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Low bone mineralization in phenylketonuria may be due to undiagnosed metabolic acidosis☆

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ABSTRACT

Background: Dietary intervention is to date the mainstay treatment to prevent toxic phenylalanine (Phe) accumulation in PKU patients. Despite success preventing central nervous system damage, there is increasing evidence of possible other unfavorable outcomes affecting other systems, e.g. kidney and bone; underlying mechanisms are yet to be fully elucidated.

Methods: This observational, cross-sectional and descriptive study investigated 20 adult with PKU evaluating biochemical parameters, BMD measurements and extrapolating data from 3-days food records and protein substitutes (PS) and special low protein foods (SLPF) composition.

Results: Blood gas venous analysis (VBG) indices were indicative of metabolic acidosis in 60% of PKU patients and VBG pH significantly correlated with BMD's *Z*-score (*p*-value = 0.022) even if its overall mean was in range (−1.29). Low bone mineral density for chronological age (*Z*-score < − 2.0) was found in 4 patients (20%). Indices of kidney function were not impaired. All used PS had a moderate excess of acidity, while SLPF were alkalizing and type/variety of consumed vegetables did not determine significant changes in acid-base equilibrium. Total intakes of potassium and magnesium were lower than expected.

Discussion: PKU patients seem to be at risk of metabolic acidosis, directly linked to possible low bone mineralization. This may be related to the acidic composition of PS, potentially capable of acidifying the entire diet. Reported low intakes of potassium and magnesium may be relevant to these observations. Further studies are needed to better address these topics.

1. Introduction

Neurotoxic effects of phenylalanine (Phe) accumulation in patients affected by phenylketonuria (PKU) are well known [1]. Dietary interventions can prevent such outcomes by maintaining Phe concentrations within a defined "safe" range (<600 umol/L in those over 12 years of age and lower for those <12 years, as defined by European Guidelines [2]) while obtaining positive outcomes both for growth and neurodevelopment [3,4]. The dietary interventions include to date the use of: 1) medical foods, characterized by low and controlled Phe content (hereinafter named "special low protein foods, SLPF"); 2) protein substitutes (PS), designed to cover the daily protein requirement necessary for growth (able to provide 50–80% of the total recommended in patients affected by PKU); 3) oral micronutrient supplements (such as vitamin D, calcium and docosahexaenoic acid (DHA), based in Italy on national recommendations for nutrients and energy intakes (LARN, "*Livelli di Assunzione di Riferimento di Nutrienti ed energia per la popolazione italiana*", https://sinu.it/tabelle-larn-2014/); 4) small quantities of natural protein foods based on tolerance. All these interventions are required lifelong, especially when no other pharmacological treatments can be used (e.g. pegvaliase or sapropterin) [5–9].

Despite success preventing neurological damage, there is increasing evidence of possible suboptimal long-term health outcomes in some PKU subjects on a low-Phe diet, including the chance to develop skeletal

Abbreviations: AA, aminoacids; BMD, Bone Mineral Density; CNS, Central Nervous System; DHA, Docosahexaenoic Acid; GMP, glycomacropeptide; PKU, Phenylketonuria; PAH, phenylalanine hydroxylase; Phe, phenylalanine; PRAL, Potential Renal Acid Load; PTH, parathormone; PS, protein substitutes; SLPF, special low protein foods.

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fragility [10–16], oxidative stress [17–20] and obesity [20–26]. A potential risk for impaired kidney function has also been described [27–29] considering the different dietary composition as a possible responsible factor. Many of these topics are still widely debated. For example, there is no clear evidence that skeletal fragility, renal impairment, or obesity are problematic in PKU or if they occur as a direct result of the underlying pathology. Likewise, there is no clear evidence of increased risk of fractures in PKU patients and bone mineral density, although normal, seems still below the population norm, which needs to be examined in greater detail [13,14,30,31]. Garcia et al. investigated 2850 children in a prospective multi-ethnic population based study where dietary PRAL was measured together with DXA investigating the question does diet in early life influence bone mineralization [32] and no association with acid load in early life was associated with bone health in childhood. Still, current knowledge on healthy subjects suggest diet composition as a major player influencing acid-base balance (providing acid or base precursors), potentially inducing metabolic acidosis when acidic [30]. Clearly, the debate on mild and chronic metabolic acidosis as a result of a diet rich in 'acid' forming nutrients and bone mineralization continues.

Based on the necessarily-changed dietary approach for PKU patients, this prospective study aims to primarily review the acid/base dietary intake of subjects with PKU and secondary discuss and deepen some potential bone and kidney influencing factors. Acid-base indices, never reported before for PKU patients, and other parameters were evaluated and reviewed in order to eventually identify possible factors to be considered and accounted for better understanding abnormal findings and eventually elucidating potential underlying pathogenic mechanisms [32–35]. Even if the strict effect of consequent acid/base corrections/interventions is still controversial, this study is the first that aims to investigate these aspects and potential implications in the management of PKU patients.

