Differences and Effects of Metabolic Fate of Individual Amino Acid Loss in High-Efficiency Hemodialysis and Hemodiafiltration

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Objective: The objective of the study was to quantify the loss and arterial blood concentration of the three main classes of amino acids (AAs)—nonessential amino acids (NEAAs), essential amino acids (EAAs), and branched-chain amino acids—as resulting from high-efficiency hemodialysis (HED) and hemodiafiltration (HDF). We moreover aimed to identify the different fates and metabolic effects manifested in patients undergoing hemodialysis and the consequences on body composition and influence of nutritional decline into protein energy wasting.

Design and Methods: Identical dialysis monitors, membranes, and dialysate/infusate were used to ensure consistency. Ten patients were recruited and randomized to receive treatment with on-line modern HED and HDF. Arterial plasma concentrations of individual AAs were compared in healthy volunteers and patients undergoing hemodialysis, and AA levels outflowing from the dialyzer were evaluated. Baseline AA plasma levels of patients undergoing hemodialysis were compared with findings obtained 1 year later.

Results: A severe loss of AA with HED/HDF was confirmed: a marked loss of total AAs (5 g/session) was detected, corresponding to more than 65% of all AAs. With regard to individual AAs, glutamine displayed a consistent increase (+150%), whereas all other AAs decreased after 12 months of HD/HDF. Only a few AAs, such as proline, cysteine, and histidine maintained normal levels. The most severe metabolic consequences may result from losses of EAAs such as valine, leucine, and histidine and from NEAAs including proline, cysteine, and glutamic acid eliciting the onset of hypercatabolism threatening muscle mass loss.

Conclusion: Dialysis losses, together with the effect of chronic uremia, resulted in a reduction of fundamental EAAs and NEAAs, which progressively led our patients after 12 months to a deterioration of lean mass toward sarcopenia. Therefore, the reintroduction of a correctly balanced AA supplementation in patients undergoing HD to prevent or halt decline of hypercatabolism into cachexia is recommended.

Introduction

TO DATE, VERY few studies have been conducted to investigate the amino acid profile of heathy subjects. Generally, the authors of published articles have based their considerations on blood samples obtained from the peripheral venous system. However, in the present study, individual amino acid (AA) concentrations were measured in arterial blood taken from the arteriovenous fistula of patients undergoing hemodialysis treatment, thus implying the need for comparison with AA levels determined in arterial blood of healthy subjects. Preanalytical procedures and devices used in these studies are highly sensitive to both storage time and temperature; indeed, if not correctly applied, these parameters may significantly influence the reliable measurement of plasma AA levels.[1] Given the lack of relevant reports in the literature, an additional difficulty is represented by the comparison of clinical records from healthy Caucasian and Oriental populations,[2] with the normal amino acid plasma profile in these populations displaying a discrepancy from the early stages (2-4) of chronic kidney disease (CKD), particularly in elderly patients.[3,4] In a comparative study of patients with CKD and undergoing dialysis (CKD5D), Duranton et al.[5] reported how 21 AAs measured in 52 patients undergoing conservative treatment (CKD 2-5) differed significantly from those of 25 patients undergoing dialysis, with CKD5D patients displaying a lower total concentration of AAs (−15.5%. $P = .01$) and essential amino acids (EAAs) (−23.0%. $P < .001$). Moreover, patients undergoing

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dialysis had lower concentrations of 16 AAs, particularly alanine, arginine, methionine, tryptophan, tyrosine, and lysine, with significantly lower plasma concentrations of all EEAs. In the same article, plasma AA concentrations in patients with CKD stage 2-3 and CKD stage 4-5 yielded similar results for total AAs, EAAs, and nonessential amino acids (NEAAs); plasma concentrations of tryptophan were significantly lower in patients with CKD stage 4-5 because of the increased catabolism of this AA by indoleamine 2.3-deoxygenase.[6] Estimated glomerular filtration rate (GFR) correlates significantly with tryptophan, thus emphasizing the connection between changes in plasma AA concentrations and residual renal function, becoming more frequent and pronounced only in advanced stages of the disease and during dialysis treatment.[7] Unfortunately, the study failed[5] to provide details on dialysis vintage, depuration effectiveness, extracorporeal technology used, ultrafiltration volume exchange, and timing of dialysis regimens; last but not least, the study was limited by an unbalanced comparison of AAs detected in the venous blood of patients with CKD to levels measured in arterial blood of patients undergoing hemodialysis. Although not necessarily constrained to a strict limited protein intake, patients undergoing hemodialysis thrice-weekly were characterized by lower AA and EAA concentrations and incipient protein energy wasting (PEW). This observation had indeed been highlighted in a previous, rigorously performed study conducted by Murtas et al.,[8] confirming the losses sustained in the different categories of AAs. Total AA loss should be carefully assessed and defined using standardized parameters and well-established hemodialysis methods such as high-efficiency hemodialysis (HED), posthemodiafiltration (post-HDF), and prehemodiafiltration (pre-HDF) applied; the same kind of monitor and membranes should be used, maintaining identical hemodialysis duration, blood flow, and dialysate flow and providing for high-precision assessment of samples of spent outflow dialysate collected for each session. In this study, compared to levels measured in the arterial blood of healthy volunteers, patients undergoing dialysis displayed a decrease in circulating TAA levels caused by low levels of NEAAs and circulating levels of EAAs and BCAAs.[9] The conclusions reached by the study are that normal levels of EAAs and BCAAs are not the outcome of an increased intake of high-quality protein by patients, but rather of an increased muscle release of these AAs. A series of factors support this hypothesis: metabolic acidosis,[10] muscle proteolysis,[11] chronic inflammation resulting from uremia exacerbated by hemodialysis,[12] and difficulty in achieving the widely acknowledged optimal protein and calorie intake in patient. It should moreover be considered how AA loss from dialysis sessions is not rapidly compensated by the metabolism of patients undergoing dialysis.[13] More specifically, AAs lost during hemodialysis belong to the category of NEAAs. The use of advanced extracorporeal depurative techniques has proved to be particularly effective on small molecules such as plasma free AAs, at times exceeding 16 g/week in patients on thrice-weekly hemodialysis.[8] Extracorporeal dialysis, and particularly high-efficiency hemodialysis, induces a state of inflammation, thus contributing toward exacerbating the degradation of muscle proteins. Briefly, concentrations of EAA and BCAA in patients on chronic dialysis may reflect a significant release of AAs from muscle, resulting in a loss of muscle mass and impossibility of maintaining a good nutritional status and stable metabolic equilibrium, with these phenomena inevitably leading to progressive establishment of PEW and pronounced cachexia.[15] Accordingly, reduced plasma levels of NEAAs may be caused by an excessive uptake of these substrates by the body. An increased body uptake of NEAAs may also be manifested during dialysis sessions, with the specific aim of decreasing protein catabolism throughout the entire body, thereby strengthening the hypothesis relating to the use of AAs released from muscle masses.[16] Indeed, a further deterioration of this metabolic profile was observed after an additional 12 months of dialysis in the same patients. Over the last 10 years, substantial technological progress has been made after the introduction of innovative technologies enabling the preparation of ultra-pure dialysis fluid from drinking water (on-line HDE and HDF) by applying a higher convective pressure gradient between plasma water/dialysate inside the dialyzer hemodialysis filter, resulting in a more significant loss of AAs and albumin than previous extracorporeal methodologies.[17] However, the use of these methods is justified by a significant increase in depuration efficiency for both small and medium-large molecules, fewer distressing side effects during the dialysis session, and lower morbidity and mortality.[18] However, in a previous study,[19] Gil et al. demonstrated a higher loss of AAs for both hemodiafiltration techniques than diffusive hemodialysis methods, observing nonsignificant differences between HDFpost and HDFpre. To the best of our knowledge, to date, very few studies have identified both the category and quantity of AAs lost during HED and HDFpost,[20,21] while no such studies have yet been carried out on HDFpre. Based on the authors' experience, a nonsignificant change up to stages CKD3-4 is hypothesized (personal observation, unpublished data). The change in nutritional and amino acid structure is first manifested in the advanced stages of CKD5 and undergoes a dramatic deterioration, particularly evident over the first few years, with the onset of hemodialysis treatment. This deterioration is almost entirely due to loss of a huge amount of amino acids through dialysis,[8] which may be difficult to balance by means of an adequate calorie and protein intake, particularly in a dialysis population with an average age of 65-70 years or more. Therefore, the aim of the present study was to ascertain and highlight the potential metabolic and nutritional consequences produced by uremia and hemodialysis syndromes, as well as by unavoidable major losses in several categories of AAs, on

