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Observational pilot study: A comparison of amino acids and derangement of intestinal function between healthy ageing subjects and patients affected by chronic kidney disease stage CKD3b-4 in conservative management

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SUMMARY

Background and aims: A comparison of the amino acid (AA) plasma profile and markers of intestinal absorption-inflammation between healthy subjects aged 65–70 years and age-matched patients affected by stage 3b–4 chronic kidney disease (CKD3b-4) was performed.
Methods: Eleven healthy volunteers were compared with 12 CKD3b-4 patients at their first outpatient control (T0) and after 12-months (T12). Adherence to a low protein diet (LPD, 0.6 ± 0.1 g/kg/day) was assessed by Urea Nitrogen Appearance. The following parameters were assessed: renal function, nutritional parameters, bioelectrical impedance analysis, plasma levels of 20 total amino acids (TAAs), both essential (EAAs) including branched-chain amino acids (BCAAs) and non-essential (NEAAs). Zonulin and faecal Calprotectin markers were used to evaluate intestinal permeability/inflammation.
Results: Four patients dropped out of the study; in the remaining 8 residual kidney function (RKF) remained stable, their LPD adherence had risen to 0.89 g/kg/day, anaemia had worsened and extracellular body fluid had increased. In comparison to healthy subjects, TAA levels of histidine, arginine, asparagine, threonine, glycine, and glutamine had all increased. No variation in BCAAs was observed. A significant increase was detected in faecal calprotectin and zonulin levels in CKD patients as the disease progressed.
Conclusions: This study confirms the finding in aged patients of an alteration in plasmatic levels of several AAs secondary to uraemia. Intestinal markers provide confirmation of a relevant alteration to the intestinal function in CKD patients.

1. Introduction

The study of alterations to the amino acid (AA) metabolism in conservatively managed chronic kidney disease (CKD) [1–4] has recently been the focus of renewed interest. CKD features evolutionary potential despite the implementation of therapeutic and nutritional procedures; an understanding of AA kinetics and metabolism may contribute towards a better perception and optimised conservation of a good nutritional status, whilst at the same time attempting to slow down the progression of CKD [5,6]. Other fundamental measures to be employed are focused on preserving key functions of the microbiota affected by the onset of uraemia

