1011645; LGC, Middlesex, UK) for plasma and urine, respectively. Operational parameters were quality controlled within and between different runs. LOD, LOQ and measured values for ultrapure water, tap-water and filtrate (PrismaSol) are presented in Table 4.

Analysis of B-vitamins: Effluent samples were analysed by liquid chromatography-mass spectrometry (LC-MS). Chromatography was run on an Agilent 1100 fitted with Phenomenex Luna 5U C18. Mass-spectrometry used a Micromass Ultima, (Manchester, UK). Vitamin B1 (thiamine hydrochloride), B2 (riboflavin), B3 (nicotinic acid), B6 (pyridoxal hydrochloride), B9 (folic acid) and B12 (cyanocobalamin) were analysed, interpolated from standard curves (0.25 to 10 ppm [0.25-10 mg/L). All B-vitamins were purchased from Sigma-Aldrich (Cheshire, UK). Parameters and conditions for chromatography and mass spectrometry are given in supplementary information. Further information on MS conditions is given in Supplementary Table S2.

Statistical Analysis: Continuous data were analysed by analysis of variance (ANOVA) or Kruskall-Wallis for skewed data and are presented as means with standard deviation

(1 SD) or standard error of the mean (1 SE) or estimated standard error of the differences between means (s.e.d.) where appropriate. 95% confidence intervals (95% C.I.) may be approximated as mean ± 2 s.e.d. Comparison of categorical or binomial data was by logistic regression and are presented as mean proportions (number [percent of total]). Comparison of treatment groups in which the data naturally followed an ordinal scale (e.g. measures of nutritional status; mild-moderate-severe) was by ordinal logistic regression. For repeated measurements, each individual was included in the model as a nested random-effect. All data are presented after correction for co-variates (e.g. dialysis dose; URR or SRI) and baseline plasma concentration. Skewed data were $\log_{10}$ transformed to normalize residual plots. Time-series data with missing measurements were analyzed by restricted maximum likelihood (REML). More stringent P-values were assumed where multiple, potentially non-independent data were analyzed, as indicated in each Table and Figure legend. All data were analysed using Genstat v17 (VSNi, Rothampsted, UK).

Results

Baseline characteristics of study population and RRT sessions

We achieved 98% consent rate. The study population tended to be elderly, male with multiple co-morbidities (Table 1). Baseline patient characteristics between RRT groups were relevant to determination of co-variates for statistical models quantifying loss of micronutrients. Overall demographics were similar for the three groups (Table 1). Patients receiving RRT (CVVH) in ICU had significantly higher C-reactive peptide, consistent with the higher proportion of sepsis-associated AKI in this group (Table 1). Patients prescribed SLEDf had lower blood pressure and plasma bicarbonate than other groups (Table 2). Most patients were malnourished (Table 2) e.g. 82% were at high risk using malnutrition universal screening tool (MUST 2, a 5 step screening tool to identify adults who are malnourished, at risk of malnutrition or obese), 71% needed nutritional support according to nutritional risk screening (NRS ≥3; NRS contains the nutritional components of MUST as well as a disease severity grading) and 77% were at least mild-moderately malnourished according to subjective global assessment (SGA B or C; Table 2; SGA assesses 10 factors in history and examination, with classification into 3 categories of nutritional status: A=well nourished, B=moderately well nourished or at risk of malnutrition, C=severely malnourished). Details of RRT sessions are given in Table 3. Effluent volume generated per session was significantly different (IHD, 54 ± 20; SLEDf, 98 ± 20; CVVH, 64 ± 21 L; $P<0.001$). Variation in dialysis dose (quantified by urea reduction ratio [URR] and solute removal index [SRI]), between RRT types justified inclusion of either as co-variate (blood and effluent outcomes, respectively) when analysing nutrient losses.

Amino acid losses

Plasma concentrations of all α-amino acids at baseline were similar for IHD and SLEDf but considerably higher for CVVH (Figure 1A). By the end of each RRT session, plasma concentration of amino acids had reduced but tended to rebound by 1-2h post-RRT (for IHD and SLED-F, CVVH for reference only; Figure 1A). This pattern was similar for all measured amino acids when considered separately but was most obvious in those present at highest concentrations in plasma (e.g. glycine and glutamine; Figure 1C & Supplementary Table 1). Minor differences in this pattern were noted for less prevalent plasma amino acids such as taurine (Figure 1E). No significant differences were noted between subgroups of amino acids (e.g. branched chain, essential or acidic groups). Corrected for plasma concentration and dose of dialysis, loss of amino acids was significantly influenced by modality (CVVH >>> SLED-F >> IHD; Figure 1B), estimated loss being similar whether sampled midway or at the end of each session. Again, the pattern of overall loss was largely driven by amino acids at highest concentration in plasma such as glutamine (Figure 1D). Nevertheless, some variation in the pattern of loss between RRT types was again noted for less abundant amino acids such as taurine (Figure 1F & Supplementary Table 1).

Trace element losses

Total measured plasma trace element composition was similar between RRT groups at baseline and did not change appreciably during or after the first RRT session (Figure 2A). This is likely attributable to patient heterogeneity for overall plasma trace element profile. Analysis of individual trace elements revealed some clear effects. Plasma selenium

(Figure 2B), zinc (Figure 2C) and copper (Figure 2D) were markedly higher in patients receiving IHD compared with other modalities. Duration of RRT, regardless of modality, reduced plasma caesium (pooled means; *from* 1.11 *to* 0.70 ± 0.30 µg/L; P_{time}=<0.001), iron (Figure 2E), molybdenum (pooled means; *from* 3.79 *to* 2.74 ± 0.41 µg/L; P_{time}=0.01) and rubidium (pooled means; *from* 242 *to* 158 ± 8.7 µg/L; P_{time}=<.001) but increased plasma chromium (Figure 2F). Significant reduction in plasma concentration of trace elements by the end of an RRT session, relative to the patient's baseline, suggests net loss from the plasma pool. Trace elements in plasma that did not vary with RRT modality or time were (all µg/L, pooled median [IQR]): Mn (1.14 [0.62-1.68]), Sr (32.8 [27.0-37.9]) and Va (1.66 [0.69-2.24]).

For calculation of net effluent trace element loss attributable to RRT, mass of each element present in effluent above limits of quantification multiplied by effluent volume was considered, corrected for plasma concentration and dose of dialysis (SRI). In addition, the quantity of trace element present in effluent at baseline i.e. when sampled post-filter but prior to patient attachment, multiplied by effluent volume, was subtracted from all values. Loss of major elements (S, Ca, P, Na, Mg and K) was calculated but data are not shown because the study hypotheses did not consider these elements. In total, net losses of 13 of 32 trace elements between modalities of RRT were assessed with significance levels adjusted accordingly. For effluent zinc, no significant net loss above baseline occurred in IHD (Figure 3A), but removal as a result of SLEDf was noted (average 848 ± 1032 µgs increase in effluent concentration from Base-to-End; Figure 3A). Apparent gain of zinc (i.e. gain to patient) resulted during CVVH (i.e. net decrease in effluent Zn from Base-to-End; Figure 3A). A statistically significant loss of strontium occurred with all modalities of RRT (Figure 3B), with effluent levels different between modalities at baseline (P for effect of RRT mode = 0.003). The latter effect was also observed for vanadium, which was lower in effluent of CVVH patients (Figure 3E). For caesium (Figure 3C) and molybdenum (Figure 3E) an effect of time only was noted. Trace elements in effluent above LOQ, but not significantly influenced by modality or duration of RRT were: Cr, Cu, Fe, Mn and Se (Figure 3E). In summary, net loss of trace elements to effluent as a consequence of RRT was negligible for IHD (net loss -369 ± 1094 µgs), statistically significantly higher for SLEDf (+1787 ± 1603 µgs), but negligible-to-reversed for CVVH (net loss -1099 ± 1012 µgs) (Figure 3D).