CKD5D patients, and to attempt to demonstrate the metabolic changes caused by an altered asset of each single AA which could result in the onset of cachexia within a few years. We therefore suggest that new options to replace AA loss should be identified, with particular focus on both patients with an expectation of lengthy dialysis vintage and no future possibility of transplant and patients undergoing hemodialysis over the age of 65-70 years.

Methods

Patients

Ten patients on a chronic hemodialysis regimen were recruited to the study. All patients were Caucasian males. The inclusion criteria were metabolic steady-state, age between 18 and 80 years, thrice-weekly hemodialysis schedule, dialysis vintage exceeding 6 months, and oligo-anuria with 24-h diuresis <200 mL/day. The exclusion criteria were absence of acute or chronic inflammatory disease, malignant tumors and/or autoimmune disease, a previous history of kidney transplant, previous treatment with steroids and/or immunosuppressant drugs, heart failure and/or ischemic cardiomyopathies, intermediate-severe changes to respiratory function, changes to liver function, no risk factor for malnutrition, and recent or ongoing administration of any form of nutritional support. The mean age of the 10 patients recruited was 70.4 ± 9.5 years (54-80 years), and the mean dialysis vintage was 77.6 ± 37.7 months. Dry weight/ideal weight of patients at the start of the study was 72.7 ± 12.3 kg (47 - 87.4). Dry weight/postdialysis target weight was established according to baseline weight and intradialytic clinical assessment and by means of bioimpedance analysis (BIA) measures.[22,23] Eight healthy volunteers (6 males, 2 females, 65 ± 9 years) were recruited as controls; a sample of arterial blood was obtained for the purpose of comparison of AA concentrations to levels measured in arterial blood drawn from the arteriovenous fistula of patients undergoing hemodialysis.

Study Design

Patients were randomized to the following extracorporeal dialysis techniques: HED, HDFpost, and HDFpre. Each patient underwent 4 sessions featuring a different sequence of the three methods: 3 patients adhered to the sequence HED-HDFpost-HDFpre; 2 patients HED-HDFpre-HDFpost, and 5 patients HDFpost-HDFpre-HED. Over the previous week, patients had undergone 3 sessions of the same procedure for each dialysis method (HED, HDFpost, HDFpre). Clinical assessment and blood chemistry tests were carried out during the fourth session, corresponding to the longest interdialytic interval. This regimen was adhered to for all 3 methods used to avoid carry-over effects from the techniques applied previously. Throughout the study, no patients received any form of nutritional supplementation, and their routine dietary intake of proteins, fats, carbohydrates, and calories remained unchanged. The study was approved by the ethics committee of the local health authorities. The study was conducted between January 2017 and February 2018.

Extracorporeal Dialysis Procedures and Study of Nutritional Status

The same dialysis monitors and same type of high- and low-performance polysulphone membranes were used for all patients as follows: for HED diffusive depuration techniques, an ultrafiltration coefficient of 18 mL/minute/mmHg/hour was used, while for high-performance ultrafiltration in HDFpost and HDFpre, a high ultrafiltration coefficient of 59 mL/minute/mmHg/hour was applied. Both membranes had a total dialysis surface of 1.8 m^2 (FX 80; Fresenius Medical Care, St. Wendel, Germany). Membranes were chosen for their high degree of biocompatibility and depurative capacity, bearing in mind another two important phenomena: increased convection in HDFpost and HDFpre is associated with a largely unquantifiable protein loss; however, as these synthetic membranes are manufactured using a mix of polymers to produce a mosaic-style membrane surface containing hydrophilic and hydrophobic components, the possibility that albumin-linked AA may be absorbed and/or retained on the surface or within the pores of the membrane should be taken into account.[24,25] Nutritional status according to age was assessed at the start of the study and after 12 months and values compared with those of a control group of 8 healthy volunteers. Comparison of nutritional status was performed using anthropometric data and based on the findings of Mini Nutritional Assessment (MNA), BIA (RenalEFG 50 KHz; EFG Diagnostic Ltd, Belfast, Northern Ireland), blood chemistry nutritional profile, and Resting and Total Energy Expenditure/kg/day (REE, TEE). TEE is obtained from REE x 1.3 for daily physical activity correction factor. In healthy subjects, assessment of protein intake can be performed from dietary records and/or food questionnaires or by calculation of average daily intake by a skilled dietician; however, for patients undergoing hemodialysis, the average calculation obtained in the first, second, and third weekly session for normalized protein catabolic rate was considered, being more precise[26] during steady metabolic state than nutritional recall. Transferrin and prealbumin were not taken into consideration as heavily affected by iron status and inflammation associated with uremia and dialysis.[27] To ensure a better collaboration in terms of nutritional adherence, a nutritionist and a medical-nursing counseling team worked in synergy with patients and their families,[28] focusing particularly on a careful assessment of nutritional status.[29,30]