2. Materials and methods

Patients with a PKU diagnosis (screened and identified at newborn screening (NBS) with identified variants in the PAH gene), in follow up at the Clinical Department of Pediatrics - San Paolo Hospital, ASST Santi Paolo e Carlo, University of Milan, Italy, agreeing to participate in this study (by signing informed consent) were included in this observational, cross-sectional and descriptive study. Inclusion criteria were set as follows: 1) age > 18 years, 2) both gender, 3) no changes in the utilized PS for the last 2 years. With regards to the latter, this was assessed at the time of the evaluation, comparing dietary habits (as referred by the patient) to medical prescription (as reported in the dietary scheme of each patient) and considering a 2 years' time limit as sufficient to exclude that any change in the obtained results could be attributed to recent changes in the used PS. Any medical condition that could possibly interfere with analyzed parameters and the use of medications that could potentially link to kidney/bone alterations were considered as exclusion criteria. Patients with ongoing treatment with sapropterin were also excluded from this study. Study design was created as shown in Fig. 1.

2.1. Chemistry parameters

Fasting early morning [2] bloods samples were collected for cystatin C, blood gas venous analysis together with a 24 h urine collection.

VBG rather than arterial blood gas analysis (ABG) was used for the easiness of puncture and lowest grade of possible complications, also since evidence that pH and pCo2 obtained via peripheral VBG well correlate with ABG measurements [31]. Metabolic acidosis was defined for venous pH < 7.32 along with the detection of venous HCO3- < 25 mEq/L. Anion Gap (AG) was also calculated using the following formula: AG = Na - (Cl +HCO3), with normal ranges considered for results equal to 12 ± 4 mEq/l [33]. Glomerular filtration rate (GFR) was determined

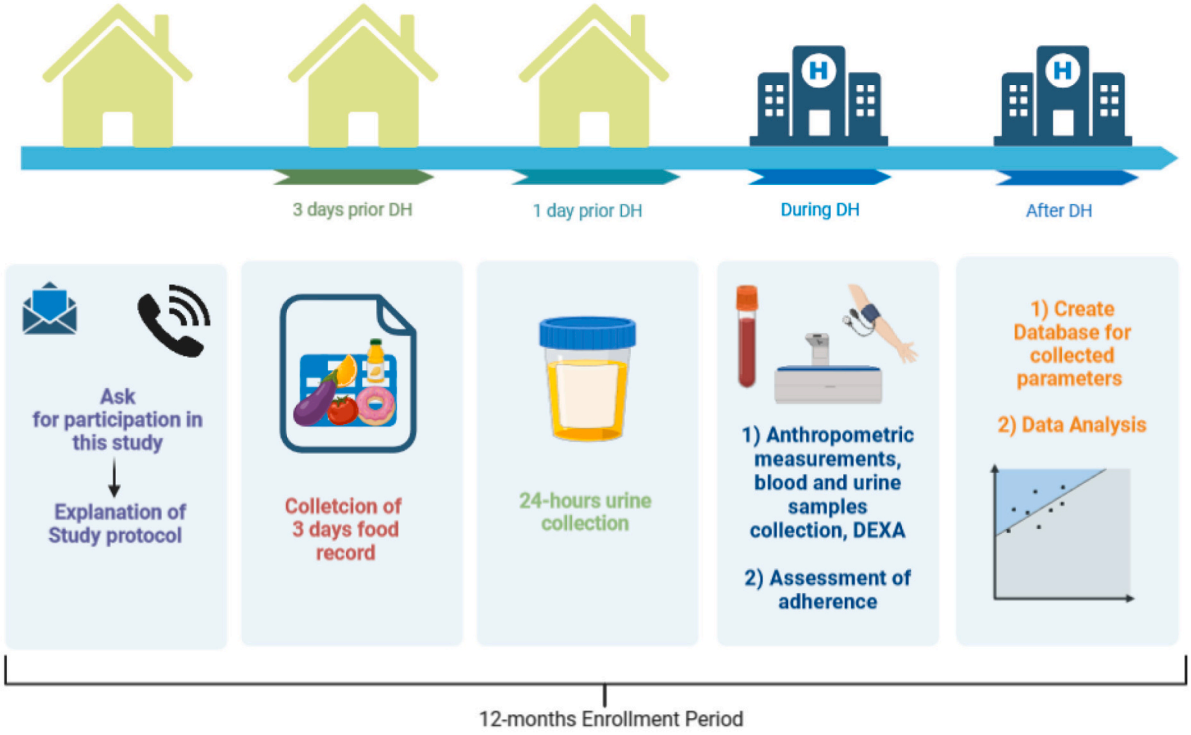

Fig. 1. *Cross-sectional structure for this study*. Patients in this study were asked to be enrolled prior annual Day Hospital (DH). If agreed to participate, the patient was asked to sign the informed consent during DH. Demographics, anthropometric measurements, nutritional data (3-days food records), chemistry parameters (including 24 h urine collection) and instrumental analysis were then collected during the DH. Adherence to diet was also assessed for all patients during DH. Data analysis was performed for all patients after the 12 months enrollment period.

using the CKD-EPI Creatinine formula, 2021[34].