B-vitamin losses

Methods for simultaneous measurement of the B-vitamin series (1, 2, 3, 6, 9 and 12) were optimised to quantify losses at <10 ppb (i.e. <10 µgs/L) but no measurable values were detected in effluent for any modality at any time point (50 samples). Reasons could include (i) dilution of signal (i.e. by 50-100 L of effluent), (ii) conversion to alternative metabolites not discriminated on targeted mass spectrometry (e.g. thiamine pyrophosphate is main form of Vit B_1 [Mwt; 425.31 g/mol], but thiamine mono/tri-phosphate and adenosine thiamine diphosphate are other active forms [Mwt; 345.33 and 674.5, respectively]) or (iii) they are not removed to effluent by RRT. The last is unlikely as the filtration coefficient for micromolecules ≤1000 Kd is ~1.0 and Vit B_{12}, the largest B-vitamin, has Mwt of 1355 g/mol. Adsorption to the RRT circuit and dialyser/filter is a possible explanation. This would represent net loss to the patient but would not appear in effluent. We therefore generated preliminary data using an *in vitro* model of RRT (using HDF440 filter for CVVH) with supraphysiological levels of B-vitamins added to

replacement solution (Prismasol for CVVH) to demonstrate net filtration and adsorption (Supplementary Figure S1A,B). After only 5 minutes of pseudo-filtration, there was a marked difference in B-vitamin concentration of replacement solution and effluent, accountable only by adsorption to the circuit and/or filter (Figure S1A,B). Similar results were obtained for trace elements (Figure S1C) and amino acids (Figure S1D). However, elution of used dialysers and filters with an acidified ethanol solution (after rinsing and clearance of blood; Figure S1E) recovered only minimal (<1g for all modes of RRT) quantities of amino acids (Figure S1F).

First vs second session of RRT

Only 28 patients (IHD, n=11; SLED-F, n=3; CVVH, n=14) received a second RRT session. For those patients, variability of end-points was generally increased but pattern of micronutrient change during RRT was similar (Figures 4 and 5). For example in plasma, amino acid concentrations tended to decline during each session (Figure 4A,B) with some declining further between end of first and start of second RRT session (e.g. plasma glutamine, Figure 4C,D). Concentration of trace elements in plasma again was largely unaffected by RRT (Figure 4E,F). Effluent losses of micronutrients generally followed the pattern observed during the first session, but with lower total losses (e.g. amino acids, Figure 5A,B). This was not the case for all amino acid groups, with little apparent effect on branched-chain amino acids (isoleucine, leucine and valine; Figure 5C,D). Similarly, comparison of effluent trace element loss during RRT2 between modalities followed a similar pattern to RRT1, with slightly lower losses (e.g. for zinc, Figure 5E,F).

Discussion

We have confirmed high prevalence of disease-related malnutrition (DRM) in patients with AKI requiring RRT. We demonstrated that acute RRT results in significant losses of amino acids and micronutrients. Such losses may contribute to DRM. We show for the first time that these losses vary both qualitatively and quantitatively with type of RRT. These data raise the possibility that tailoring of nutritional supplements to mode of RRT might be indicated in the future, if further evidence is acquired.

Loss of amino acids: three different methods of nutritional assessment were used pre-RRT [9, 10]. The proportion of patients at risk of malnutrition was very high, 82% in this study using MUST. Contributing factors to pre-RRT DRM in AKI patients are likely to include (i) reduced food intake, because of anorexia or malabsorption, (ii) increased catabolism associated with acute illness and sepsis, (iii) increased gastrointestinal losses. Micronutrient loss from RRT has previously been considered but, prior to the present study, none had compared three different modalities. We demonstrate marked amino acid loss for all 3 modalities, with greatest losses for CVVH (~14-22g per session), followed by SLEDf (~7-10g) then IHD (~3-6g). This difference between modalities remained, though attenuated, after adjustment for delivered dose of RRT and baseline plasma amino acid concentration (both differed considerably between RRT groups). It is likely that plasma amino acids levels were greatest in the CVVH group due to enteral or parenteral supplements [11]. These data suggest that amino acids are lost more from convection-based RRT than from diffusion-based treatment. Modes of RRT using a combination of both mechanisms were predictably intermediate for amino acid loss. Previous studies have demonstrated significant amino acid loss during CVVH [12, 13] and also during haemodialysis **[14],** but the latter studied patients with ESRD, which has important pathophysiological differences from AKI and should not be compared directly. We suggest that nutritional assessment of patients receiving RRT might need to take into account the mechanism of solute removal. Specifically, convection-based RRT, increasingly being used in renal units, may have a greater effect on metabolism and nutritional homeostasis than diffusion-based RRT.

Reductions in total serum/plasma amino acids during RRT, as observed in this study (with the exception of IHD) are common, but some ambiguity exists [14, 15]. Such variability may relate to differences in the study cohorts in terms of their nutritional or metabolic state. Sampling of the plasma amino acid pool is a snap-shot of overall protein turnover (composite of protein assimilation/supplementation versus degradation). For individual amino acids, those at relatively high concentration in plasma (e.g. glutamine, glycine) tended to dominate the overall pattern of loss between modes of RRT. For some such as taurine, however, important differences were noted such as more being lost in SLEDf than CVVH (a decrease of ~30-50 µmol/L from baseline of 62 ± 45 µmol/L). Acute or chronic taurine deficiency has been associated with heart failure [16]. We were particularly interested in assessing losses of essential and conditionally essential amino acids because we assumed that their deficiency might be more likely to result in adverse clinical consequences. Losses varied with mode of RRT but, for example in SLEDf, plasma concentration decreased from 821 to 545 ± 114 umol/L, illustrating the effect that RRT can have on important micronutrients that can be restored only through diet.

Loss of trace elements was variable between elements and between modes of RRT for the same element. Differing behaviours of individual elements is not surprising as they vary in size, charge, protein binding and movement between extra- and intracellular compartments. We were particularly interested in selenium, copper, zinc and iron because of their roles in immune response to critical illness and in oxidative stress [17], which is a feature of AKI [18]. Small clinical and in vitro studies have demonstrated loss of trace elements in haemofiltrate [19-21]. Tonelli et al suggested that marginal Se status is strongly associated with risk of death and hospitalisation, although this was a study of incident haemodialysis patients with ESRD rather than AKI [22]. Compared with published reference ranges for healthy adults, baseline plasma Se, Cu and Zn levels were low in our study, again consistent with DRM in AKI and critical illness [23, 24]. Zn effluent losses varied markedly between RRT modalities, with adjusted loss greatest for CVVH. Rate of loss appeared to increase with duration in SLEDf but remain unchanged for IHD. A possible explanation is that Zn is removed more by convection because this mechanism can remove larger, protein-bound, molecules to some extent. Furthermore, our *in vitro* data suggest that Zn adsorption could be considerable (Figure S1C) and this would remain unaccounted for in any analyses.