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and by changes in absorption of the intestinal wall underlying a further rise in toxicity of the uremic milieu [7,8]. To date however, literature reports relating to the effect of AA metabolism and kinetics on CKD progression have failed to produce validated findings. Likewise, the effect elicited by a direct action of the amino acid profile and metabolism in CKD on intestinal function and uremic microbiota remains to be clarified, although undoubtedly conditioned by a reduction of protein dietary intake [9]. It should be highlighted how, at the current state-of-the-art, patient populations aged 65 years and over affected by mild-moderate CKD display a 20–25% prevalence of Protein Energy Wasting (PEW), which gradually increases to 30–40% in the more severe forms of CKD. The most widely acknowledged causes include: CKD-induced protein hypercatabolism with loss of muscle mass, reduced amino acid intake, loss of appetite, reduced physical exercise and a range of depressive syndromes [10,11]. Literature reports relating to the study of AA plasma levels in healthy elderly subjects or elderly CKD patients at various stages are somewhat lacking and poorly defined. Greater attention should focus on the higher percentage of nephrology outpatients over the age of 65–70 years affected by CKD stages 3b and 4 [12]. Indeed, even ten years ago, the percentage of conservatively managed stage 3b–4 CKD patients over the age of 65 years exceeded 80% [13]; as these patients age, they become increasingly fragile and vulnerable and undergo uraemia-induced changes to the protein-metabolism and intestinal function. Moreover, in healthy elderly subjects, more coherent ranges should be taken into account in establishing age-adjusted values compared to those adopted by conventional K-DIGO criteria [12], particularly as values for estimated Glomerular Filtration Rate (eGFR) [12,13] should routinely allow for the well-known physiological phenomena of functional kidney ageing in these subjects [14–17]. Accordingly, metabolic variations and differences in AA plasma levels based not only on the degree of chronic renal insufficiency but also to variations induced by renal ageing should be taken into account [17]. Kinetic amino acid metabolism will subsequently vary markedly once patients start replacement dialysis treatment [18–20]. The difficulty of establishing alterations to AA kinetics should also be underlined, with regard not only to the discrepancies encountered in establishing the actual normal values of AAs in healthy subjects over the age of 65–70 years, but also in ascertaining the level of variations to AA plasma profile in age-matched CKD patient populations. Additional factors may complicate studies in this field, including the well-known differences in eating habits (meat-eaters, vegans, fish-eaters and vegetarians) [21] and cultural differences between populations worldwide [22], in addition to a lack of consistency in the use of laboratory methods used to determine plasma levels of free amino acids [23], including pre-analysis storage procedures. Further to the differences detected in AA levels in arterial and peripheral venous blood, the analytical methods used are highly sensitive to storage times and temperature, particularly as blood samples are extremely susceptible to a wide variation in AA levels if taken whilst the patient is fasting or shortly after meals [24]. AA kinetics are subject to irregular deviations caused by a gradual loss of glomerular-tubular function resulting in reduced power of the anabolic cellular pathway. Hypercatabolism and anabolic resistance in CKD are highly compromised in the elderly, particularly at a mitochondrial level, mainly muscle cells, due to increased insulin resistance, metabolic acidosis and to the role played by the products of glycosylation [25,26]. It is an acknowledged fact that to overcome this barrier and adequately maintain muscle mass, protein intake of at least 1.0–1.2 g/kg/day [27] is required, although in the context of CKD, protein intake is strictly limited, based on CKD stage of the patient, to a range of 0.8 to 0.3 g/kg/day, with the intent of preserving Residual Kidney Function (RKF) [5,28]. Moreover, a high percentage of elderly uremic patients suffer from anorexia and display initial or advanced signs of Protein Energy Wasting (PEW), even in industrialized nations, due to the difficulty of maintaining an adequate calorie intake at levels as low as 30 KCal/kg/day [29–31]. A limited intake of high-quality proteins and acceleration of the protein-amino acid catabolism with subsequent reduction of protein synthesis produced through the action of anorexigenic hormones and activation of pro-inflammatory cytokines, inevitably impinge on the amino acid metabolism. In states of CKD-associated malnutrition with an important caloric reduction the AAs are used as source of energy, particularly [31], at the expense of a progressive decrease of body fat and, particularly, lean muscle mass. To slow down the progressive worsening of CKD, protein intake should therefore be drastically reduced to 0.6 g/kg/day (Low Protein Diet, LPD) or 0.3–0.4 g/kg/day (Very Low Protein Diet, VLDP), although essential AA supplementation is mandatory [32,33]. Plasma amino acid alterations may relate to individual AAs or groups of the same, although data obtained from comparison of healthy subjects over the age of 65 with subjects affected by stage 3b–4 CKD are currently lacking. Garibotto et al. reported how the branched-chain AA metabolism, in particular leucine and valine, is compromised by an excessive catabolism of muscle mass, with plasma and tissue levels of arginine, tyrosine, tryptophan, cysteine and other EAAs also affected [23,34]; however, these observations do not relate to the more representative, and prevalently affected, age group of the over-65s. Numerous studies have detected altered plasma levels of a series of AAs including valine and phenylalanine; unfortunately, also in this case, the populations studied did not relate to patients over the age of 65. Moreover, the patients studied were prevalently diabetic and/or affected by other conditions and severe comorbidities. Rare reports relating to normal ranges for plasma AAs comprise a study published by Tan et al. [35], who analysed populations featuring different eating habits but with a mean age of 38.7 years. Plasma ranges relating to arterial and venous AA levels in CKD patients are provided in a paper by Aquilani et al. [36]; the Author compared arterial and venous blood parameters from 11 patients over the age of 65 years affected by cardiorenal syndrome having an eGFR <60 mL/min/1.73 m^2 versus those detected in the arterial blood of 8 healthy subjects; in this study, levels of all AAs were found to be lower in CKD patients compared to healthy subjects, although the detrimental effect of heart failure should be taken into account. An additional aspect of notable importance in patients with CKD is represented by the progressive onset of severe dysbiotic alterations affecting the microbiota and intestinal absorption, targeting particularly the tight-junctions of the intestinal wall [37–39]. On the one hand, a reduced protein intake results in a decrease in production of highly toxic uremic molecules such as PBUTs (p-cresyl-sulfate, p-cresyl-glucuronide, indoxyl-sulfate, indole-3-acetic acid). PBUTs are more readily absorbed due to lack of containment by the intestinal barrier [40,41]; moreover, inflammation and toxicity associated with uraemia alter the intestinal microbial balance, thus enhancing formation of PBUTs through the transformation of aromatic AAs such as tyrosine and phenylalanine and degradation of tryptophan [42,43]. Since the ranges of plasma amino acids remain to be fully clarified in both healthy elderly subjects and CKD patients at stages 3b–4 over the ages of 65–70 years, we set up an observational study to compare, although in a limited number of cases, the differences between healthy subjects and CKD patients, and to assess potential changes to the amino acid profile and blood chemistry and nutritional parameters in the groups studied. We simultaneously investigated the impact of CKD on intestinal inflammation by means of faecal calprotectin assay and on intestinal absorption by measuring levels of zonulin, which appears to be involved in a series of functions, including movement of fluid, macromolecules, and leukocytes

between the bloodstream and intestinal lumen, and vice versa, and protection against microorganism colonization of the proximal intestine [44]. Indeed, faecal zonulin levels had not previously been studied and used as valid indicators in the course of moderate-advanced stages of CKD, as had conversely been the case for other chronic conditions that result in metabolic "inflammaging" [45,46]. Increased zonulin levels were correlated with an alteration of the tight-junction of the intestinal barrier, thus facilitating transfer of microbial antigens and a subsequent increase in release of lipopolysaccharides (LPS). Furthermore, zonulin levels correlated with cellular resistance to the action of insulin [47,48]. LPS, particularly those deriving from Gram-negative bacteria mobilised in blood circulation, induce an inflammatory response elicited by the release of pro-inflammatory cytokines produced as a result of lympho-monocyte activation. Conversely, calprotectin is a protein expressed by neutrophils, the faecal quantity of which acts as a sensitive indicator of gastrointestinal inflammation [49–52]. Levels of these faecal markers illustrate the state of well-being of regulatory intestinal functions, proving to be valid tools not only in the follow-up assessment of chronic pathological conditions such as CKD, but also in stimulating the efficacy of therapies aimed at restoring the microbiota and absorptive function of the intestinal wall. The aims of this observational study of the protein and amino acid metabolism focus on a comparison between healthy volunteers and conservatively managed patients with stage 3b–4 CKD over the age of 65–70 years. We are confident that this study may indeed further encourage research in the specific field and an exchange of views between the authors of similar studies, somewhat lacking in literature, in order to implement the study of the kinetics, fate and variation of plasma amino acid levels, whilst not overlooking the important alterations of intestinal function in the course of CKD.