Kinetics and Determination of Amino Acids

To avoid interferences with AA plasma concentrations, patients on dialysis were not allowed to eat for a period of 6 hours before or during dialysis session on blood and dialysate test days. To ensure the highest degree of reliability of results, accurate methods were applied in the sampling and

collection of plasma from arterial blood for use in AA concentration assays: before and after the dialysis session, 10 mL of whole blood were collected in 2 heparinized test tubes and stored at room temperature (to avoid issues of thermal hydrolysis). Plasma was separated within 2 hours of collection by centrifuging at 3000 rpm for 10 minutes. The plasma thus obtained was frozen in 2-mL cryogenic test tubes at a temperature of 220°C. Dialysate outflux was sampled by means of continual spillage[31,32] to quantify AA loss via a high-precision volumetric pump used for drug administration (Agilia®; Fresenius Kabi, Bad Homburg, Germany) situated at dialysate outflow filter and featuring a constant rate of aspiration throughout the 4-hour treatment session. The flow rate of dialysate sampled corresponded to 1% of total dialysate flow. The fluid collected was then mixed to obtain a homogenous solution, and 4-mL samples were obtained and stored in a freezer in two 2-mL test tubes. Within 2 days of collection, samples were transferred on dry ice to the labortaory for final storage. These substrates were determined for each dialysis session in arterial blood and dialysis fluid. The analytical method required "pre-column" derivation of free amino acids by ortho-phthalaldehyde and 9-fluorenyl-methyl-chloroformate for the recognition of primary and secondary amino acids, respectively. Derivates were separated by means of reverse-phase liquid chromatography and revealed using a fluorometer X-LC (model 3020FP). Analysis was carried out on a 1-μL sample of a standard mixture or serum. Sample testing was invariably preceded by analysis of a standard mixture to verify system efficiency. Graduated concentrations (from 29 to 233 μM/μL) of the standard mixture were used to establish the calibration curve for subsequent use in quantitative analysis. To increase reliability of the results, each sample was analyzed in triplicate and each amino acid was quantified based on the mean obtained from three determinations. The results were obtained by injecting 1 mL of derived mixture and simultaneously measuring absorbance at 338 nm and 262 nm. Samples were tested using an amino acid analyzer HPLC X-LC-Jasco linked to an HP ProDesk elaborator. AA concentrations were expressed in both μl/L and mg/dl and compared with standard values in our laboratory. The data obtained in this study were subsequently compared statistically using the same laboratory procedure, with arterial AA concentrations determined in 8 healthy volunteers. Twenty AAs were determined as TAAs and EAAs, including the branched-chain AAs and NEAAs. Aminoaciduria was not taken into consideration because of the presence in all patients of diuresis <200 mL/day.

Statistical Analysis

Statistical analyses were performed by means of a linear mixed model in which patient identification was introduced as a random effect. This operation allowed us to establish the potential differences resulting solely from the decode and remove them from the model, thereby increasing potency and precision. All analyses were performed using the R software version 3.4.1. The disconnection/connection difference, the percentage loss between disconnection and connection, and the results of statistical tests have been reported for all comparisons. For comparison data, the Mann-Whitney test was used, considering a valid significance only when $P < .05$.

Results

Dialysis Adequacy

A thrice-weekly dialysis regimen with 240-minute sessions was adopted for all patients and all methods used. All 10 study patients underwent scheduled hemodialysis sessions in line with the relevant study design: HED, HDFpost, or HDFpre. Patients' mean age was 70.4 ± 9.5 years; dialysis vintage 77.6 ± 33.7 months, and dry weight/ideal weight 72.7 ± 12.3 kg. For all three methods, dialysis efficiency was provided by an equilibrated Kt/V of 1.37 ± 0.2[33] and a urea reduction rate of 72.5 ± 4.5%. In HED plasma, water replacement corresponded to 4.4% of body water (equal to water increase during the interdialytic interval), in HDFpost to 29.8%, and in HDFpre to 64.5%.

Nutritional Status

Table 1 illustrates the anthropometric measurements, clinical assessment of nutritional status using MNA, body composition assessed by BIA, resting energy expenditure (REE), and total energy expenditure (TEE). Protein intake (protein catabolic rate) and dialysis patients' biohumoral parameters at the start of the study and after 12 months are highlighted. Moreover, the comparative statistical analysis performed between the healthy controls and patients at the time of recruitment and 12 months after the start of the study to assess the outcomes achieved over time is reported.

Comparison Between Healthy Subjects and Patients on Hemodialysis at the Time of Recruitment (T0)

No differences in body mass index were observed between the groups, and the MNA test revealed a normal nutritional status. However, analysis of body composition in patients undergoing hemodialysis revealed a significant reduction in body cell mass (Phase Angle, A°) and lean body mass (BCM), including muscle mass. These changes were linked to redistribution of intercompartmental body water characterized by an increased extracellular water (ECW)/intracellular water (ICW) ratio caused both by an expansion of ECW and a reduction in ICW. These contrasting changes resulted in a conservation of total body water (TBW), although muscle mass index (MMI, Kg/m^2) of patients highlighted a state of moderate sarcopenia[34,35] with values of 9.37 ± 1.07 kg/m^2. REE and TEE of patients were similar to those observed in healthy subjects. Protein intake contributed to 15.6% of TEE in patients. Reduced

Table 1. Nutritional Data differences between Healthy Subjects and Hemodialysis Patients at the Recruitment (T0) and After 12 Months