We optimised methods to detect B-vitamins in the ppb range but were unable to detect any >LOD in effluent. We attribute this to the dilution effect of large effluent volumes. B-vitamin deficiency can occur in patients with ESRD receiving regular haemodialysis. Standard practice is to prescribe supplements of water-soluble vitamins. It is not known whether clinically significant losses of water-soluble vitamins occur in RRT for AKI where patients usually undergo only a limited number of RRT sessions and are likely to have different pre-treatment pathophysiological status. We assume that significant vitamin loss does occur based upon molecular size, charge and lack of protein binding but we have been unable to quantify it. Our *in vitro* study, using supraphysiological but easily measurable concentrations of B vitamins added to filtrate, suggested that significant adsorption to the haemofilter occurs. Such an effect would partly explain the undetectable levels in RRT effluent.

This study has several limitations. Firstly it is an observational study in which prescription and details of each RRT were determined by clinicians independent of the study. RRT modality, duration, pump speeds and delivered dose therefore varied. We could not randomise patients to RRT groups and we recruited fewest patients to the SLEDf group. Unsurprisingly the CVVH group, recruited from ICUs, had the worst baseline clinical nutritional status. Secondly we measured only plasma levels of nutrients, which may not reflect total body status of a nutrient. The volume of distribution, degree of protein binding and kinetics of transfer between fluid compartments is likely to vary between the nutrients which we studied. We have adjusted our results for plasma concentrations but we have not attempted to incorporate two-compartment kinetic modelling into our calculations. It might be that two compartment modelling would be appropriate for some individual nutrients, including those for which we noted a degree of post-RRT rebound in plasma concentration (such as glutamine). We acknowledge that comparison between RRT modalities is particularly complex for such solutes. Nevertheless we believe that we have adopted a pragmatic approach to the calculation of total nutrient losses and comparison of these losses between RRT modalities.

Another limitation was the inability to quantify enteral and parenteral input of individual nutrients, though all patients in the CVVH group received some supplementation. We provided indirect evidence for adsorption of some micronutrients from an *in vitro* experiment, but we were unable to quantify the extent of adsorption of amino acids, trace elements or B vitamins in the clinical study. This will be an important challenge in future studies. It will also be of interest to study differences in adsorption of micronutrients to different RRT membrane types e.g. polyacrylonitrile (a component of the AN69 hemofilter) and polysulphone (a component of the FX60 dialyzer). Markedly different degrees of adsorption of specific micronutrients between membrane types might confound interpretation of the corresponding effluent concentrations.

Our data derived mainly from patients receiving their first and only session of RRT. Only 28/72 patients in our cohort required a second RRT session so our data may reflect qualitative and quantitative differences at this time, rather than being representative of a prolonged schedule of RRT. However, our analysis of patients receiving a second RRT session broadly corresponded to their first, with evidence for gradual nutritional depletion. The observation that less than 40% of patients required a second RRT session was perhaps surprising but was similar to data from our unit for similar years. One possible explanation would be a low threshold for initiation of RRT, which was a clinical decision independent of the study. Such an approach might increase reported renal recovery rate. It was not surprising that the highest rate of second RRT was in the CVVH group (15/24), as these were the most inwell patients.

The fact that most RRT sessions in the study represented a first treatment of patients with AKI, together with the observational design with no control over RRT prescription, explains the low blood pump speed for IHD. Indeed it is similar to those for SLEDf and CVVH, though dialysate flow rates are markedly different. It would be of interest to acquire data from several consecutive RRT treatments, likely with increasing blood pump speeds, but this was not feasible within the design of this study.

To our knowledge, this is the first study to compare loss of amino acids and micronutrients in IHD, SLEDf and CVVH. These 3 RRT modalities are used commonly to manage AKI and were chosen to allow comparison of mechanisms of solute clearance: diffusion, combined diffusion + convection, and convection. Even though patient numbers were small, this is one of the largest studies investigating loss of micronutrients in acute RRT, a potentially important research area so far largely neglected. The study was not designed to investigate clinical consequences of micronutrient loss, but it has provided data which will facilitate design of such studies. Further work is required to investigate the detail of individual micronutrient clearance with different RRT modalities. Thereafter, if larger studies suggest evidence of clinically significant losses and adverse clinical outcomes (or surrogates such as markers of inflammation), a future step might be to design interventional studies with bespoke nutritional supplements.

In conclusion, we have demonstrated significant amino acid and trace element loss in 3 RRT modalities commonly used to manage AKI. The pattern of nutrient losses varies considerably between modalities (convection >> haemodiafiltration > diffusion). The type of RRT used to manage AKI might influence the patient's risk of malnutrition and their nutritional requirements. Far more research is required in this important area.

Disclosure

This paper presents independent research funded by the NIHR under its Research for Patient Benefit programme, grant reference PB-PG-0613-31042. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health and Social Care. Early stages of the work were funded by a pump priming grant from Nottingham University Hospitals Charity. The authors have no competing financial interests to disclose.

Acknowledgements

The authors gratefully acknowledge the help and support of Dr Scott Young for ICP-MS analyses, Dr Liu Miu for B-vitamin analyses, Dr Dongfang Li for amino acid analyses, Dr Jim Craigon for statistical advice on the manuscript (all School of Biosciences, Sutton Bonington Campus, University of Nottingham). We thank nursing staff at NUH Renal and Transplant Unit and Intensive Care Units, Mr Mike Pikett for technical input with haemodialysis machines and the patients who kindly consented to participation in the study. We thank the Nottinghamshire Kidney Units Appeal for ongoing support of our research programme.

Supplementary Material

1. Table S1: the change in plasma and effluent amino acids during RRT

Table legend: Δ Plasma losses: Data reflect average decrease over the course of each RRT session (concentration at End minus baseline). Statistics (P-value, plasma) are for the effect of time on plasma concentration. Effluent losses: Since there was no significant difference between losses estimated at mid or end RRT session, data reflect pooled average losses (for Mid and End samples). Statistics (P-value, effluent) are for the effect of RRT on net loss in effluent. 95% confidence interval for the data may be estimated as 1.96 × s.e.d. Some α-amino acids were measureable but at limits of quantification, and unreliable (anserine, betalanine, carnosine, ethanolamine, gammaaminobutyricacid, homocysteine, hydroxylysine, phosphoserine, phosphoethanolamine, sarcosine). Key: 's.e.d', standard error of the differences between means. P-value testing for effect of RRT mode only (as timepoints are pooled estimates).

2. Table S2: LC-MS/MS parameters and limits of detection for B vitamins in effluent

2 tables: Experimental Details: Standards were prepared for LC-MS/MS as described in Methods but briefly; 400ppm of standards, prepared as stock solution using mobile phase solvent A. These were diluted serially using mobile phase A to achieve a concentration range of 10ppm, 5ppm, 2ppm, 1ppm, 0.5ppm, 0.25ppm. The folic acid stock solution was dissolved in 0.1% NaOH before serial dilution. A standard calibration curve was plotted for each vitamin. Stock vitamins were stored at -20 C. All were purchased from Sigma-Aldrich.

LC conditions (Agilent 1100 series):

MS conditions (MicroMass Ultima MS)

3. Figure S1: an *in vitro* model of micronutrient adsorption to RRT circuits

Figure legend: Micronutrient adsorption to RRT circuits: A; An HDF440 was primed (30mins) using standard PrismaSol solution before addition of supraphysiological concentration of B-vitamins (B), trace elements (C) and amino acids (D, all purchased from Sigma-Aldrich). Replacement solution was sampled before (Base, -5mins) and after (time zero, 0mins) addition of micronutrients and amino acids and subsequently at 5, 30 and 60mins of 'pseudo'-RRT. Data are mean ± S.E. (standard error of the mean) of 3-4 independent experiments. E, In a separate experiment n=15 spent filters (IHD & SLED-F, Fx60; CVVH, AN69) were rinsed of blood contamination (for 1-2h with PBS plus 3mM EDTA at 80mls/min) and flushed with an eluting fluid (100 mLs, 50:50 EtOH:Water + 0.1M HCl for 15mins at 80mls/min). The resulting elute (50-80mls) was evaporated to dryness before reconstitution in 1ml amino acid running buffer for measurement of adsorbed amino acids (F).