2. Materials and methods

Eleven healthy volunteers aged 65 years and over (age 72.2 ± 3.7–7 males and 4 females) were compared with a group of twelve age-matched patients (age 74.6 ± 4.2–7 males and 5 females) affected by chronic kidney disease who had been preselected on the basis of eGFR values ranging between 29 and 44 mL/min/1.73m2 (CKD stages 3b–4). CKD patients were subjected to careful monitoring of prescriptions relating to conservative-nutritional management of the disease both at the start of the outpatient study (T0) and at 12-month follow-up (T12), a period of time deemed sufficient to enable achievement of metabolic stability following adherence to a new dietary regimen and a new lifestyle. Patients were excluded from the study based on absence throughout the specific period of a metabolic steady state defined as: presence of acute or chronic inflammatory diseases, type I or II diabetes, malignant tumours, autoimmune diseases, treatment with steroids and/or immunosuppressant drugs, chronic pulmonary disease, malnutrition, cardiomyopathy with heart failure, liver diseases. Moreover, CKD patients with an eGFR >44 and <15 mL/min/1.73m2 and healthy volunteers with an eGFR <80 mL/min/1.73m2 were excluded from the study. Between T0 and T12, patients recruited to the study were not provided with any form of nutritional/amino acid supplementation. From the start of the study, a dietary program based on protein intake of 0.6 ± 0.1 g/kg/day, calorie intake of >30 kcal/kg/day and phosphate intake of <1000 mg/day was prescribed. Protein intake was made up of approximately 50% animal proteins; to boost compliance with the diet prescribed a full range of highly palatable protein-free food products were used. Healthy volunteers were asked to stick to their standard diet following initial assessment by an expert renal nutritionist. The following parameters were taken into account when assessing the two populations: bioelectrical impedance analysis performed by the same operator using the same instrument (Renal EFG50 KHz; EFG Diagnostic Ltd, Belfast, Northern Ireland), Body Mass Index, blood count, iron pool, electrolyte panel, 24-h proteinuria, Urea Nitrogen Appearance (UNA), corresponding to the sum of nitrogen present in the urine, together with nitrogen output from faeces and sweat and distribution in body water to provide a precise indication of daily protein intake in grams [53,54], PTHi, C-Reactive Protein (CRP), plasma protein profile, immunoglobulins, pH and plasma bicarbonataemia. Following the preselection assessment of eGFR, a stricter estimation of RKF was carried out based not only on creatinine clearance [55,56], but also on calculation of GFR obtained from 24 h urine collection and estimation of residual creatinine clearance (KrCr) and urea (KRUREA) using the formula (KRCr + KRUREA)/2 (defined Measured Glomerular Filtration rate - MGFR) [57,58]. A comparison was also conducted between healthy volunteers and CKD patients at MGFR to assess values obtained for the equations of the Prediction Modification of Diet in Renal Disease Study Group (MDRD) and the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) [59]. Both eGFR and MGFR were normalised to the body surface area of each patient and healthy volunteers. Samples were obtained in the morning on an empty stomach 12 h after the last meal. The amino acid panel was deemed as comprising 20 amino acids (EAAs, NEAAs, BCAAs). Faecal zonulin was determined using an ELISA kit (Zonulin Stool - ELISA, DRG Instruments Gmbh, Germany): normal faecal values in healthy volunteers were <60 ηg/ml. Calprotectin was determined by means of immunoenzymatic assay and measured using a Chorus TRIO instrument (DIESSE Diagnostica Senese S.p.A, Italy): normal values of faecal Calprotectin were <50 mg/g faeces.

This study was based on an observational design and each patient signed an informed consent form agreeing to use and processing of his or her personal data. The study was approved by the Ethics Committee of the local Health Authorities.

2.1. Method of determination of amino acids

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 [18–21]. 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 h of collection by centrifuging at 3000 rpm for 10 min. The plasma thus obtained was frozen in 2 mL cryogenic test tubes at a temperature of − 20 °C. The plasma 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 laboratory for final storage. 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 mL 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 mM/mL) 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 analysed 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 ml/L and mg/dl and compared with standard values in our laboratory. Twenty AAs were determined including Total AAs (TAAs), Essential AAs (EAAs) including Branched-Chain AAs (BCAAs) and Non-Essential AAs (NEAAs).

2.2. Statistical analysis

The Shapiro–Wilk test supported by visual inspection was used to assess the normality of distribution of continuous variables. Most variables violated the normality assumption, but violations were not marked. Accordingly, the central tendency and dispersion of continuous variables were reported as mean ± SD and hypothesis testing was based on non-parametric statistics. Descriptive statistics for categorical variables were reported as N (percent frequency). Between-group comparisons (CKD patients at T0 vs healthy subjects and CKD patients at T12 vs healthy subjects) were carried out by Mann–Whitney U-test and by the Chi-square test for continuous and categorical variables, respectively. The association between couples of variables was assessed by correlation analysis, computing the Spearman correlation coefficient r and Pearson coefficient. We opted to strengthen statistical confidence by using a Spearman correlation to compare the ranks of the two quantitative variables and a Pearson correlation to compare real numbers of the two variables. A p-value <0.05 was considered statistically significant. All analyses were carried out using the SAS/STAT statistical package, release 9.4 (SAS Institute Inc., Cary, NC, U.S.A.).

3. Results

Four patients affected by stage 3b–4 CKD dropped out of the study: one due to a deterioration of MGFR to stage 5 CKD (MGFR <15 mL/min/1,73 m^2), two following a refusal during the first month of the study to adhere to the new dietary regimen and one due to onset of an intercurrent subacute/acute lung infection. Statistical comparisons were therefore carried out on the eleven healthy volunteers and the remaining eight patients affected by stage 3b–4 CKD, aged 73.5 ± 6.8 (4 females & 4 males). Two of the healthy control subjects and 5 CKD patients were taking antilipemic agents (statins). Calcaemia and phosphoremia levels were well controlled; CKD-elicited derangement of mineral balance linked to the calcium–phosphorus equilibrium was unmasked by a progressive secondary hyperparathyroidism manifested as a result of renal enzyme activity in transforming vitamin D (25-OH-D3) into its active metabolites (1,25-OH-D3). Pathological levels of CRP (normal ranges 0.8–1.0 mg/L) were evident at 12 months (T12), although compared to T0, the difference did not reach statistical significance (Table 1).