(GFR $= 141 \times min\left(\frac{\text{serum creatinine}}{k}, 1\right)^{\alpha} \times max\left(\frac{\text{serum creatinine}}{k}, 1\right)^{-1,209} \times 0,993^{Age} \times 1,018[\text{if female}] \times 1,159[\text{if black}]$)where: $k = 0,7$ if female or $k = 0,9$ if male and: $\alpha = -0,329$ if female or $k = -0,411$ if male.

Tubular maximum reabsorption of phosphate by GFR (TmP-GFR), expressing the theoretical lower limit of serum phosphate below which all filtered phosphate is reabsorbed, was calculated using the following Eqs. [35], depending on the tubular reabsorption of phosphate (TRP):

$TRP = 1 - \left\{\frac{U_{phosphate}}{P_{phosphate}}\right\} \times \left[\frac{P_{creatinine}}{(U_{creatinine} \times 1000)}\right]$.If TRP was ≤0,86, TmP-GFR was calculated as TmP/GFR=$TRP \times P_{phosphate}$ otherwise TmP/GFR=$0,3 \times TRP/[1 - (0,8 * TRP)] \times P_{phosphate}$

Phenylalanine (Phe) values with which the correlations were made for this study are those collected and emerged at the dosage performed on the occasion of the Day Hospital.

2.2. Nutritional data

3-days food diaries were reviewed to record food and drink amounts consumed during the 3 days prior samples collection. Data regarding macro and micronutrients dietary contents from natural sources as well as from PS were thus collected by food records and calculated using the analysis software MètaDieta®. Data regarding oral ongoing micronutrient supplements were also collected. The potential renal acid load (PRAL) was calculated for every item analyzed, including both diet and PS, using the following formula: PRAL = (2 × (0.00503 × mg Met/d)) + (2 × (0.0062 × mg Cys/d)) + (0.037 × mg phosphorus/d) + (0.0268 × mg chloride/d) − (0.021 × mg potassium/d) − (0.026 × mg magnesium/d) − (0.013 × mg calcium/d) − (0.0413 × mg sodium/d) [36].

Mineral/ vitamin content of special low protein foods (SLPF) and PS was also analyzed; data for protein-free products were collected from labeling of different brands, whereas data for regular foods were collected from the Food Composition Database for Epidemiological Studies in Italy [37].

2.3. Instrumental analysis results

Bone mineral density of the lumbar spine (L2–L4) was measured using dual energy X-ray absorptiometry (bone densitometer DPX-L, prodigy equipment with "fan beam" technology), as recommended by European Guidelines [2]. Results were reported and interpreted according to the criteria suggested by the International Society for Clinical Densitometry [38].

BMD measurements were expressed as BMD *Z*-score, as suggested for healthy young men and/or premenopausal women [39]. According to The International Society for Clinical Densitometry (ISCD), a Z-scores of −2.0 or lower was defined as "*low bone mineral density for chronological age*" and "*below the expected range for age*" while those above −2.0 were defined as "*within the expected range for age*" [40].

Information regarding recently performed physical activity were also gathered, using the IPAQ questionnaire (International Physical Activity Questionnaire) [41], which investigates lifestyle habits thus stratifying patients into three categories of physical activity: low/inactive, moderate and high [42].

This study was approved by the internal ERB, protocol number: 0028924, and performed in accordance with the Declaration of Helsinki. Informed consent was obtained from all participants.

2.4. Statistical analysis

Sample size estimates were based on precision of 10% with an assumed prevalence <5% (as PKU prevalence in Italy is <1/10′000 [43]) and the level of confidence was set at 95% [44]. All statistical analyses were performed using R version 4.0.3. After collecting data, confounding variables such as age, gender, exercise levels and BMI were tested in a generalized linear model in relation to predicted variables. Mean's difference was assessed with *student' t-test* for unpaired sample, correlations were calculated using Pearson's correlation coefficient if variables were normally distributed (Shapiro–Wilks test) and described by mean ± standard deviation; otherwise, Spearman's rank-order correlation was used and results were described as median and min–max. Statistical significance was set for $p < 0.05$.

3. Results

20 PKU patients were recruited (mean age 27 years [18–45]; 8 females; all Caucasian), reviewed and analyzed (please refer to Table 1 for Demographics). All had classical PKU [45] based on available genetic results identifying variants in the *PAH* gene [46] and were in good health; the use of no concurrent medication was reported for at least 30 days prior to the collection of blood/urine samples. Mean BMI was 24.15 kg/m2 ± 4.33. Concerning physical activity, all subjects only took light to moderate exercise during days prior to analysis thus categorized as subjects with low to moderate physical activities by IPAQ. Based on reviewed 3-days food diaries, patients did not made any changes to dietary habits over the analyzed time period. As female patients menstruated regularly at the time of evaluation, they were not considered in menopausal transition.

3.1. Chemistry parameters

Main chemistry parameters results are shown in Table 2. Mean Phe levels were 778,55 ± 218,94 umol/L (vs. recommended for age < 600 umol/L [2]) and significantly correlated with daily intakes of natural protein (*p value = 0.007*), as expected; tyrosine (Tyr) resulted within reference levels (54,69 umol/L ± 13,69).