4. Expanded materials and methods:

(i) clinical study
(ii) analysis of free amino acids in plasma and effluent
(iii) analysis of major and trace elements in plasma and effluent
(iv) analysis of B vitamins in effluent
(v) statistical analysis
(vi) estimation of dose of dialysis
(vii) *a priori* power calculation for sample size

Supplementary Material available at KI Reports website

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Tables

Table 1. Patient characteristics at admission stratified by type of renal replacement therapy

Characteristic	All (n=72)	IHD (n=33)	SLEDf (n=15)	CVVH (n=24)	P-value*
Age, yr	65 ± 13	66 ± 14	70 ± 11	62 ± 12	0.17
Weight, kg	84.9 ± 21.8	91.4 ± 24.2	77.4 ± 20.2	80.6 ± 16.9	0.057
BMI	26.8 ± 6.1	27.6 ± 6.3	25.7 ± 7.6	26.4 ± 4.8	0.47
Male	54 (75)	25 (75)	10 (66)	19 (79)	0.43
Systolic blood pressure, mm Hg	128 ± 27	140 ± 25	99 ± 20	121 ± 23	<.001
Diastolic blood pressure, mm Hg	68 ± 12	75 ± 11	59 ± 9	62 ± 9	<.001
Ethnicity (% Caucasian)	71 (99)	33 (100)	14 (93)	24 (100)	-
Inotropic support (%)	24 (33)	0	0	24 (100)	-
Co-Morbidities					
Septic AKI	38 (52)	10 (30)	8 (53)	20 (83)	<.001
Multiple AKI causes	25 (35)	8 (24)	6 (40)	11 (45)	0.21
CKD	31 (43)	16 (48)	9 (60)	6 (25)	0.06
Diabetes	35 (43)	18 (54)	8 (53)	9 (37)	0.40
Hypertension	41 (56)	22 (66)	8 (53)	11 (45)	0.27
Serum parameters					
Creatinine, µmol/L	575 ± 313	692 ± 307	592 ± 388	404 ± 175	<.001
Urea, mmol/L	34.3 ± 15.5	36.7 ± 9.6	30.5 ± 12.5	29.8 ± 15.2	0.08
Sodium, mmol/L	133 ± 7	132 ± 7	133 ± 7	135 ± 7	0.28
Potassium, mmol/L	5.24 ± 1.00	5.15 ± 1.0	5.57 ± 0.95	5.15 ± 1.03	0.25
Bicarbonate, mmol/L	17.8 ± 5.9	17.7 ± 5.5	14.8 ± 5.7	19.9 ± 5.9	0.019
Phosphate, mmol/L	2.37 ± 1.05	2.56 ± 1.02	2.59 ± 1.19	1.99 ± 0.96	0.08
Albumin, g/L	22.5 ± 7.7	23.8 ± 6.9	22.8 ± 8.9	20.4 ± 7.8	0.21
C-reactive peptide, mg/L	140 ± 118	114 ± 112	112 ± 99	196 ± 122	0.01

Table 1. Data are Mean ± 1SD for continuous variables and number of patients (% of group total) positive for each category. *Statistical differences between groups of patients on admission were assessed by Kruskall-Wallis One-Way ANOVA for continuous variables and chi-squared test for categorical data. All data analyses were conducted using Genstat v17 (VSNi, UK). Statistical significance was accepted at $P < 0.05$.

Table 2. Nutritional Status of Study Participants stratified by type of renal replacement therapy

Characteristic		All (n=73)	IHD (n=33)	SLEDf (n=15)	CVVH (n=24)	P-value*
Dietetic assessment						
	MUST 0	11 (15)	7 (21)	2 (13)	2 (8)	0.06
	1	2 (3)	2 (6)	0 (0)	0 (0)	-
	2	60 (82)	25 (73)	13 (87)	22 (92)	0.13
	NRS $\leq$2	21 (29)	16 (47)	2 (13)	3 (12)	<0.001
	NRS $\geq$3	52 (71)	18 (53)	13 (87)	21 (88)	0.55
	SGA A	17 (23)	10 (29)	3 (20)	4 (17)	0.09
	B	36 (49)	20 (59)	7 (47)	9 (38)	0.03
	C	20 (28)	4 (12)	5 (33)	11 (46)	0.21
Serum parameters						
	Plasma glucose, mmol/L	6.97 $\pm$ 3.58	7.13 $\pm$ 3.87	7.54 $\pm$ 4.02	6.55 $\pm$ 3.06	0.78

Table 2. Values are Mean $\pm$ 1SD for continuous data and number (% of group total) for categorical data. *Statistical differences between groups of patients on admission were assessed by Kruskall-Wallis One-Way ANOVA for continuous data and chi-squared test for categories of nutritional assessment tools. All data analyses were conducted using Genstat v17 (VSNi, UK). Statistical significance was accepted at $P < 0.05$. Key: Subjective Global Assessment (SGA) tool assessed using a 7-point scale: A (6-7) = well nourished, SGA B (3-5) = mild-moderately malnourished, SGA C (1-2) = severely malnourished; Malnutrition Universal Screening Tool (MUST) on a 3-point scale: MUST 0 = low risk, MUST 1 = medium risk, MUST 2 = high risk of malnutrition; Nutritional Risk Screening (NRS) was assessed using a 6-point scale with $\leq$2 points = nutritional support not indicated and NRS $\geq$3 = nutritional support indicated.

Table 3. RRT characteristics of study participants

Characteristic	IHD (n=33)	SLEDf (n=15)	CVVH (n=24)	P-value*
RRT (First session, T1)				
Prescribed RRT time, mins	120 ± 5	344 ± 42	1440 ± 0	-
Actual RRT time, mins	122 ± 22	282 ± 105	1225 ± 341	-
Blood flow rate, ml/min	207 ± 17	214 ± 29	234 ± 25	<.001
Plasma flow rate, ml/min	145 ± 13	148 ± 21	169 ± 25	<.001
Effluent flow rate, ml/min	462 ± 121	209 ± 26	†35 ± 0	-
Serum urea pre-RRT, mmol/L	36.7 ± 9.6	35.9 ± 24.1	29.8 ± 15.2	0.03
Serum urea post-RRT , mmol/L	28.5 ± 8.4	22.7 ± 24.3	18.7 ± 9.0	<.001
Urea reduction ratio (URR)	0.31 ± 0.14	0.54 ± 0.18	0.38 ± 0.16	<.001
Kt/v	0.41 ± 0.21	0.94 ± 0.42	0.85 ± 0.41	<.001
Solute removal index (SRI)	0.28 ± 0.14	0.45 ± 0.51	0.51 ± 0.21	<.001
RRT (Second session, T2)	**(n=11)**	**(n=4)**	**(n=15)**	
Prescribed RRT time, mins	180 ± 26	360 ± 0	1440 ± 0	-
Actual RRT time, mins	185 ± 30	323 ± 56	1244 ± 319	-
Blood flow rate, ml/min	225 ± 25	203 ± 5	234 ± 28	-
Plasma flow rate, ml/min	159 ± 24	140 ± 7	140 ± 58	-
Effluent flow rate, ml/min	516 ± 65	200 ± 0	†35 ± 0	-
Serum urea pre-RRT, mmol/L	25.8 ± 11.6	25.8 ± 19.5	13.6 ± 5.1	-
Serum urea post-RRT , mmol/L	15.7 ± 8.0	10.0 ± 6.4	10.1 ± 4.1	-
Urea reduction ratio (URR)	0.17 ± 0.09	0.35 ± 0.17	0.38 ± 0.16	-
Kt/v	0.21 ± 0.11	0.51 ± 0.25	0.83 ± 0.34	-
Solute removal index (SRI)	0.40 ± 0.11	0.26 ± 0.01	1.40 ± 0.93	-