Table 2 highlights the onset of CKD-elicited anaemia featuring reduced iron levels and concomitant impoverishment of iron reserves. Nutritional deficit was emphasized by a downward, although not statistically significant, trend in total plasma proteins and a progressive decrease of C3. Inflammaging present in the CKD stages studied was indirectly confirmed by the λ component of serum proteins, despite the absence of serious acute, subacute or chronic inflammatory conditions throughout the 12-month follow-up period. Bioelectrical impedance analysis continues to represent a valid assessment tool for nutritional status for use in routine monitoring: the results yielded confirm whether uraemia has affected the nutritional-calorie status, demonstrated by a significant decrease of the phase angle due prevalently to increased extracellular body water and a non-significant tendency towards reduction of muscle mass (Table 3). It should also be taken into account that, despite prescription of a low-protein diet of 0.6 ± 0.1 g/kg/day, patients did not comply fully with the diet prescribed, as attested to by UNA. Indeed, at T12, although only 3/8 (37.5%) patients had adhered rigorously to the prescribed diet, a substantial tendency to adapt to the desired protein intake was observed. The manifest decrease in RKF was confirmed by MGFR, based on both eGFR derived from calculation of MDRD and CKD-EPI. The parameters used to calculate eGFR (MDRD, CKD-EPI) based on creatine alone, displayed a predictable overestimation versus MGFR in CKD patients. However, the correlations between MGFR and eGFR in healthy subjects at T0 and T12 were aligned (R Spearman >0.8; $p < 0.0001$). Plasma pH was maintained unchanged by metabolic/respiratory compensatory phenomena, although the decrease in plasma bicarbonates revealed onset of relatively well compensated metabolic acidosis (Table 4).

Table 5 illustrates and confirms how in the moderate-advanced stages of CKD intestinal function undergoes a series of alterations linked to the uremic setting. Faecal zonulin and calprotectin act as ideal markers for intestinal dysbiosis produced by an altered intestinal absorption and inflammation that follows a significant upward trend in line with progression of CKD. The panel of 20 AAs examined displayed several variations: a significant increase in 4/20 NEAAs (20%) including asparagine, aspartic acid and serine, which maintained high plasma levels as CKD progressed; glycine showed a continued tendency towards a delayed, although significant, at T12. Four out of twenty AAEs (20%) displayed a significant, stable increase of histidine in line with the evolution of RKF, whilst deterioration of MGFR levels of threonine, arginine and tryptophan were characterised by a delayed onset. All other AAs, in particular BCAAs, remained unaffected by uremic status compared to healthy subjects, conversely, TAAs displayed a modest, although significant, rise (approx. 17%) as uremic status worsened (Table 6).

The ratio between AAEs (including BCAAs)/NEAAS remained unchanged in healthy subjects (ratio: 0.67), CKD patients at T0 (ratio: 0.66) and CKD patients at T12 (ratio: 0.62). Lastly, Table 7 (see supplementary material) provides a summary of the correlations detected between AAs and MGFR. Only three AAs displayed a significant correlation with RKF. Finally, we assessed the association between AAs and MGFR in CKD patients at T0, at T12 and between the difference (Delta) between values at T12 and values at T0. Correlation analysis revealed no significant associations between AAs and MGFR at T0 (p values ranging from 0.22 to 0.98). Conversely, several significant associations were observed at T12, as reported in Table 7. As far as the association between Deltas is concerned, only the difference (value at T12 - value at T0) in four amino acids was significantly associated with the difference in MGFR (Spearman's $r = 0.88$, $p = 0.007$).

4. Discussion

It is widely acknowledged, particularly in elderly patients, that CKD produces: a tendency towards hyperuricemia, secondary hyperparathyroidism, anaemia caused by a deficit in renal activation of endogenous erythropoietin and progressive iron deficiency, decrease in total plasma proteins and reduction of the phase angle due to an increase of extracellular water and decrease of body fat and muscle mass [60–62]. The prescription of a low-protein diet produces a significant impact on the intake and kinetics of AAs. Generally, protein intake in uremic stages 3–5 CKD patients is reduced and a very low protein diet (VLPD) or low protein diet (LPD) is prescribed. Regrettably, methods employed to monitor dietary compliance are frequently based on the use of nutritional interviews or questionnaires, with the absence of expert renal nutritionists in hospital wards worldwide playing a key contributory role. Very few authors take into account the well-validated

Table 1
Ematochemical plasmatic assesment.