	Healthy Volunteers	Hemodialysis Patients at the Recruitment of the Study (T0)	Hemodialysis Patients After 12 months
Nutritional parameters			
Body weight, Kg	75.0 ± 10.0	72.7 ± 12.3$^{\square\square}$	70.5 ± 11.8$^{\square\square}$
Body mass index, Kg/m^2	27.1 ± 2.2	25.7 ± 3.4	25.0 ± 3.3
Minimal nutritional assessment, score	25.7 ± 4.5	25.5 ± 5.3	24.9 ± 5.1
Protein intake, g/Kg/day	1.1 ± 0.2	0.93 ± 0.12	0.91 ± 0.17
Bioelectrical impedance analysis			
Total body water, %	53.6 ± 11.3	51.7 ± 6.6$^{\square\square\square\square}$	55.2 ± 7.0$^{\square\square\square\square}$
Extracellular body water, %	43.4 ± 14.5$^{\#}$	55.2 ± 4.5$^{\#\square}$	57.5 ± 4.7$^{\square}$
Intracellular body water, %	37.3 ± 15.5$^{\#}$	44.8 ± 4.6$^{\#\square}$	42.5 ± 4.8$^{\square}$
Body cell mass, %	48.4 ± 4.8$^{\diamond}$	43.8 ± 4.9$^{\diamond\square\square\square}$	41.1 ± 5.0$^{\square\square\square}$
Muscle mass, %	43.6 ± 6.3$^{\#}$	36.9 ± 4.8$^{\#}$	37.7 ± 5.5
Fat free mass, %	66.1 ± 17.3	65.9 ± 8.1$^{\square\square\square}$	68.8 ± 8.4$^{\square\square\square}$
Fat mass, %	33.9 ± 17.3	34.1 ± 8.1$^{\square\square\square}$	29.7 ± 8.9$^{\square\square\square}$
Phase angle, (A°)	5.8 ± 0.5$^{\diamond}$	4.3 ± 0.7$^{\diamond\square\square\square}$	4.0 ± 0.6$^{\square\square\square}$
Resting energy expenditure			
Resting energy, KCal/Kg/day	19.5 ± 2.1	18.6 ± 3.1	19.3 ± 3.3
Total energy expenditure, KCal/Kg/day	25.3 ± 2.8	24.1 ± 4.1	24.6 ± 4.2
Total energy expenditure, KCal%	17.3 ± 3.1	15.5 ± 1.9	14.8 ± 0.1
Ematochemical parameters			
Total protein, g/dL	6.6 ± 8.9	6.7 ± 0.6	6.6 ± 0.4
Albumin, g/dL	4.1 ± 0.3	3.9 ± 0.2	3.8 ± 0.3
Creatinine, mg/dL	0.9 ± 0.4§	9.9 ± 1.6$^{\S\square\square\square\square}$	10.4 ± 1.8$^{\square\square\square\square}$
Total Cholesterol, mg/L	185 ± 25$^{\diamond}$	128 ± 24$^{\diamond\square\square\square}$	131.2 ± 26$^{\square\square\square}$
C_3, mg/dL	95.0 ± 5.5	94.8 ± 9.4	87.7 ± 7.5
Hb, g/dL	13.5 ± 0.6	12.3 ± 2.3$^{\square}$	11.9 ± 0.7$^{\square}$
C reactive protein, mg/L	3.5 ± 0.7§	5.8 ± 2.4$^{\S\square\square\square\square}$	7.0 ± 6.3$^{\square\square\square\square}$
Lymphocytes, mm3	3200 ± 1200§	1918 ± 309$^{\S\square\square\square\square}$	1180 ± 537$^{\square\square\square\square}$

Comparison: healthy versus start of the study. §: $P < .001$; ◇: $P = .03$; #: $P = .02$; ⊿: $P > .05$; C reactive protein normal values < 5 mg/L. Comparison between start of the study versus 12 months later. □□□□: $P < .001$; □□□: $P = .03$; #, □□: $P = .02$; □: $P < .05$.

protein intake corresponding to 1.2-1.4 g/kg/day was in line with quantities recommended in the guidelines.[36] Patients' biohumoral profile showed a significant reduction of circulating lymphocytes and an increased serum complement activity, indicating a state of systemic inflammation. Serum concentrations of protein and albumin were similar to those detected in healthy subjects, although patients undergoing hemodialysis were characterized by reduced hemoglobin levels. Briefly, at the start of the study, compared with healthy subjects, patients on dialysis presented with a state of systemic inflammation, decreased specific immunity, altered intercompartmental body water ratio, and sarcopenia with inadequate protein intake. These alterations were all associated with normal body weight and normal score at clinical assessment of nutritional status (MNA).

Variations Over Time (T0 vs. T12) of Dialysis Patient Parameters

After 12 months' dialysis, patients presented with a 2.5% loss of body weight and deterioration of body composition; this was mainly evident in the reduction of the phase angle, while ICW and BCM were further decreased compared with values obtained at the start of the study, and ECW rose significantly. Moreover, despite the further reduction of active oxygen-consuming cells, REE remained virtually unchanged. These findings all pointed toward the presence of a hypermetabolic state. Protein intake tended to decline (0.91 g/kg; $P = .06$). A significant decrease in circulating lymphocytes was indicative of a reduced immune defense. In addition, in spite of constant monitoring and administration of iron and erythropoietin supplements, a fall in hemoglobin levels was observed, although these remained within the recommended range.[37] A worsening of sarcopenia was observed (8.79 ± 1.0 kg/m^2). To summarize, over a 12-month hemodialysis period, patients underwent weight loss, worsening of sarcopenia, and decline of specific immune defenses, in addition to a rise in ECW and decrease of ICW.