Table 3. Values are Mean ± 1SD for continuous data and number (% of group total) for categorical data. Only a proportion of study participants received a second RRT session (IHD, n=11, SLED-F, n=4, CVVH, n=15) in this cohort. Hence information is included for comparison (to first session, T1) but was not formally compared. For T1, *statistical differences were assessed by Kruskall-Wallis One-Way ANOVA for continuous data. All data analyses were conducted using Genstat v17 (VSNi, UK). †, fixed filtration fluid rate (mls/kg min^{-1})

Table 4. Limits of detection, quantification and baseline reference samples for ICP-MS

Major element (ppm, mg L^{-1})	CRM Seronorm L2 Plasma (% recovery)	CRM Seronorm L2 Urine (% recovery)	LOD (ppb)	LOQ (ppb)	Ultrapure Water (18 MΩ cm)	Tap Water	Filtration fluid (PrismaSol)
Sulphur	1335 (105)	671 (101)	8.5	21.3	13.41±0.93	37.4±2.14	<LOQ
Calcium	119 (102)	119 (94)	0.08	0.20	<LOD	52.0±1.54	48.5±4.4
Phosphorus	110 (113)	807 (102)	0.08	0.22	<LOD	0.95±0.11	<LOQ
Sodium	3531 (102)	2815 (89)	0.08	0.21	0.88±0.45	35.8±1.90	2804±165
Magnesium	33.9 (96)	78 (89)	0.02	0.05	0.04±0.00	10.4±0.54	13.8±1.3
Potassium	221 (104)	1921 (92)	0.09	0.22	0.48±0.07	6.76±0.34	127±13
Trace element (ppb, µg L^{-1})							
Zinc	1532 (115)	1281 (104)	11.0	27.5	1.9±0.19	489±273	94.8±27.8
Iron	2150 (95)	13.9 (147)	0.70	1.76	9.83±2.7	17.7±2.5	15.0±5.5
Copper	1925 (91)	56.3 (108)	0.12	0.29	1.53±0.22	100±32	4.5±2.5
Manganese	14.5 (106)	9.3 (109)	0.09	0.23	2.12±0.79	1.12±0.15	<LOD
Strontium	110 (107)	120 (97)	0.09	0.22	0.45±0.11	247±2	22.4±2.0
Selenium	136 (106)	71.7 (117)	0.34	0.86	0.79±0.41	10.0±3.4	2.0±0.5
Chromium	5.7 (101)	30.1 (95)	0.15	0.39	<LOD	<LOD	<LOD
Vanadium	1.1 (149)	26 (99)	0.05	0.13	<LOD	<LOD	0.68±0.06
Molybdenum	1.21 (149)	48 (92)	0.21	0.52	<LOD	<LOD	<LOQ
Rubidium	8.7 (109)	1150 (113)	0.10	0.25	0.20±0.02	4.95±0.65	<LOQ
Lithium	9689 (116)	100 (98)	0.05	0.13	<LOD	7.27±0.58	<LOQ
Caesium	0.02 (233)	6.6 (110)	0.02	0.05	<LOD	0.15±0.00	<LOD

Table 4: Certified reference materials (CRM) were obtained from LGC Standards, Bury, UK. Limits of detection (LOD) and quantification (LOQ) were calculated from calculating standard deviation (SD) of 10 operational blank samples as LOD = 3.25 (Students t-test, *df*=9, 99%CI) × SD and LOQ = 2.5 × LOD. Elemental composition of ultrapure water (n=6), tap water (n=6) or PrismaSol filtration fluid (n=18) was determined by ICP-MS. Percentage recovery is mean recovery from 12 independent runs. Aluminium, Arsenic, Beryllium, Cadmium, Cobalt, Lead, Nickel, Silver, Titanium, Thallium and Uranium were measurable above LOD in plasma, but not in effluent. As these elements were not a focus of our hypotheses, values are not reported. Barium and Boron were quantifiable but were not included in the NIST CRM and are therefore also not reported.