	Healthy Subjects (11)	CKD patients T0 (8)	CKDpatients T12 (8)
Total Cholesterol, mg/dL	222.27 ± 23.09[ǂ]	197.25 ± 39.57[ǂ]	193.13 ± 34.69[ǂ]
HDL Cholesterol, mg/dL	64.18 ± 18.87	70.13 ± 22.99	77.86 ± 32.19
LDL Cholesterol, mg/dL	137.09 ± 22.52[✧§]	110.63 ± 22.20[✧§]	102.50 ± 20.79[§]
Triglycerides, mg/dL	98.91 ± 69.06	75.63 ± 35.48	75.50 ± 19.57
Glycemia, mg/dL	94.36 ± 8.03	94.50 ± 10.41	94.50 ± 11.21
AST, U.I.	20.55 ± 3.39	20.38 ± 6.72	18.63 ± 2.97
ALT, U.I.	16.64 ± 2.58	17.38 ± 6.67	13.50 ± 4.66
γGT, U.I.	11.87 ± 1.46[✧]	13.75 ± 2.94	15.81 ± 2.74[✧]
Creatine phosphokinase, U.I.	100.27 ± 59.73	141.38 ± 94.21	130.63 ± 122.35
Sodium, mmol/L	141.03 ± 2.12	141.91 ± 1.78	141.53 ± 1.16
Potassium, mmoL/L	4.31 ± 0.41	4.38 ± 0.42	4.35 ± 0.41
Calcemia, mg/dL	9.73 ± 0.55	9.19 ± 0.48	9.35 ± 0.68
Phosphate, mg/dL	3.57 ± 0.58	3.41 ± 0.33	3.70 ± 0.58
Ionized Calcium, mmol/L	5.06 ± 0.38	4.94 ± 0.27	4.84 ± 0.18
Uric Acid, mg/dL	4.24 ± 0.49[✧§]	5.16 ± 1.12[✧]	6.01 ± 3.01[§]
C Reactive Protein, mg/L	1.45 ± 1.85	1.76 ± 1.26	3.04 ± 5.42
PTHi, pm/mL	64.35 ± 22.40[✧†]	171.35 ± 163.58[✧]	202.31 ± 98.70[†]

ǂ: p = 0.022; ✧: p = 0.01; §: p < 0.003; †: p = 0.008; T0: CKD study start; ALT: Alanine Transaminase; AST: Aspartate Aminotransferase; γGT: Gamma-glutamyl Transferase.

Table 2
Hematological and Immunological plasmatic parameters.

	Healthy Subjects (N = 11)	CKD patients at T0 (N = 8)	CKD patients at T12 (N = 8)
Hb, g/L	14.16 ± 1.20	11.91 ± 1.01[§]	11.85 ± 1.12[§]
RBC, mm^3	5.39 ± 6.95	4.23 ± 2.84[§]	4.25 ± 3.22[§]
Iron levels, μg/dL	106.09 ± 26.69[‡]	104.63 ± 46.03[‡]	72.38 ± 32.76[‡]
transferrin, mg/dL	268.95 ± 54.98	252.75 ± 50.09	244.75 ± 36.17
ferritin, μg/L	175.60 ± 93.28[†]	80.06 ± 63.91[†]	78.97 ± 60.65[†]
WBC, mm^3	6270.9 ± 2055.40	6568.8 ± 1509.7o	7258.8 ± 1413.20
Lymphocytes, mm^3	2123.6 ± 745.11	1543.9 ± 843.51	1875.0 ± 656.33
IgG, mg/dL	1080.5 ± 263.70	1134.6 ± 219.09	1146.0 ± 259.62
IgA, mg/dL	202.48 ± 79.95	194.91 ± 115.62	250.14 ± 138.91
IgM, mg/dL	120.61 ± 72.28	79.94 ± 65.23	85.51 ± 88.43
C3, mg/dL	120.13 ± 28.05	108.74 ± 37.62	95.89 ± 21.93
C4, mg/dL	26.61 ± 6.94	27.09 ± 10.90	25.76 ± 8.58
Total Protein, g/dL	7.25 ± 0.39[†]	6.73 ± 0.48[†]	7.07 ± 0.57
Albumin, g/dL	4.21 ± 0.23	3.99 ± 0.22	4.13 ± 0.18
Protein λ-zone, %	11.87 ± 1.46[§]	15.39 ± 1.00[§]	15.02 ± 2.74[§]

[†]: p < 0.05[‡]; p < 0.01;[§]: p < 0.001 for the comparison with Healthy subjects at T0; Hb: hemoglobin; RBC: Red Blood Cells; WBC: White Blood Cell; Protein λ-zone: λ Protein λ of serum protein electrophoresis.

Table 3
Anthropometric and Bioimpedance measures.

	Healthy Subjects (N = 11)	CKD patients at T0 (N = 8)	CKD patients at T12 (N = 8)
Age, years	72.27 ± 3.74	73.56 ± 6.90	74.56 ± 6.90
Weight, Kgs	64.50 ± 12.80	62.44 ± 9.43	62.76 ± 9.05
Height, cm	164.55 ± 7.62	156.00 ± 13.26	156.00 ± 13.26
Rz, Ohm	536.27 ± 39.97	497.00 ± 72.28	515.50 ± 113.19
Xc, Ohm	52.15 ± 12.42	42.25 ± 10.82	42.25 ± 17.38
Phase Angle, grades	5.16 ± 0.60[‡]	4.50 ± 0.73	4.22 ± 0.67[‡]
Total Body Water, %	35.65 ± 16.60[†]	53.55 ± 8.74[†]	55.93 ± 9.20[†]
Extracellular Body Water, %	19.25 ± 8.48[§]	44.36 ± 14.88[§†]	55.75 ± 4.39[§†]
Intracellulare Body Water, %	44.65 ± 4.10	41.09 ± 14.62	40.69 ± 10.06
Body Fat Mass, Kgs	21.91 ± 2.40[§]	16.44 ± 3.84[§]	19.97 ± 9.96
Fat Free Mass, Kgs	27.55 ± 2.85[§]	44.30 ± 8.42[§]	44.00 ± 10.03[§]
Body Cellular Mass, Kgs	24.86 ± 2.79[†]	20.78 ± 4.20[§]	19.84 ± 6.52[§]
Body Muscle Mass, kgr	27.55 ± 2.85	26.35 ± 5.14	24.21 ± 6.07
Body Mass Index	24.86 ± 2.79	24.84 ± 1.98	25.31 ± 2.31
Resting Metabolic Rate, Kcal	1385.1 ± 68.02	1355.0 ± 121.52	1298.8 ± 144.81