Considering other evaluated laboratory parameters, VBG pH levels were below normal ranges in 60% of patients ($n = 12$) (mean 7,31 [7,35]), as also bicarbonate with 95% of patients demonstrating low concentrations (mean 22,40 ± 1,43 mmol/L). These results did not correlate with analyzed confounding variables and were consistent with evidence of metabolic acidosis in PKU patients. Mean AG levels, although still in the range, resulted at the upper limit (mean 13,9 ± 2,9) of normal values and, in 25% of cases ($n = 5$), exceeded it. These results did not correlate with gender.

With regards to kidney function: 1) 40% of patients ($n = 8$) had a 24 h urinary creatinine clearance excretion lower than reference, even if creatinine and urea plasma levels were in range (mean 0,72 ± 0,14 mg/dl and 9,75 ± 3,16 mg/dL, respectively); 2) 24 h abnormal proteinuria was detected in 2 patients (400 mg/24 h and 390 mg/24 h), with a calculated GFR below reference range in the first case (74,4 mL/min/1.73 m^2); 3) 35% of patients ($n = 7$) presented low urinary phosphorus levels (mean 0,56 ± 0,34 g/24 h), even if plasma inorganic phosphorus was not altered; 4) 24 h urinary magnesium excretion was above reference range in 15% of patients, even if serum magnesium was in the normal range; 5) in 5 patients TmP/GFR was below the recommended range (<2,6 mg/dL) [47]. There was no correlation between plasma mineral levels and corresponding urinary levels.

3.2. Nutritional information

All subjects had a normocaloric intake (mean calories 1742,1 ±

Table 1
Demographic characteristics.

	Mean ± SD or n (%) or Median [min-max]
Age (years)	27 [18.00–45.00]
Gender (males)	12 (60)
< 35 years old	18 (90)

Table 2
Chemistry parameters results. Overview of biochemical and urinary parameters results for PKU patients reviewed in this study, including reference values. Abnormal results are bolded within the table. Used abbreviations: V = venous blood; S = serum; U = urinary; GFR = Glomerular Filtration Rate; AG = Anion Gap.

Parameter	Mean ± SD	Reference values
VpH	**7,30 ± 0,04**	**7,32–7,42**
V Bicarbonates, mmol/L	**22,40 ± 1,43**	**26–32**
V Ionized Calcium, mmol/L	1,22 ± 0,04	1,15–1,27
VpO2, mmHg	27,00 ± 6,11	24–40
VpCo2, mmHg	49,72 ± 6,65	41–51
V lactic acid, mmol/L	1,67 ± 0,77	0,4–2
AG, mEq/L	13,86 ± 2,95	12 ± 4
S-Calcium, mg/dL	9,68 ± 0,41	8,4–10,2
S-Inorganic phosphorus, mg/dL	3,41 ± 0,62	2,5–4,5
Alkaline phosphatase, U/L	61,65 ± 13,53	38–126
N-telopeptide, nEq/mMcrea	63,63 ± 33,23	M: 21–83; F: 17–94
S-Vitamin D, ng/mL	40,78 ± 9,89	30–100
S-Creatinine	0,72 ± 0,14	0,52–1,25
S-Parathormone, pg/mL	40,17 ± 12,23	13,6-85,8
S-Phenylalanine, umol/L	778,55 ± 218,94	37–115
S-Tyrosine, umol/L	54,69 ± 13,69	21–87
GFR_CKP-EPI Creatinine	122,39 ± 14,30	> 90 mL/min/1.73 m^2
Cystatin C, mg/L	0,74 ± 0,12	0,56-0,99
U-Creatinine Clearance mL/min	87,84 ± 29,18	80–160
U-Microalbuminuria 24 h	6,37 ± 2,85	<30
U-Proteinuria, mg/24 h	172,60 ± 90,82	42–225
U-Calcium mg/24 h	221,38 ± 83,46	100–300
U-Phosphorus g/24 h	0,56 ± 0,33	0,4–1,3 g/24 h
U-Magnesium mg/24 h	98,81 ± 33,09	73–122
TRP	91,43 ± 2,75	85–95%
TmP/GFR (mg/dL)	3,20 ± 0,59	2,6-3,8

385,9 kcal/day). Mean total protein intake was 80,7 ± 22.3 g/day (mean 1,18 ± 0,26 g/kg/day), meeting the Italian dietary guidelines [48]; PS provided a mean of 67,5% ± 12,23 of total protein intake.

3.2.1. PRAL (potential renal acid load)

PRAL calculations resulted positive for all the PS consumed by patients (mean 26.0 ± 12.6 mEq/day) Table 3. Patients consumed 10 different PS, with up to 4 different PS mixed within the same diet. Three PS contained no with minerals or vitamins.

Together all food sources except for PS, PRAL resulted negative (mean − 5.22 ± 18.16) in 65% of patients ($n = 13$); remaining part of patients had positive results that correlated to the larger amounts of common cereals and/or of natural protein (such as dairy) they were consuming.