Figures

Figure 1. Plasma amino acids and effluent loss during different modes of RRT

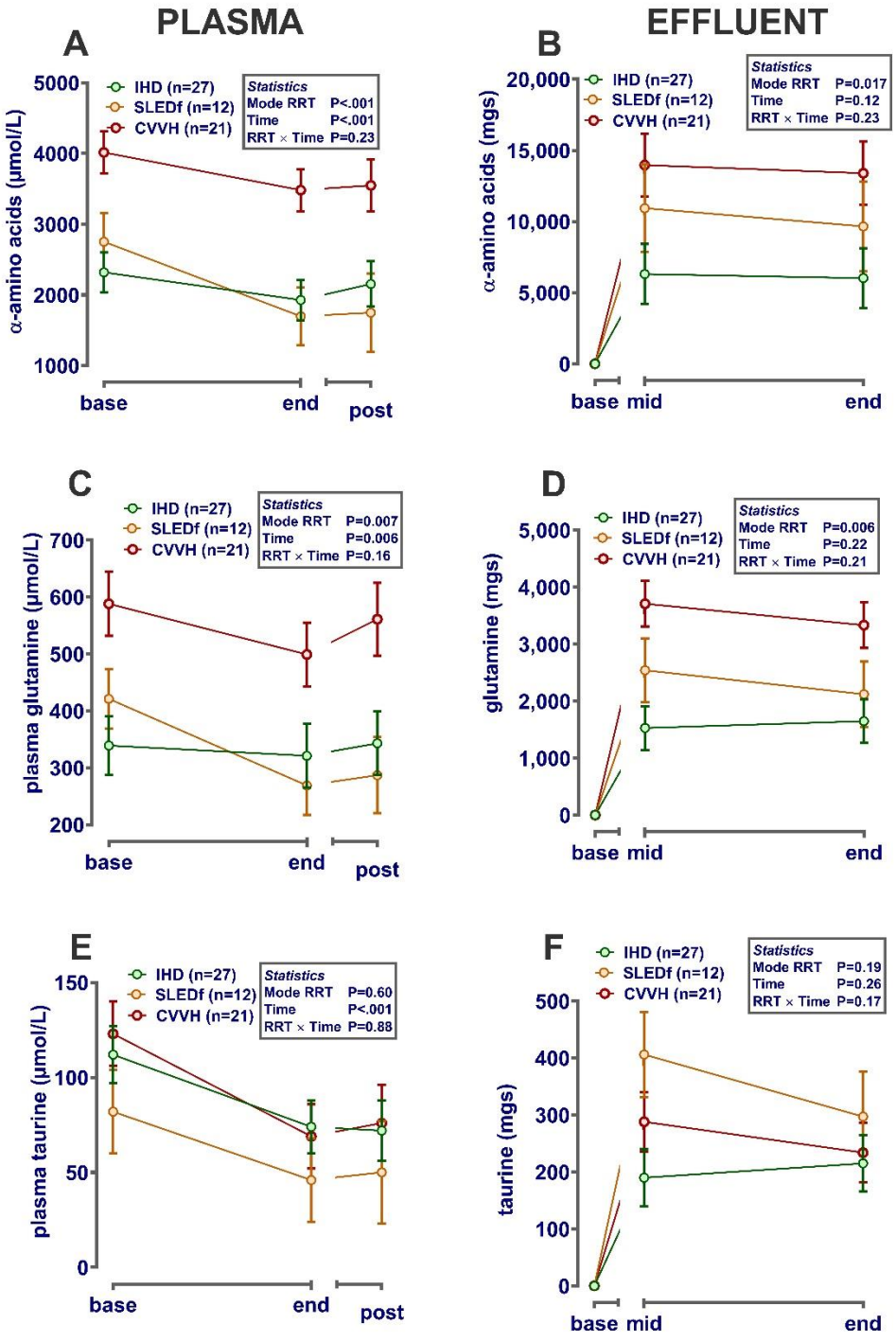


Figure 1. Plasma amino acids and their loss in effluent during different modes of RRT. Plasma (**A,C,E**) and effluent (**B,D,F**) were sampled prior to, at the end, and 1-2h after the end of each RRT session. Alpha amino acids (x20 standard proteogenic plus a further 18 amino acids) were measured after deproteinisation and derivatisation using a Biochrom 20 (Biochrom, Cambridge, UK). Data (means ±S.E.) are presented corrected (i.e. included as co-variates in the statistical model) for dose-of-dialysis (urea reduction ratio for plasma levels; solute removal index for effluent losses) and plasma concentration (for calculation of effluent losses only). If necessary, to normalise residual error before statistical analysis, data were $\log_{10}$ transformed. Graphs were generated in Graphpad Prism 6 (Graphpad Software Inc, Ca, USA). Analysis was by RM-ANOVA or mixed effect models, as appropriate, with RRT mode and time as fixed effects and patientID as a nested random effect, using Genstat v18 (VSNi, Rothampsted, UK). Statistical significance was accepted at $P<0.05$.

Figure 2. Plasma trace elements during different modes of RRT

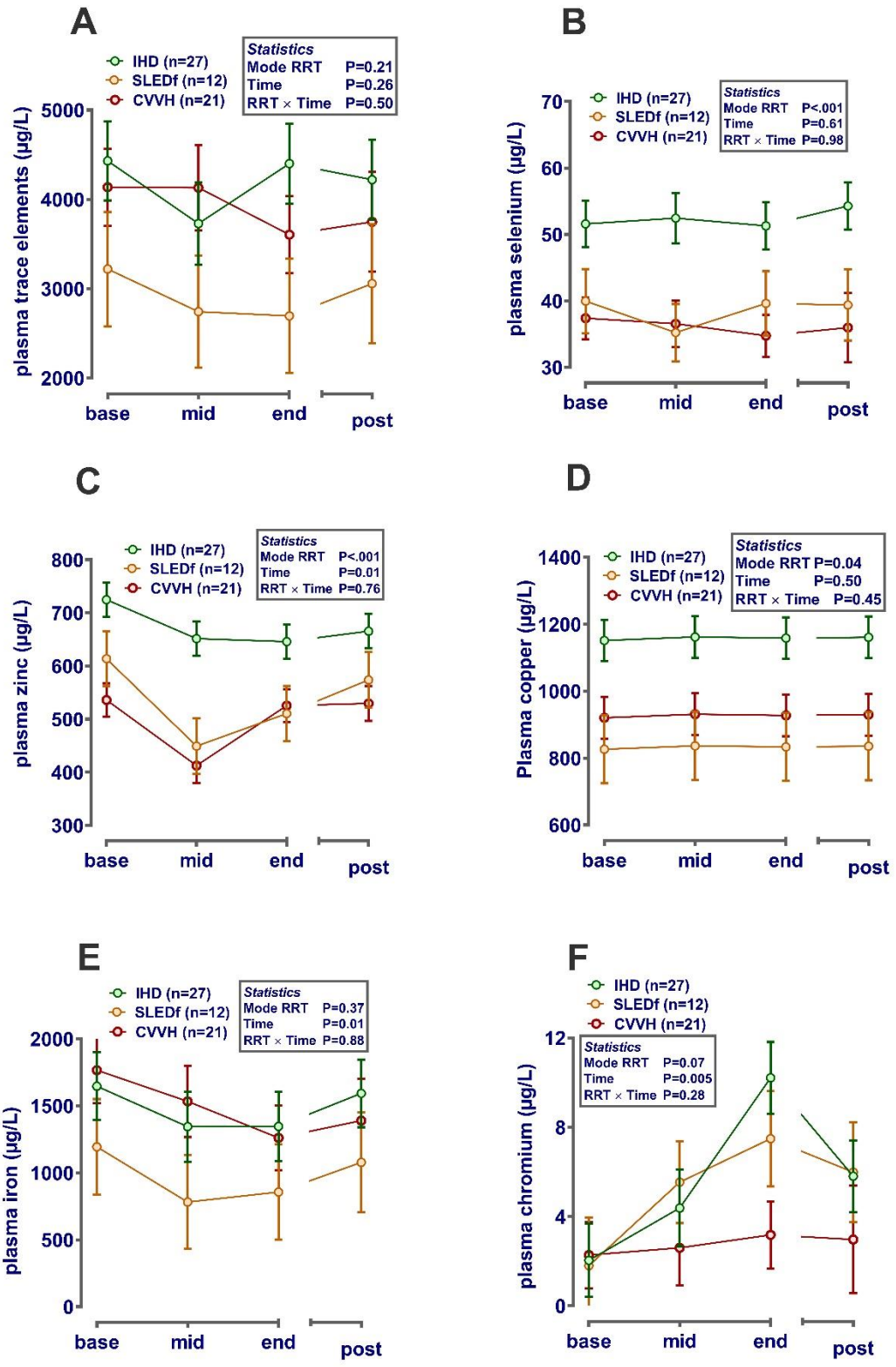
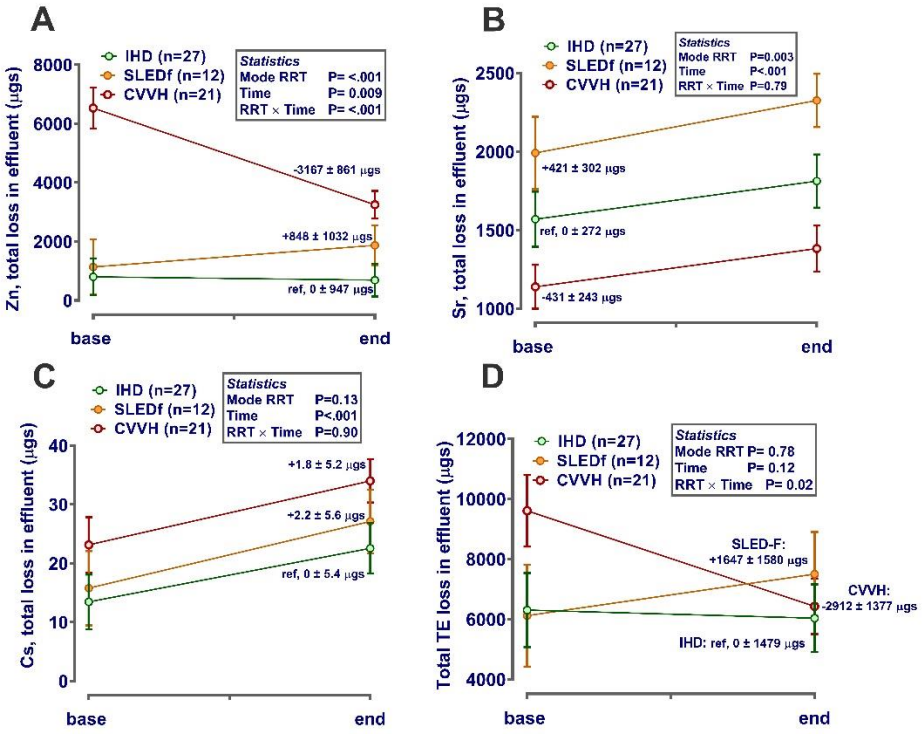