[†]: p < 0.05; [‡]: p < 0.01; [§]: p < 0.00; RZ: Electric Resistance; Xc: Electric Reactance.

calculation of UNA, which should indeed always support estimation and follow-up in CKD [53,54]. Accordingly, the prescription of a VLPD is associated with the administration of large amounts of tablets or sachets as an AA supplement, with these compounds containing calcium salts and a mix of amino acids and their keto-analogues containing both EAAs and BCAAs but not NEAAs. Compliance with the prescribed diet may be negatively affected by this large number of tablets or sachets to be taken daily, mainly at low-protein meal times (a 65 kg patient will take 10–12 tablets/day/365 days/year), which may elicit gastrointestinal side effects and alterations to the calcium metabolism. Our group routinely prescribes a LPD of 0.6–0.7 g/kg/day. The nutritional prescription of

Table 4
Renal Function evaluation parameters.

	Healthy Subjects (N = 11)	Patients with CKD at T0 (N = 8)	Patients with CKD at T12 (N = 8)
Plasmatic Blood Urea Nitrogen, mg/dL	11.47 ± 3.52	42.44 ± 15.07§	43.86 ± 16.09§
Plasmatic Creatinine, mg/dL	0.81 ± 0.16	1.99 ± 0.53§	2.12 ± 0.51§
diuresis 24 h, mL	1640.9 ± 417.02	2131.3 ± 633.51	1993.8 ± 526.09
UNA, g/Kg/day	1.17 ± 0.13†	1.05 ± 0.27	0.89 ± 0.21†
MGFR, mL/min./1.73 m^2	87.70 ± 7.45	26.99 ± 2.80§	22.93 ± 3.25§
MDRD, mL/min/1,73 m^2	85.97 ± 17.40	31.85 ± 9.40§	30.83 ± 7.88§
CKD-EPI, mL/min./1.73 m^2	85.91 ± 16.64	30.41 ± 10.05§	30.06 ± 8.17§
proteinuria 24 h, mg/24 h	94.73 ± 76.39‡	533.63 ± 469.93‡	324.04 ± 316.12‡
Blood pH	7.36 ± 0.02	7.34 ± 0.03	7.35 ± 0.04
plasmatic bicarbonates, mmol/L	26.69 ± 2.18‡	23.76 ± 0.89‡	23.03 ± 1.42

†: $p < 0.05$; ‡: $p < 0.01$; §: $p < 0.001$; U.N.A. Urea Nitrogen Appearance, g/kg/day; MDRD: Modification of diet in renal disease equation CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration equation.

Table 5
Fecal intestinal markers.

	Healthy Subjects (N = 11)	CKD patients at T0 (N = 8)	CKD patients at T12 (N = 8)
Fecal Calprotectina, ng/mL	30.25 ± 27.62‡	78.37 ± 87.42‡	95.60 ± 53.26‡
Fecal Zonulina, mg/g	54.96 ± 32.73‡	142.67 ± 114.42‡	231.43 ± 181.54‡

‡: $p < 0.01$.

Table 6
Plasmatic amino Acid Levels, μmol/mL.

	Healthy Subjects (N = 11)	CKD patients at T0 (N = 8)	CKD patients at T12 (N = 8)
Asparagine	4.84 ± 1.78	17.08 ± 4.32§	14.39 ± 5.50§
Glutamic Acid	159.49 ± 21.75	180.29 ± 23.74	179.92 ± 46.72
Aspartic Acid	10.51 ± 2.12	18.28 ± 6.31†	19.57 ± 15.65†
Serine	31.60 ± 6.77	49.51 ± 11.34†	40.41 ± 7.35†
Glutamine	147.22 ± 27.46	156.69 ± 95.37	229.41 ± 131.79
Hystidine	22.24 ± 3.72	33.49 ± 18.47‡	63.13 ± 31.61‡
Glycine	123.63 ± 34.81†	157.44 ± 49.75	176.66 ± 66.84†
Threonine	65.18 ± 14.40†	76.26 ± 14.96	89.34 ± 29.27†
Alanine	348.94 ± 84.88	340.82 ± 58.70	316.25 ± 39.76
Arginine	95.73 ± 15.79‡	112.37 ± 34.34‡	158.22 ± 61.73‡
Tyrosine	54.69 ± 12.41	48.75 ± 10.93	47.66 ± 7.48
Cysteine	207.54 ± 56.71	217.92 ± 36.15	208.18 ± 54.18
Valine	172.90 ± 19.25	183.10 ± 46.29	168.03 ± 35.51
Methionine	19.12 ± 3.48	20.39 ± 5.89	21.40 ± 5.23
Tryptophane	34.53 ± 4.95†	36.15 ± 8.05	48.61 ± 21.17†
Phenylalanine	45.11 ± 4.76	51.33 ± 8.20	48.63 ± 6.45
Isoleucine	47.62 ± 8.39	51.11 ± 18.97	50.46 ± 11.84
Leucine	90.32 ± 15.38	91.20 ± 29.24	88.59 ± 20.23
Lysine	125.23 ± 15.70	139.14 ± 22.40	134.89 ± 17.86
Proline	196.71 ± 63.87	208.06 ± 55.76	233.29 ± 125.77
Total Amino Acids	2003.2 ± 239.89†	2189.4 ± 300.11	2337.1 ± 419.61†
Branched Amino Acids	310.85 ± 40.79	325.41 ± 91.84	307.08 ± 66.30
Esential Amino Acids	622.25 ± 66.72	682.17 ± 121.71	713.08 ± 140.98
Non Essential Amino Acids	1380.9 ± 218.82	1507.2 ± 222.71	1624.0 ± 317.33

†: $p < 0.05$; ‡: $p < 0.01$; §: $p < 0.001$.