Behavior of Amino Acids According to Category

Plasma AA concentrations were determined before dialysis sessions. The study revealed a reduction in total AAs in patients compared with values obtained from the arterial blood of healthy controls (Table 2). Moreover, compared with healthy controls, study patients displayed a significant decrease in NEAAs, with similar levels of EAAs (leucine,

Table 2. Differences of Arterious Blood Levels AA Between Healthy Subjects and Patients on Hemodialysis

	Healthy Controls (n = 8)	CKD Patients (n = 10)
TAA, mg/dL	33.1 ± 0.8	25.8 ± 4.3*
EAA, mg/dL	8.9 ± 0.2	8.6 ± 1.9
NEAA, mg/dL	24.4 ± 0.6	17.4 ± 3.2†
BCAA, mg/dL	3.5 ± 0.2	3.4 ± 0.7

AA, amino acid; BCAA, branched-chain amino acid; CKD, chronic kidney disease; EAA, essential amino acid; NEAA, nonessential amino acids.
*$P < .001$.
†$P < 0.02$.

isoleucine, valine, threonine, lysine, methionine, phenylalanine, tryptophan) and no differences in BCAAs (leucine, isoleucine, valine). The study highlighted a loss of AAs in dialysis fluid for each treatment method. The results obtained indicated a different quantity of AAs lost per session between the three hemodialysis methods used. Overall total AA loss during dialysis was essentially higher, although not significantly, in HDF than in HED (Table 3).

Mass balance of total amino acid loss from dialysis fluid filter outflux in a patient undergoing thrice-weekly dialysis amounts to approx. 16 g/week, resulting therefore in a mean yearly loss of free AAs of 836 g.

Essential Amino Acids

Table 3 also provides a list of the losses in dialysate of all free amino acids from the EAA category in descending order. In particular, valine and histidine exceeded a mean loss of 900 mg/week for all three methods on a thrice-weekly dialysis regimen. Both hemodiafiltration methods clearly resulted in greater losses in outflow dialysate of histidine, lysine, and threonine. Patients on thrice-weekly dialysis displayed a mean yearly loss of EAAs of more than 270 g.

Nonessential Amino Acids

Table 3 lists the losses in dialysate of all free amino acids from the NEAA category in descending order. In particular, cysteine, proline, and glutamic acid exceeded a mean loss of 1800-2400 mg/week for all three methods on a thrice-weekly dialysis regimen. In these classes of AA, pre-HDF methods resulted in the highest losses in outflow dialysate. The quantities of serine, asparagine,

Table 3. Loss of Amino Acids (AAs) in mg per Hemodialysis Session in the Three Different Extracorporeal Methodologies

	HED	HDF post	HDF pre
Essential Amino Acids (EAAs)			
Valine	319 ± 131$^{\Delta}$	334 ± 188$^{\Delta}$	339 ± 102$^{\Delta}$
Histidine	282 ± 142$^{\square}$	320 ± 146$^{\square}$	342 ± 118
Leucine	221 ± 85$^{\square\Delta}$	246 ± 101$^{\square\Delta}$	237 ± 85$^{\Delta}$
Phenylalanine	209 ± 87	217 ± 79$^{\square}$	186 ± 87$^{\square}$
Lysine	196 ± 81$^{\square\square\square\square}$	246 ± 87$^{\square\square\square\square\Delta}$	237 ± 51$^{\Delta}$
Threonine	147 ± 74$^{\square\square}$	174 ± 72$^{\square\square\Delta}$	172 ± 88$^{\Delta}$
Isoleucine	147 ± 61$^{\Delta}$	159 ± 58$^{\Delta}$	152 ± 34$^{\Delta}$
Tryptophan	98 ± 36$^{\square}$	87 ± 72$^{\square\Delta}$	85 ± 31$^{\Delta}$
Methionine	37 ± 12$^{\square\square\square}$	43 ± 13$^{\square\square}$	34 ± 10$^{\Delta\square\square}$
Total AA loss, mg	1656 ± 87$^{\square\square\square}$	1826 ± 98$^{\square\square\square\Delta}$	1784 ± 103$^{\Delta}$
Nonessential amino acids (NEAAs)			
Proline	802 ± 507$^{\Delta}$	818 ± 434$^{\Delta}$	876 ± 403$^{\Delta}$
Cysteine	896 ± 328$^{\square\square}$	746 ± 260$^{\square\square\Delta}$	779 ± 270$^{\Delta}$
Glutamic acid	550 ± 196$^{\Delta}$	570 ± 173$^{\Delta\square\square}$	680 ± 135$^{\square\square}$
Glutamine	491 ± 245$^{\Delta}$	434 ± 246$^{\Delta}$	423 ± 220$^{\Delta}$
Alanine	405 ± 147$^{\Delta}$	392 ± 130$^{\Delta}$	389 ± 102$^{\Delta}$
Glycine	184 ± 98§	217 ± 87$^{\square\square\square}$	152 ± 51$^{\S\square\square\square}$
Arginine	184 ± 56$^{\square\square}$	132 ± 75$^{\square\square\S}$	169 ± 88§
Tyrosine	135 ± 49$^{\Delta}$	130 ± 43$^{\square\square\Delta}$	102 ± 34$^{\square\square}$
Serine	61 ± 23$^{\Delta\Delta}$	58 ± 29$^{\Delta}$	51 ± 16$^{\square}$
Asparagine	37 ± 19$^{\Delta}$	43 ± 15$^{\Delta\square}$	34 ± 17$^{\square}$
Aspartic acid	25 ± 12$^{\Delta}$	29 ± 14$^{\Delta\square\square\square\square}$	51 ± 17$^{\square\square\square\square}$
Total AA loss, mg	3573 ± 284$^{\Delta}$	3566 ± 286$^{\Delta\square\square\square}$	3701 ± 314$^{\square\square\square}$
Branched-chain amino acid (BCAA)			
Valine	319 ± 131$^{\Delta}$	334 ± 188$^{\Delta}$	339 ± 102$^{\Delta}$
Leucine	221 ± 85$^{\square\Delta}$	246 ± 101$^{\square\Delta}$	237 ± 85$^{\Delta}$
Isoleucine	147 ± 61$^{\Delta}$	159 ± 58$^{\Delta}$	152 ± 34$^{\Delta}$
Total loss, mg	687 ± 69$^{\square}$	739 ± 74$^{\square\Delta}$	728 ± 73$^{\Delta}$
Total AA loss	6096 ± 15$^{\Delta}$	6131 ± 155$^{\Delta}$	6213 ± 161$^{\Delta}$

HDF, hemodiafiltration; HED, high-efficiency hemodialysis.
$^{\square\square\square\square}$: $P < .001$; $^{\square\square\square}$: $P < .03$; $^{\square\square}$: $P < .02$ $^{\square}$, §: $P < .05$; $^{\Delta}$: $P > .05$.

and aspartic acid lost were modest. Patients on thrice-weekly dialysis displayed a mean yearly loss of EAAs over 56 g.