Figure 2. Plasma trace elements during RRT. Plasma was sampled prior to, during, at the end and 1-2h after each RRT session for measurement of trace elements. Data (means ±S.E.) in plasma are presented corrected (i.e. included as a co-variate) for dose-of-dialysis (urea reduction ratio). If necessary, to normalise residual error before statistical analysis, data were $\log_{10}$ transformed. Statistical analysis was by RM-ANOVA or mixed effect models, as appropriate, with RRT mode and time as fixed effects and patient ID as a nested random effect, using Genstat v18 (VSNi, Rothampsted, UK). Statistical significance was accepted at $P \leq 0.002$ (adjusted for the number of comparisons). Fig 1A is sum of all measurable trace elements >LOQ (Cs, Cr, Cu, Fe, Li, Mn, Mo, Rb, Se, Sr, V, Zn).

Figure 3. Effluent trace elements during different modes of RRT.



E

	IHD		SLED-F		CVVH			Effect size				P-value		
	Base	End	Base	End	Base	End	s.e.d.	IHD	SLED-F	CVVH	s.e.d.	Mode	Time	M × T
Potassium	6828	7514	8067	10186	6317	8117	1312	0	1433	1113	874	0.28	<.001	0.21
Zinc*	802	690	1135	1871	6527	3246	909	0	849	-3167	963	**<.001**	0.009	**<.001**
Copper	612	524	870	709	1361	1295	385	0	-72	21	349	0.058	0.48	0.96
Strontium*	1570	1812	1991	2326	1139	1383	235	0	93.3	2.62	146	**0.003**	**<.001**	0.79
Selenium	34.3	44.5	79.6	75.3	128	106	25.5	0	-14.4	-31.3	13.7	0.03	0.74	0.06
Chromium	109	100	125	79	77	59	36	0	-36.6	-8.71	19.8	0.08	0.04	0.47
Caesium*	13.4	22.5	15.7	27.1	23.1	33.9	6.4	0	2.2	1.8	5.5	0.13	**<.001**	0.90
Aluminium	4890	4549	5176	4862	1357	2991	1540	0	27	1974	1329	0.16	0.70	0.27
Iron	1641	1605	973	1732	1336	1110	717	0	794	-190	665	0.69	0.70	0.37
Manganese	50.1	57.5	70.1	57.3	27.2	26.0	16.9	0	-20.2	-8.5	11.6	0.07	0.98	0.22
Molybdenum	58.0	70.7	50.6	88.5	51.0	58.5	21.6	0	25.1	-5.20	16.0	0.64	**0.007**	0.19
Vanadium	86.0	87.0	103	101	48.4	50.0	15.0	0	-2.47	0.54	7.09	**0.004**	0.86	0.91
Barium**	158	139	292	323	2525	3271	494	0	49	764	461	**<.001**	0.29	0.21

Figure 3. Effluent trace elements during different modes of RRT. **A-D;** Concentrations of trace elements were measured in spot samples of post-filter baseline effluent after 'priming' of each RRT machine before patient was connected ('BASE') or at the end of each session ('END'). Total losses were estimated by multiplying measured concentrations in BASE or END sample (µgs/L) by the total volume in liters of effluent produced for each patient for that session. Data are estimated mean after correction (i.e. included as co-variates in the statistical model) for dose-of-dialysis (solute removal index) and plasma concentration. If necessary, to normalise residual error before analysis, data were $\log_{10}$ transformed. Analysis was by RM-ANOVA or mixed effect models, as appropriate, with RRT mode and time as fixed effects and patient ID as a nested random effect, using Genstat v18 (VSNi, Rothampsted, UK). Estimated effect size was calculated by the statistical package with IHD as referent category for the effects of mode and time (as indicated on eacg graph). Statistical significance was accepted at $P \leq 0.003$ (adjusted for the number of comparisons). **E**; Table, Potassium included for orientation of RRT effects, as it is known that SLED-F removes more K^+ than IHD. *data for above graphs also included on table for comparison to other trace elements.**Barium levels were quantifiable but since values are not included in the CRM (Seronorm L2 Urine) data should be interpreted with caution.

Figure 4. Comparison of plasma amino acids and trace elements between first and second RRT session