Table 7
Correlation coefficients between selected plasmatic amino acids and MGFR.

	Spearman's r	Pearson's r
Cystine	0.88 (p = 0.007)	0.85 (p < 0.007)
Histidine	0.55 (p = 0.17)	0.67 (p = 0.007)
Glycine	0.64 (p = 0.10)	0.71 (p = 0.047)

LPD, although failing to yield optimum results in our study, was supplemented by the use of highly palatable protein-free foods and products. It should therefore be borne in mind that protein intake will need to be monitored and assessed during nutritional follow-up. Indeed, studies focusing on the protein and amino-acid metabolisms frequently report high numbers of dietary non-compliance right from the start of treatment, with percentages exceeding 50% in the case of VLPD using alpha-kappa keto-analogues [63–69]. Monitoring conducted by means of dietary interviews is frequently unreliable due to the negative motivation in patients in referring the precise outcome of dietary changes in the hope of avoiding dialysis or in the inaccurate interpretation of changes made to their diet, often caused by the lack of support and assistance from family members [67]. In this study, we associated dietary monitoring conducted by means of interview with the patient and caregivers of both healthy subjects and CKD patients, with an analytic process aimed at quantifying the output of urinary and non-urea nitrogen, thus yielding reliable findings with regard to protein intake. Likewise, correlations between protein intake and amino acids may be deceptive in accurately estimating RKF, therefore justifying why we chose to use MGFR based on the formula (KRCr + KRUREA)/2. This determination should be only be

performed in patients in a metabolic steady state to avoid the negative interference of hypercatabolic states that may result in alterations to the generation and clearance of urea. Furthermore, KRUREA amortizes the overestimation of values obtained by means of KRCr as urinary creatinine, even in the more advanced stages of CKD, and continues to be raised due to the well-known tubular secretion of creatine in the urine and concomitant decrease in plasma levels. It is moreover crucial to bear in mind that eGFR calculated on the basis of MDRD and CKD-EPI, calculation that can be achieved on numerous websites, is based solely on plasma creatinine, age, sex and ethnic origin, whilst failing to account for anthropometric parameters such as height, weight, BMI and body surface area. Recourse to a personalized rather than standardized form of precision medicine and nutrition should be advocated, bearing in mind that healthy subjects over the age of 65–70 years fail to conserve a mean renal function of >100 mL/min due to the acknowledged phenomena of renal senescence resulting in a GFR loss of at least 0.73 mL/min/year after the age of 30–40 years [70–73]. With regard to plasma AA levels, considering that no literature reports conducted to date have referred to the validation of normal ranges in patients over the age of 65–70 years, and even less so in stage 3b–4 CKD patients, we felt duty-bound to undertake this pilot study. Moreover, it should be highlighted how an additional inhibition of mitochondrial cellular activity in elderly patients due to a decreased sensitivity to insulin and, in CKD patients, to hypercatabolism and inflammation, hinders the adequate incorporation and use of AAs at a cellular level, particularly in myocytes, thus resulting in an increase in plasma levels of AAs accompanied by impoverishment of muscle mass [25] and increased release of AAs into the venous system, further impeding reliable estimation of the fate of AAs in the moderate/advanced stages 3b–4 of CKD. In the course of CKD, this phenomenon is associated with a reduced residual renal clearance and tubular reabsorption of small molecules, including AAs, all of which amplified by a state of acidaemia/metabolic acidosis [73,74]. In the present study, metabolic/nutritional balance was reasonably maintained as shown by the ratio between AAEs/NEAAs which remained unchanged in healthy subjects and CKD patients at both T0 and T12. Other authors have reported a correlation between kidney function and increased cysteine levels, largely caused by mitochondrial dysregulation associated with uraemia, although these studies were performed in younger patients with non-advanced stages of CKD [75]; this phenomenon was not confirmed in our study. Mahbub et al. [76] conducted a study on a vast population, subsequently highlighting the wide inconsistency and evident contrasts between AA plasma levels, with particular regard to BCAAs and phenylalanine, although also in this case the data obtained related to a young population affected by lesser degrees of kidney failure: indeed, in the worst percentile examined in this study, mean age corresponded to 59 years, with an eGFR of 70.9 mL/min/1.73m2, leading the authors to conclude that an increase in leucine and tryptophan correlated with a decline in GFR resulted in these three AAs being taken as indicators of CKD progression. These findings have not been confirmed by other authors [77–82] or by data obtained in our study. Further studies have reported increased levels of glycine, cystine and valine, although in the presence of different stages of CKD [83,84]; in our patients glycine correlates with a decline in GFR. Plasma levels of BCAAs remain unchanged at the CKD stages we studied, likely due to the fact that our patients' kidneys still exerted an avid uptake of BCAAs and no significant influences were observed [84] as a result of a contained level of CKD "inflammaging" and, despite the advanced age of patients, outpatient monitoring had been successful in preventing onset of an overt state of malnutrition/sarcopenia [49,80]. It is likely therefore that the correlations observed between AAs and RKF were not statistically relevant at these stages of CKD, although an association was detected between MGFR and glycine, histidine and cystine (Table 7). Due to the presence of micro-inflammation, CKD has the potential to alter the mitochondrial-lysosomal function of cystine, although in our case studies, plasma levels of cystine, in line with previous findings, maintained similar levels compared to the group of healthy volunteers [85]. Glutamine is an amino acid of paramount importance in maintaining a normal anabolic–catabolic equilibrium in view of its crucial role in acid-base balance in the kidney as a self-defence mechanism against deamination and particularly in limiting the presence of ammonia in the brain [26]; even in CKD patients, glutamine reserves derive largely from protein degradation in muscle mass, thus representing a compensatory process against CKD [86]. Mair et al. [87] recently reported a finding of compromised tubular secretion of glutamine which increased progressively as CKD advanced towards more severe stages 4–5; in our study, we observed a 56% increase in glutamine at T12 compared to healthy subjects and a statistical significance was reached. An increase in histidine levels was detected in CKD patients versus healthy controls of +184% and in glycine of +43% after 12 months of conservative management; however, histidine levels have only rarely been reported during studies investigating CKD; a few specific studies do however mention high levels of histidine in the context of CKD [83,88]. Despite the beneficial role of arginine in protecting the endothelial system as a vasodilator through its action on nitric oxide and its singular insulin genetics, there is still ongoing debate as to the negative effects ascribed to methylation of arginine and consequent proteolysis resulting in a potential increase of toxic molecules such as asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA) and N-monomethyl-L-arginine (NMMA) [89]; moreover, no literature reports to date have described alterations to arginine plasma levels. To the best of our knowledge, no studies have addressed the issue of threonine levels, whilst tryptophan levels have been studied by Konje et al. [90] in a group of patients affected by stages 3–5 CKD. This study found a positive correlation between tryptophan plasma levels and incidence of cardiovascular events, although the tryptophan plasma levels detected in patients with a mean age ranging from 54 to 57 years were far higher (61.4–71.4 μmol/mL) than those observed in our study (T0: 36.15 ± 8.05, T12: 48.61 ± 21.17 μmol/mL). The main limitation of our study is represented by the small number of patients studied, who demonstrated a modest, but statistically significant 17% increase in level of TAAs but at present there are no similar works relating to patients aged >70 years in CKD3b-4 stages with our working in progress ongoing intent to implement the number of patients to be studied; however, it is clear that these values display a tendency towards increasing, and it may therefore follow that other AAs could behave in a similar manner as RKF falls below 15 mL/min. It would be of interest to study AA levels and intestinal absorption in stage 5 of CKD, although at this stage it may be more complicated to investigate AAs and dietary compliance due to the availability of a potentially brief follow-up period on the basis of increased mortality and high incidence of drop-out by patients progressing to dialysis treatment [91]. Furthermore, the effectiveness of VLPD compared to LPD on RKF progression is still debated [92]. With regard to the impact of CKD on intestinal function, to date, no studies have reported on the variations observed in intestinal permeability on the basis of levels of faecal zonulin in the course of CKD, with even the most recent publications referring solely to determination of plasma levels. Likewise, no studies have been published relating to the presence of intestinal inflammation with uremic dysbiosis during any stage of CKD, detected using a sensitive marker of inflammation such as faecal calprotectin, in the course of CKD.