Branched-Chain Amino Acids

Finally, in Table 3, although comprised in the category of EAAs, we deemed it opportune to highlight the loss of BCAAs in outflow dialysate. It should be pointed out how valine exceeded mean dialysis loss of 600 mg/week. All three BCAAs are hydrophobic. A significant modest prevalence of HDFpost versus HED was observed for BCAAs. Patients on thrice-weekly dialysis displayed a mean yearly loss of BCAAs over 110 g.

Changes in Plasma Amino Acids After 12 Months of Dialysis

Plasma levels of each single amino acid were also measured in both healthy subjects and patients at the start of the study and after 1 years' follow-up (Tab. 4). It was evident that of the first six AAs featuring a percentage reduction in plasma levels of >30% in arterial blood, five were NEAAs, with the exception of tryptophan. Changes observed in arterial AA concentrations of 8 healthy volunteers compared with levels detected for dialysis patients at the start of the study and after 12 months of dialysis treatment were as follows: glutamine levels had exceeded concentrations measured in healthy subjects after 12 months of hemodialysis; cysteine maintained higher levels than healthy volunteers, although registering an approximately 30% decrease over 12 months of dialysis; arterial levels of arginine and phenylalanine did not vary significantly between healthy subjects and patients either at the start of the study or after 12 months' dialysis. It should also be highlighted how all BCAA concentrations remained unchanged at all three time points, displaying values in line with those detected in healthy subjects. Even after 12 months' treatment, patients' proline levels were higher than those recorded in healthy controls.

Discussion

This study shows how at the time of recruitment patients were already affected by a state of malnutrition/inflammation typical of the terminal stages of uremia in patients on dialysis.[38] Indeed, this serious metabolic situation represents a key component of the so-called "malnutrition-inflammatory complex syndrome"[39,40] linked in patients on dialysis to a reduced quality of life, increased number of hospitalizations, higher incidence of coronary heart disease and depression, and an increased mortality rate.[41-45] Malnutrition initially presents as a somatic protein type with the onset of sarcopenia. However, a significant depletion of active cell mass, including muscle tissue, may at times contribute to a change in body water, with a rise in ECW and decrease of ICW, as reported previously in stressed postoperative and depleted patients.[46] After a 12-month follow-up period, malnutrition evolves into PEW characterized by further deterioration of nutritional status, weight loss, and nonsignificant inflammation. The inability of clinical assessment of nutritional status (MNA) to reveal the presence of malnutrition in patients recruited to the study is deemed of interest. Nonetheless, the pathogenesis

Table 4. Comparison of Arterial Blood Amino Acids Concentrations in Hemodialysis (HD) Patients at the Start of the Study and After 12 months of Dialysis

Average Amino Acids Blood Levels of the Three Treatments, mg/dL	Healthy Subjects	Hemodialysis Patients: Start of the Study	Hemodialysis Patients after 12 Months from the Start	Delta, mg/dL	Delta, %
Glutamine	6.8 ± 0.20□□	3.21 ± 1.09§	8.20 ± 1.47§□□	+4.98	+154.80
Aspartic acid	0.80 ± 0.02*	0.10 ± 0.03*	0.04 ± 0.01*	−0.06	−60.05
Serine	0.93 ± 0.05*	0.33 ± 0.11□□□	0.18 ± 0.10*□□□	−0.17	−48.44
Glutamic acid	2.92 ± 0.16*	1.91 ± 0.34*	1.08 ± 0.15*	−0.84	−43.85
Tryptophan	1.28 ± 0.18*	0.48 ± 0.19□□□	0.28 ± 0.02*□□□	−0.21	−42.90
Asparagine	0.80 ± 0.02*	0.30 ± 0.20□□□	0.19 ± 0.01*□□□	−0.12	−38.36
Cysteine	0.93 ± 0.06*	3.42 ± 0.89*	2.34 ± 0.21*	−1.08	−31.57
Lysine	1.71 ± 0.15□	1.47 ± 0.33	1.14 ± 0.14□	−0.36	−23.81
Arginine	1.03 ± 0.13	1.10 ± 0.43	0.85 ± 0.17	−0.25	−22.92
Alanine	2.78 ± 0.14&	2.25 ± 0.58□□□	1.76 ± 0.69&□□□	−0.49	−21.69
Threonine	1.28 ± 0.11*	0.67 ± 0.29*	0.54 ± 0.19*	−0.14	−20.80
Isoleucine	0.58 ± 0.05	0.69 ± 0.15	0.57 ± 0.13	−0.14	−19.86
Valine	1.78 ± 0.17□	1.62 ± 0.35	1.33 ± 0.17□	−0.31	−18.73
Tyrosine	1.0 ± 0.11*	0.67 ± 0.15*	0.55 ± 0.18*	−0.12	−18.58
Methionine	0.15 ± 0.02	0.28 ± 0.07	0.23 ± 0.01	−0.05	−18.06
Histidine	0.90 ± 0.09 □□	1.53 ± 0.68□□	1.29 ± 0.15	−0.23	−14.94
Leucine	1.02 ± 0.08	1.04 ± 0.21	0.90 ± 0.13	−0.15	−14.58
Proline	2.40 ± 0.12□□	3.44 ± 1.37□□	3.11 ± 1.11	−0.33	−9.70
Glycine	2.01 ± 0.09*	0.77 ± 0.26*	0.71 ± 0.17*	−0.07	−9.08
Phenylalanine	0.76 ± 0.07	0.82 ± 0.14	0.78 ± 0.16	−0.05	−5.67

§: $P < .001$; (*): $P < .0001$; &: $P < .05$: □□□: $P < .03$; □□: $P < .02$ □: $P < .05$.