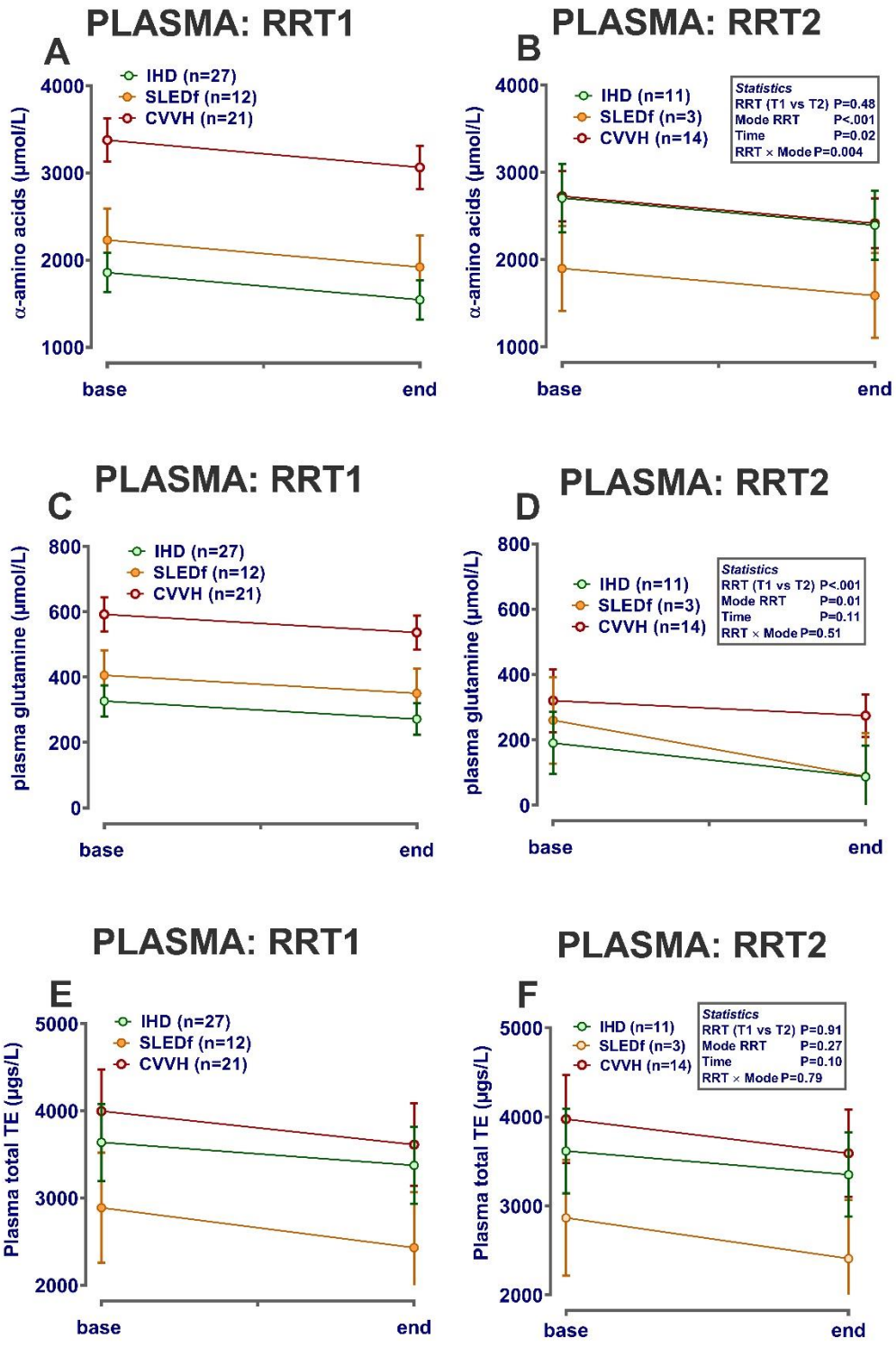


Figure 4. Plasma amino acids and trace elements during the first (RRT1) and second (RRT2) session of renal replacement therapy. Plasma was sampled before, mid-way through and at the end of each RRT session. Micronutrients were measured in spot samples of plasma as described in Methods. Data (means ±S.E.) are presented corrected (i.e. included as co-variates in the statistical model) for dose-of-dialysis (urea reduction ratio). If necessary, to normalise residual error before statistical analysis, data were $\log_{10}$ transformed. Graphs were generated in Graphpad Prism 6 (Graphpad Software Inc, Ca, USA). Analysis was by RM-ANOVA or mixed effect models (Genstat v18; VSNi, Rothampsted, UK), as appropriate, with RRT mode, time and session as fixed effects. Patient ID was included as a nested random effect. Statistical significance was accepted at P≤0.002 (adjusted for the number of comparisons).

Figure 5. Comparison of effluent amino acid and trace element loss between first and second RRT

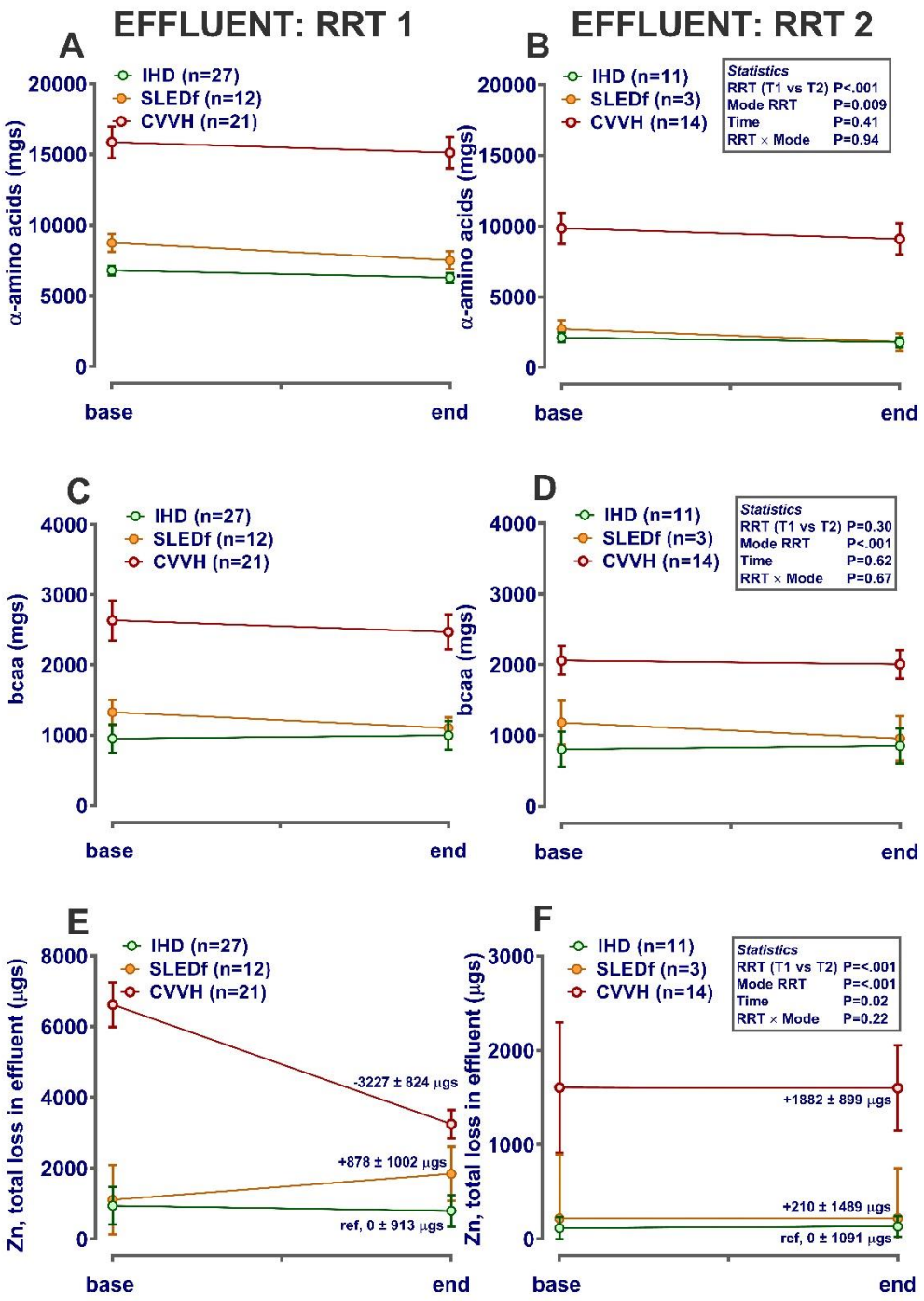


Figure 5. Comparison of effluent amino acid and trace element loss during the first (RRT1) and second (RRT2) session of renal replacement therapy. Effluent was sampled before, mid-way through and at the end of each RRT session. Concentrations of micronutrients were measured in spot samples of baseline effluent (i.e. after 'priming' each dialyser; IHD, SLED-F) or in replacement solution (e.g. PrismaSol for CVVH). Total losses were estimated by multiplying all measured concentrations (µgs/L) by the total volume of effluent produced for each patient. Data (means ±S.E.) are presented corrected (i.e. included as co-variates in the statistical model) for dose-of-dialysis (urea reduction ratio for plasma levels; solute removal index for effluent losses) and plasma concentration (for calculation of effluent losses only). If necessary, data were $\log_{10}$ transformed before statistical analysis to normalise residual error. Graphs were generated in Graphpad Prism 6 (Graphpad Software Inc, Ca, USA). Analysis was by RM-ANOVA or mixed effect models (Genstat v18; VSNi, Rothampsted, UK), as appropriate, with RRT mode, time and session as fixed effects. PatientID was included as a nested random effect. Statistical significance was accepted at $P \leq 0.002$ (adjusted for the number of comparisons).

Figure 6. Reference diagram