Evaluating possible determinants of the PRAL value, it was possible to observe that this significantly correlated ($p < 0.05$) with the protein content of the foods consumed (intended as SPLF and natural foods), but, separating the data, only between PRAL and natural foods, not for SPLF taken alone. The correlation was confirmed with the protein content of the protein substitutes consumed ($p < 0.001$), (please refer to Fig. 2 for further details).

With regards to micronutrients, recommended intakes of calcium, phosphorous and vitamin D (based on Italian recommendations, LARN) [49] were generally met, but this was not the case for magnesium and potassium, which were found to be insufficient within current intakes Table 4.

Analyzing dietary composition, we could highlight that SLPF tend to have a significantly lower content of magnesium ($p < 0.001$), calcium ($p < 0.001$) and vitamin D ($p < 0.05$) compared to regular products Table 5.

As a limit, minerals content of PS could not be analyzed as means, since used PS were differently supplemented in micro and macro nutrients and working on an average of contents would not have been representative of the possible extremes (e.g. mixtures that contain only amino acids and macronutrients vs. mixtures that are fully supplemented with vitamin D, magnesium, phosphorous, potassium, etc.).

Different micronutrient supplementations were ongoing in all patients. These included vitamin D (25.000 IU/2,5 mL, monthly), DHA (10 mg/kg/day) and vitamin B12 (10 μg/day). The large consumption of fruit and vegetables ensured the need for folate without the need of micronutrient supplements.

3.3. Instrumental analysis results

A "low bone mineral density for chronological age" (*Z*-score < − 2.0) was detected in 4 patients (20%) with a mean Z-score in the studied population of −1.29 (± 0,76). Positive significant correlation between BMD and VpH could be found, as shown in Fig. 3.

BMD did not correlate with other analyzed parameters, including nutritional habits.

4. Discussion

It was at first in 1977 that, in the context of the first attempts to implement specific dietary interventions for PKU patients, metabolic acidosis was reported by Manz et al. as a possible deleterious effect of the use of synthetic amino acids in the diet [50]. Since then, much has been done to improve the composition of amino acid substitutes to reduce the acid impact [51]. Still, there is growing evidence of possible unfavorable outcomes in patients on dietary treatment and few studies tried to re-investigate such aspects throughout nowadays PS. With this cross-sectional, observational and descriptive study we reviewed data from 20 PKU patients (mean age 27 years [18–45]) to investigate such data along with possible indices of impaired kidney and bone function, evaluating possible correlations [27–29,52].

Metabolic acidosis was found in 60% of PKU patients (mean VpH 7,31 [7,35], mean bicarbonate 22,40 ± 1,43 mmol/L, mean AG 13,9 ± 2,9). Phe levels did not correlate. Possible reasons for an increase in the production of fixed acids were ruled out. Same for altered renal function (indices of kidney function were in range including creatinine, urea and

Table 3
Protein Substitutes (PS) composition, as used among patients analyzed.

PS	Energy (kcal)	L (g)	CHO (g)	Met (mg)	Cys (mg)	P (mg)	Chl (mg)	K (mg)	Mg (mg)	Ca (mg)	Na (mg)	Vit D (μg)	PRAL (mEq/day)
1	542,9	0,0	35,7	1642,9	2500,0	2000,0	0,0	1607,1	642,9	2514,3	214,3	42,9	29,53
2	560,1	5,1	18,4	2008,5	2843,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	55,46
3	982,75	2,41	21,03	1724,1	2413,8	1117,2	0,0	803,4	365,5	1958,6	69,0	33,9	33,9
4	830,60	1,86	101,09	1693,9	2568,3	1382,51	103,83	491,80	536,61	1786,89	103,83	39,89	51,03
5	422,78	0,0	5,69	2101,3	3227,8	0,0	0,0	0,0	0,0	0,0	0,0	0,0	61,2
6	500,0	5,0	13,0	2250,0	3050,0	1820,0	695,0	1220,0	550,0	2000	520	50,0	50,3
7	490,0	2,1	19,0	1944,4	3055,6	1377,8	6,9	34,7	533,3	1777,8	27,8	40,0	69,8
8	600,00	2,2	44,0	1687,5	2562,5	1381,3	125,0	500,0	535,0	1781,3	125,0	40,0	50,5
9	756,52	2,95	60	2260,9	3043,5	1782,6	1217,4	1573,9	626,1	2000,0	852,2	50,43	48,5
10	1339,28	44,64	114,28	2607,1	6785,7	2528,6	1600,0	1750,0	439,3	3850,0	1100,0	75	103,2

Nutrients has been calculated based on 100 g of protein. Used abbreviations: L = Lipid; CHO = Carbohydrates; Met = Methionine; Cys = Cysteine; P = Phosphorus; Chl = Chlorine; K = potassium; Mg = Magnesium; Ca = Calcium; Na = Sodium; Vit D = Vitamin D.