of malnutrition in patients on dialysis is well acknowledged and documented, comprising additional hypercatabolic factors, including chronic infection secondary to indispensable dialysis procedures, metabolic acidosis during interdialytic intervals, hormonal changes, and frequent inadequacy of nutritional intake, particularly in elderly patients. The progressive malnutrition observed in patients recruited to the study undeniably indicated the inadequacy of protein and calorie intakes to meet metabolic demand. Indeed, the initial protein deficit registered corresponded to 22-34% of recommended values of 1.2 - 1.4 g/kg/day,[36] with a tendency toward worsening at the 12th month of follow-up. Protein deficit was however actually considerably higher because of the loss of amino acids into the dialysis bath at each session, even exceeding 800-900 g yearly for the 20 main AAs.[8] A limitation of this study may unfortunately be represented by our decision to not ask patients on dialysis to keep a food journal, which would have allowed us to quantify calorie intake. The use of 48-h or weekly recall as a method of dietary assessment led to some issues with data capture. Many patients were unable to recall a full period, and therefore, meals reported over a mid-long recall period were a patchwork providing an average estimation of nutritional intake[47] at the time of assessment, the accuracy of which remains questionable. This was in addition to the well-known problems of dietary assessment including overestimation and underestimation of intake and a tendency for participants to inform the investigator of food intake that they feel is correct rather than their actual intake.[48,49] Accordingly, calculation of normalized protein catabolic rate[26] has since been applied which, particularly in patients on dialysis thrice-weekly, provides a more precise estimation of protein intake. In view also of the advanced age of patients, the presence of a calorie deficit versus the recommended intake of 30-35 kcal/kg/day is highly likely; however, the weight loss, reduced appetite, and worsening observed over the 12-month period of observation further supported our opinion of a somatic protein form of malnutrition aggravated by a calorie deficit. Indeed, a relative hypermetabolic state, as observed in the present study, may contribute toward determining a discrepancy between calorie intake and demand. Accordingly, at the start of the study, our patients undergoing hemodialysis, who consumed +6% calories per kg of body cell mass, displayed a 12.4% lower body cell mass than healthy controls (relative hypermetabolic state). At 12 months, the normalized overconsumption had increased in line with a further decrease in body cell mass and virtually stable REE. These findings all indicate a lack of compensation of calorie deficit in patients on dialysis. The authors hypothesize therefore that an insufficient protein intake is of greater relevance than a calorie deficit. This hypothesis is based on the finding of a worsening over time of two body compartments: the muscular system and immunocompetent cell network. Immunocompetent cells, including lymphocytes, are indeed active and avid consumers of amino acids.[50] Our study suggests that an excessive muscle release of amino acids[8] may maintain albumin synthesis but fail to support lymphocyte proliferation. The latter is an important clinical observation which should be further investigated to verify whether this deterioration of lymphocytes may be associated with the availability of different types of amino acids. Twelve months after the start of the study, an increase in degree of inflammation, although not significant, may lead to serious metabolic consequences, as demonstrated by the time trend observed in the findings obtained.

Amino Acid Loss and Metabolic Consequences

A series of factors may have influenced amino acid loss in dialysate outflux, thus justifying the discrepancy in changes observed in plasma concentrations over a 12-month period. Amino acid/albumin association, in the same way as all plasma molecules, may affect the transfer of AAs through dialysis membrane pores; their absorptive effect however should not be underestimated. No studies have been conducted to date to investigate this phenomenon in view of quantification encountered in patients on dialysis because of intradialytic variations resulting from a marked change from acid pH at the start of the session to basic pH on completion. It should also be noted how AAs possess a series of side chains (CH3, OH, NH2, SH), thus hampering determination of the effect produced by these factors on the different AA categories with neutral nonpolar loads (alanine, phenylalanine, glycine, isoleucine, leucine, methionine, proline, tyrosine, tryptophan, valine), neutral polar side chains (asparagine, glutamine, serine, threonine, cysteine), acid side chains (aspartate, glutamate), and basic side chains (arginine, histidine, lysine). Factors that may potentially affect binding of some AAs with plasma proteins are in turn influenced by the presence of PEW in patients on dialysis, implying a significantly reduced serum concentration of albumin. No specific data to this regard have been reported in literature. As an example, in CKD 4-5 normoalbuminuria patients, protein binding of tryptophan is negatively correlated with GFR, which likely competes to bind to this AA because of the increase in organic acids present in the uremic environment.[51] It is therefore probable that the higher concentrations of radical acids present at the time of sampling, coinciding with acidemia peak in the patients studied, may result in an increased propensity to dialytic loss during the first 2 hours because of higher plasma concentrations of free AAs. The patients included in this study manifested a change in pH from mean predialysis plasma values of 7.360 $\pm$ 0.05 versus 7.460 $\pm$ 0.06 at the end of the session, possibly accounting for an increase in the number of free AAs, particularly over the second half of the hemodialysis session. Regrettably, data relating to the protein-binding of AAs in patients with CKD are virtually nonexistent in the literature; in 1969, Burzynski[52]