5. Conclusions

Whilst parameters linked to the evolution of CKD are indisputable, a series of discrepancies are present in literature with regard to levels of single AAs or classes of AAs in CKD. These reports are significantly affected by: gender discrepancies, diverse methods used in analytical determination, differences between western and eastern eating habits, nutritional status and variations, pre-analytical and analytical procedures employed in determination of plasma AAs. In the present study, advanced age beyond 65–70 years in both healthy subjects and CKD patients likely represented the most significant factor when compared to the metabolism of younger patients and controls. It remains to be clarified which and to what extent AAs are implicated at an anabolic level in protein synthesis. The findings highlighted following use of intestinal faecal markers demonstrating a negative impact of uraemia on intestinal permeability, thus affecting absorption of numerous, increasingly toxic uremic molecules of low and medium molecular weight, are of particular interest. Finally, although the protein intake prescribed in the present study was exceeded, reaching slightly less than 0.9 g/kg/day, after a year one stabilisation period RKF had not deteriorated further in any of the patients studied. These findings therefore may encourage the undertaking of further research in the field using larger patient populations stratified according to age and CKD stage, with the aim of establishing the most appropriate therapeutic strategies for use in restoring optimal metabolic use of AAs and/or rectifying intestinal absorption compromised by a microbiota damaged by the presence of uraemia.

6. Practical application statement

The aim of our pilot study was to start to fill a gap in nephro-nutritional literature by comparing the qualitative and quantitative metabolic amino acid asset in ageing patients with CKD3b-4 versus healthy subjects of the same age. Specifically, we have attempted to identify metabolic changes, and particularly derangements, of all amino acids following a one-year adaptation of CKD patients to the low-protein diet, highlighting the negative impact of uraemia on the uremic microbiota by monitoring specific markers of intestinal inflammation-absorption. This study seeks to pave the way towards stimulating researchers to verify the data obtained with a view to identifying tailored therapy geared at rebalancing the amino acid metabolism and fundamental functions of the microbiota in CKD stages 3b–4.

Author contributions

Conceptualization, methodology, writing—original draft preparation, writing—review and editing, supervision, data curation P.B.; conceptualization, validation, R.A.; software, validation, formal analysis, RM.; investigation, resources M.P.E., M.L.D., M.C., R.S., B.C. and A.S.; methodology, formal analysis P.I. and M.D.; conceptualization, methodology, supervision, data curation, validation, supervision, resources, S.M. **A**ll authors have read and agreed to the published version of the manuscript.

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Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Local Ethics Committee of Health Public Institute (protocol code MRC Project: 74/2018, approved on 29 May 2018).

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

Data Availability statement

The data presented in this study are available on request.

Declaration of competing interest

The authors declare no conflict of interest.

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