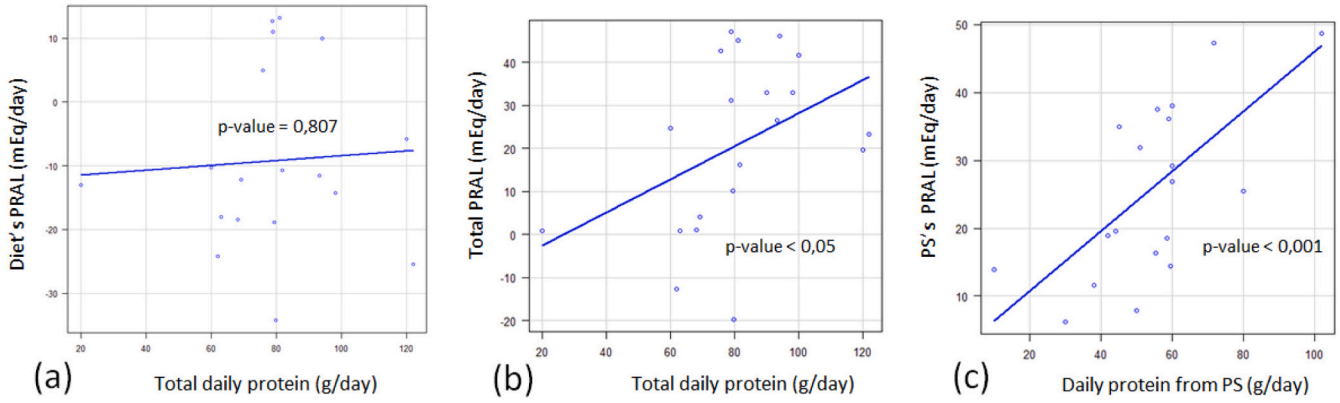

Fig. 2. *Correlations between PRAL and daily protein amount from different sources.* From the left: (a) dietary PRAL (resulting from all foods consumed except PS) related with total daily protein intakes (considering all sources); (b) evidence of correlation between total PRAL (considering dietary sources including PS) and total daily protein; (c) evidence of correlation between PS's PRAL (considering the PS one alone) and daily protein intakes as obtained from PS (meaning protein content of PS). Used abbreviations: PS = Protein Substitutes.

Table 4
Calcium, Potassium, Phosphorus, Vitamin D and Magnesium intakes from different sources vs. recommendations. The average content derived from different sources is analyzed and compared: dietary intakes taken alone (excluding the PS), intakes from PS alone and total intakes (including both dietary and PS intakes and also micronutrient supplements). One-sample *t*-test has been used to determine whether the total intake mean differed from recommended; **p*-value <0.05; ***p-value<0.0001. Used abbreviations: PS = Protein Substitutes; LARN = Reference Nutrient and Energy Intake Levels for the Italian population [49].

	Dietary intakes (excluding PS) Mean ± SD	Content in PS Mean ± SD	Total intake Mean ± SD	Recommended (LARN)
Calcium (mg/day)	308,9 ± 139,15	886,90 ± 415,47	1366.23 ± 198.66	1000
Potassium (mg/day)	2006,55 ± 544,67	423,23 ± 453,28	2273,86 *** ± 691,59	3900
Phosphorus (mg/day)	530,02 ± 205,30	603,90 ± 298,86	892,36 ± 410,25	700
Vitamin D (μg/day)	0,65 ± 0,94	15,34 ± 7,52	21.05 ± 13.48	15
Magnesium (mg/day)	111,82 ± 56,37	227,38 ± 77,75	208,92* ± 91,66	240

Table 5
Comparison of nutritional composition of Special low-protein foods (SLPF) vs. corresponding regular products. Comparison of contents of some regular products compared to same special low-protein counterpart are represented (for every 100 g of product). Unpaired *t*-test has been used to detect differences, if present. **p*-value <0.05, **p-value <0.001.

	Common foods			Special low-protein foods		
	Bread	Pasta	Biscuits	Bread	Pasta	Biscuits
Magnesium, mg	15,1 ± 9,8	75,5 ± 24	15,6 ± 4,8	6,7 ± 0,3**	Traces**	6,8 ± 1,9**
Calcium, mg	64,3 ± 42,4	24,8 ± 3,7	53 ± 39,4	14,2 ± 5,9**	13,9 ± 5,5*	10,2 ± 3,7**
Vitamin D, μg	0,11 ± 0,2	0,17 ± 0,17	0,5 ± 1,1	0*	0*	0*

cystatin C) [53]. Besides that, TmP/GFR resulted impaired in 5 patients while PTH vitamin D and TRP were normal. Considering the evidence of good control of tubular renal phosphate reabsorption, this would suggests a phosphate renal wasting not due to a tubular defect, even though