reported difficulties in comparing the concentration of 10 AAs and a wide range of variability, although observing how a comparison of adults (aged 19-66 years) affected by CKD with high plasma urea levels with subjects having a normal kidney function revealed a high percentage of albumin-bound glycine of +48.1%, aspartic acid +34.5%, glutamic acid +22.8%, lysine +31.5%, and threonine +21.5%. However, in the present study, free AA levels were determined on dialysate outflux, with dialytic losses seemingly unaffected by or correlated with protein-binding; indeed, the correlation between protein-bound AA levels and plasma losses of the same proved to be overdispersive and nonsignificant ($r = 0.39$, p: n.s.). We should however point out how the amino acid profile of our patients on hemodialysis at the start of the study was the result of a series of interacting, although at the same time partially contrasting, factors. Changes to the AA metabolism during the predialysis stage of kidney disease, systemic inflammation present in CKD, dialysis replacement treatment with specific focus on adequacy and methodological differences, nutritional supplementation of proteins/AAs, and anomalies present in plasma amino acid levels both on fasting and after meals, have all been well documented in conservatively managed patients with CKD.[53] More specifically, an increase in urea synthesis as a result of major loss of kidney function may induce both an increase of sulphurated AAs[54,55] and a reduction of tyrosine and tryptophan.[56-60] A state of chronic inflammation in the study patients on dialysis was key in eliciting a further deterioration of plasma amino acid content, in turn resulting in increased protein and amino acid turnover with prevalence of catabolism on protein synthesis and excessive uptake of AAs, particularly in muscle tissue.[61] This aspect has long been underestimated in clinical practice. Subsequently, these losses close and extend the vicious circle by exacerbating both systemic inflammation and plasma levels of each single amino acid. A further contribution is provided by chronic inflammation of the gastrointestinal tract,[62] frequently associated with advanced stages of CKD up until dialysis treatment; dialysis sessions *per se* are known to further worsen the state of systemic inflammation.[40] In addition, continual AA losses in dialysis outflux contribute after every session to the abnormalities registered in AA plasma concentrations. It should be underlined how a mean of 6 g of amino acids are lost in each hemodialysis session,[19] as confirmed in both a previous[8] and present study. Currently, however, there is no plausible hypothesis to account for the observed increased plasma levels of histidine, although a key contribution may possibly be ascribed to the reduced activity of protein synthesis associated with dialysis treatment. Indeed, in patients with severe kidney failure, the concentration and activity of proteins with a high histidine content such as hemoglobin, myosin heavy chains, and cytochrome c-oxidase are reduced.[63] Metabolic hyperactivity of the intestine may justify the low levels of glutamine detected in our study because this amino acid is generally widely used by intestinal epithelial cells and immune cells implicated in chronic inflammation. Theoretically, the nutritional intake of AAs through proteins may contribute toward reducing changes in plasma AAs; however, in the patients studied, in the same way as the majority of patients undergoing hemodialysis, protein intake is frequently insufficient and markedly lower (by 25%-35%) to recommended intake for patients on dialysis.[36] Consequently, a combination of prevalently catabolic metabolic activities and an insufficient nutritional intake of AAs, in addition to changes in amino acid metabolism during the predialysis stage of the disease, may explain the changes in circulating AAs observed in the present study. After 12 months' follow-up, a deterioration in AA plasma levels was highlighted: with the exception of glutamine, all other AAs displayed reduced plasma concentrations, with the phenomenon particularly evident in the reduction of lysine, a fundamental essential amino acid, the concentration of which was lower than that observed in healthy controls. An increase in catabolic processes is sustained by at least three factors supporting this hypothesis: (1) Both hypercatabolism and anabolism are metabolic processes that require extensive availability of energy (particularly in the form of ATP), the production of which is based on AA activity within the cell cycle of tricarboxylic acids.[64] A further deterioration of lean body mass at 12 months was clearly the result of an increase in catabolic processes, particularly in lean muscle tissue; (2) indeed, an important role in muscle catabolism is played by metabolic acidosis, notably in the presence of uncompensated acidosis, as the former stimulates the ubiquitin-proteasome pathway of protein degradation[14]; (3) although no nutritional supplementation was prescribed throughout the study, glutamine was found to have increased significantly after 12 months' follow-up, with the increase detected in our study being a direct consequence of the intense buffer activity of ammonia on skeletal muscle produced through proteolysis and oxidation of AAs, as well as through the nucleotide metabolism in messengers such as cyclic adenosine monophosphate and cyclic guanosine monophosphate needed to ensure regular functioning of the Krebs cycle and transduction of cell signals, ionic conductance channels, and glycogenolysis. Moreover, the increase in catabolic processes and formation of glutamine would appear to exceed intestinal uptake of this amino acid and losses in dialysis; this might explain the diphasic behavior of glutamine, switching from low plasma concentrations at the start of the study to high concentrations 12 months later. Lysine reduction may be secondary to an excessive intake aimed at supporting protein synthesis and promoting the production of aerobic cellular energy. The results of this study highlight the existence of an apparent paradox: although representing on average 65% of total nutritional AAs, plasma levels of nonessential AAs displayed a progressive decrease. On the

contrary, in the present study, branched-chain AAs, featuring a nutritional intake ranging from 12 to 20%, maintained normal plasma concentrations. The paradox however is merely apparent because of the fact that the normality of plasma concentrations of branched-chain AAs is likely the result of an increased release from muscles secondary to intense proteolysis, as demonstrated by Murtas et al. by means of accurate BIA measures.[8] The decrease in nonessential AAs is however likely due to an excessive intake, which occurs in particular during anabolic metabolic cycles and energy production. In spite of the observational and prospective nature of this study, the potential complications deriving from changes to the amino acid profile should be taken into account; accordingly, we hereby provide a few considerations that may be of use in routine clinical practice: (1) If the changes to the amino acid profile remain uncorrected, the risk of developing, worsening, or accelerating the onset of cachexia and/or PEW will rise considerably, thus affecting both quality of life and survival rates; (2) a decrease in plasma tyrosine levels may elicit a reduction in the cerebral production of adrenergic neurotransmitters and consequent risk of motor dysfunction, cognitive impairment, and mood and behavior disorders; this picture is further complicated by low levels of tryptophan resulting in the decreased cerebral production of serotonin[65,66]; (3) amino acid abnormalities hamper myocardial energy production[67] with a risk of reduced pump function and/or increased arrhythmias; (4) an increase of methionine, and consequent increase of homocysteine synthesis, may produce widespread damage to the vascular structures, in part subsequent to a compromised microbiota produced by a state of uremia, in the presence of a slightly compromised GFR and poor intake of vegetable proteins[68]; (5) in the light of the findings of the present study, patient adherence to recommended protein intake is essential in attempting to correct and/or limit amino acid abnormalities. Should the recommended protein intake not be sufficient to correct amino acid abnormalities, it might be hypothesized that the dialysis patients' nonessential AAs behave and carry out their metabolic function as semiessential AAs. To conclude, the loss of amino acids through hemodialysis is unavoidable irrespective of the type of dialysis method used, although extracorporeal convective methods may contribute toward an increased protein and amino acid loss, amounting to more than 800-900 g yearly. The amino acid profile, even when compared to those of healthy controls, reveals unexpected results, never to date described in literature, relating to the metabolic derailing of the amino acid profile in line with the uremic environment. The frequent nutritional deficiencies and insufficient calorie intake observed in patients undergoing hemodialysis, particularly the elderly, and consequent deficit of essential AAs, imply a need to supplement losses by implementing a balanced intake of BCAAs, thus ensuring supply of almost all NEAAs which, in patients undergoing dialysis with chronic uremia, assume a fundamental and essential compensatory role.

Pratical Application

It is an acknowledged fact that the use of currently available hemodialysis technologies elicits a dramatic loss of amino acids during dialysis. The amounts of amino acids lost vary, although inevitably patients will continue year on year to experience losses targeting particularly the muscular mass and progressively leading to a state of protein energy wasting and subsequently overt cachexia. In the authors' opinion, the only means of slowing down or arresting this severe metabolic decline is to ensure that losses are constantly replaced by the administration of products containing doses tailored to meet the deficit of each individual amino acid. Further studies should be undertaken to support the validity of this hypothesis.[42-44].

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