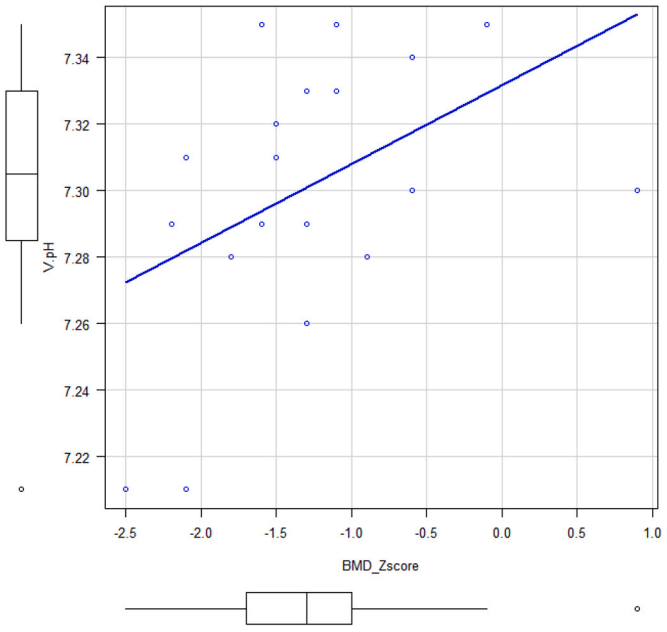

Fig. 3. Correlation between VpH and DEXA Results. positive significant correlations between DEXA Z-score and V-pH ($p = 0.022$) are shown. Used abbreviations: VpH: venous blood pH; DEXA: Dual-energy X-ray absorptiometry.

the metabolic acidosis could play a major role in that and further studies will be needed to better explain these results.

Possible non-renal causes of metabolic acidosis were hypothesized. 4 patients (20%) had BMD's *Z*-scores $< -2,0$, with an overall mean of $-1.29 \pm 0,76$. Evidence of a BMD reduction in PKU patients is well reported in many systematic reviews and meta-analysis. However, none of our patients had a history of fracture, which is in contrast with reports of a fracture rate of 20% among PKU subjects (even if controls are lacking in most studies reporting it) [16], suggesting a less relevant clinical significance of this data in our sample. Also in contrast with other studies [14–15], no other parameters significantly correlated with BMD, including Phe levels, vitamin D, PTH or nutrient intakes. This was not true for VpH that showed a positive correlation with BMD ($p = 0.022$) suggesting a possible strict correlation to metabolic acidosis, even though it still has to be considered that bone health depends on many factors including genetic variability [54,55].

Dietary habits were also evaluated including analysis of derived

parameters, such as the potential renal acid load (PRAL) which is based on nutrient intakes and known to possibly alter the acid-base balance (Fig. 4) [30].

Different protein substitutes (none of whom was glycomacropeptide, slow-release PS or large neutral aminoacids) were daily used among our patients. Adherence to treatments was confirmed on Tyr dosages. All PS presented a positive, thus acidic, PRAL charge based on composition (26.0 ± 12.6 mEq/day) that significantly correlated to protein content (p ⟨0,001). Such observations suggest to consider PS as a potential influencing factor of the total dietary acid load therefore future studies that aim to compare PKU patients according to type of used substitute (PS vs GMP).

PKU diets are normally characterized by the large consumption of vegetables and fruits, which is known to carry a negative PRAL mainly due to the higher content of potassium. Vegetables and fruits were normally consumed in our patients based on 3 days food records. Even though plasma potassium levels resulted normal in our sample Table 2, analyzing dietary composition (based on consumed foods/SP) we could highlight that its amounts were not sufficient to ensure the recommended by Italian guidelines daily intakes Table 4 (2273,86 ± 691,59 mg/day vs 3900 mg/day recommended [49]). Given that normally potassium exchanges H^+ in the distal part of the nephron (inducing reduced calcium urinary excretion [56]) and is capable of suppressing calcium reabsorption and/or bone mineral dissolution [57–60], it can be speculated how increasing their consumption may be relevant in countering the positive PS charge, potentially resulting in an alkalizing protective effect [30,61]. This could be further implemented by favoring the choice of foods with a higher potassium content. Same observations can be done for magnesium, which is also well known to play a major role for bone mineralization [62,63] and resulted low in intakes Table 4.

Along with that, SLPF are reported to contain less potassium than common foods, along with less quantities of sodium, phosphorus and magnesium (since mostly created for patients with renal failure) Table 5 [64–66]; yet, to date they have not been commented as possible contributors to PRAL.

SLPF's PRAL resulted neutral/negative in our sample (mean − 5.22 ± 18.16 mEq/day). Still, the overall total dietary PRAL resulted positive (mean 20,75 ± 20,05 mEq/day). This is in contrast with data for general population, which are conflicting but either way mostly indicating a neutral or negative charge in most cases (−1,7 ± 7,8 on a sample of 257 healthy subjects; − 11,1 ± 18,6 on another sample of other 2369 healthy subjects [67,68]), with large variations among different countries (mean score of −23.0 mEq/day in France, − 22.0 mEq/day in Iran, − 21,8 mEq/day in Korea, − 14,6 mEq/day in Netherland, 10.4 mEq/day in Japan and 22.1 mEq/day in China [67]). These data would eventually make only healthy Chinese subjects comparable with our PKU subjects for a mean PRAL >20,20 mEq/day in chinese vs 20,75 ± 20,05 mEq/day in our sample.

Considering the only factor accounting for a positive charge in this study was the PS, we suggest that this factor may be the main one to determine the total PRAL, overcoming the negative charge of natural protein and SLPF and strongly influencing the acid/base equilibrium. This may be potentially due to the fact that the total dietary PRAL is linked to the daily total protein intakes, which in PKU patients is mostly carried by PS instead of natural proteins consumed by healthy population.

All these results taken together further emphasize and open discussion about how the composition of the dietary treatment of PKU patients needs to be strictly monitored taking into account also and above all the mineral composition of the proposed foods (SP, natural foods, SLPF), which could be decisive in favoring or worsening the usual acid load of the entire diet. With this goal, further studies exploring possible adaptations of PS in terms of potassium content may be useful.

Unfortunately we couldn't succeed finding correlations between VBG and PRAL, but we think that there may be relevant bias in that. This was still a relatively small number of patients and overall variability of consumed PS, natural protein sources and different amounts/composition of SLPF may have played a major role in such downfall. The impossibility of determining, moreover, the content of potassium and magnesium in SLPF due to the lack of data on that, may have been the main contributing factor in our failure to identify a significant correlation in support to the “acid load hypothesis”. Furthermore, we were only evaluating a one time window and, considering the potential role of protein intakes during skeletal development with an anabolic effect that may be diminished if the dietary acid load is high, longitudinal studies could be of interest evaluating when and how possible alterations can happen. At last, hand-grip force has not be evaluated with regards to bone health assessments and this could have implement results interpretation for correlations found.

What is to intervene first in explaining the alterations found in our PKU patients is yet to be fully elucidated. No doubt that there may be a vicious circle between bone and renal adaptations, tending to feed continuously and difficult to understand considering the renal system as a whole, with the activation of the acid base reserve and consequent excretion/reabsorption of bicarbonates and the so-called “bone reworking” cycle [57,69]. Increasing sample size, standardizing the dietary approach, including PS, and the availability of accurate data on SLPF content may turn out to be the key to determine other possible hidden correlations.

Despite all these possible limitations, we believe that this data cannot be ignored and that may strongly add relevant thoughts about possible long term consequences of dietary interventions in PKU.

5. Conclusion

Optimizing outcomes for PKU patients on dietary treatment is still an

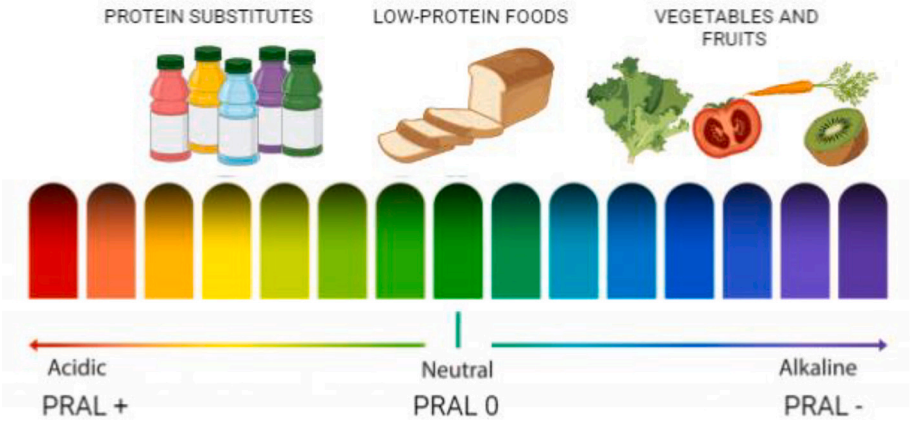

Fig. 4. *Graphical representation of the Potential Renal Acid Load (PRAL) charge of substrates consumed within the PKU diet.* On the left, protein substitutes represented as contributors to a positive charge of PRAL; in the middle, Special low-protein foods that seem to have a mostly neutral PRAL, even though not fully known in their mineral content therefore data not fully reliable to date; on the right, fruits and vegetables which are known as contributors to a negative charge of PRAL. PRAL was calculated for all PS used among patients (which included the analysis of 10 different PS's contents); natural protein PRAL was obtained from the Food Composition Database for Epidemiological Studies in Italy (http://www.bda-ieo.it/wordpress/en/). Used abbreviations: PS = Protein Substitutes.

open challenge. Evidence of metabolic acidosis and low bone mineralization, linked one another as described in this paper, needs further evaluation and attention as it may have relevant consequences on a long-term basis. Positive PRAL of PS may influence metabolic acidosis but doesn't seem to be the sole cause in this imbalance. The importance of food quality and content control is also essential in order to assess correct requirements and related desired contents.

This study brings the attention to skeletal fragility, possible renal impairment and metabolic acidosis in PKU and also reopens the debate to nutrients requirement in this population that need special diets.

Although vitamin D and Calcium are often measured in order to prevent deficiency, other minerals associated with bone-health, such as potassium and magnesium, need further exploration.

However, further studies on metabolic acidosis and renal impact will be needed to confirm these hypotheses.

CRediT authorship contribution statement

Valentina Rovelli: Conceptualization, Writing – original draft, Writing – review & editing. **Vittoria Ercoli:** Data curation, Methodology, Software. **Alice Re Dionigi:** Visualization, Investigation. **Sabrina Paci:** Supervision. **Elisabetta Salvatici:** Supervision. **Juri Zuvadelli:** Supervision. **Giuseppe Banderali:** Software, Validation.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

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