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Bone mineral density in French adults with early-treated phenylketonuria

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ABSTRACT

Phenylketonuria (PKU) treatment requires a low-phenylalanine (Phe) diet limiting natural protein intake, using medical low-protein foods and Phe-free amino acids (AA) supplements along with micronutriments' supplies. Current recommendations suggest maintaining this diet for life to prevent neuro-psychological effects of high Phe concentrations. The long-term consequences of such a diet are poorly understood, particularly on bone health. Our study aimed to assess the prevalence of low bone mineral density (BMD) (*Z*-score ≤ -2, for vertebral and/or femoral site) in adults with PKU and to investigate associated risk factors, in the French ECOPHEN cohort.

The study included 171 patients with 67.3 % of women and a median age of 27 years old. The median femoral and vertebral Z-scores of BMD were both – 0.6. Only 11.4 % of patients had a low BMD. Compared to patients with normal BMD, patients with low BMD had a lower body mass index (BMI) (median 20.4 versus 24.4 kg/m^2, $p = 0.002$), and were more likely to have never stopped their diet (58.8 % versus 31.8 %, $p = 0.029$). They also had higher total protein intake (1.1 versus 0.9 g/kg/day, $p = 0.015$), with more proteins from AA supplements (0.80 vs 0.53 g/kg/day, $p = 0.010$). In multivariate analysis, younger patients born after 1990 and who never stopped diet had a 4.7 times risk to have low BMD ($p = 0.005$), after adjustment on age, sex, BMI.

Abbreviations: AA, Amino Acid; BMD, Bone mineral density; BMI, Body mass index; CTX-1, Cross-linking telopeptide of type I collagen; DXA, Dual energy X-ray absorptiometry; PFAAS, Phenylalanine free amino acids supplements; GMP, Glycomacropeptide; ISCD, International Society for Clinical Densitometry; PAH, Phenylalanine hydroxylase; Phe, Phenylalanine; PKU, Phenylketonuria.

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In summary, our study identified a subgroup of PKU adult patients with low BMD and showed that prolonged low natural protein diet is associated with low BMD.

1. Introduction

Phenylketonuria (PKU, OMIM 261600, ORPHA79254) is the most prevalent inborn error of metabolism, of autosomal recessive transmission, caused by a wide range of variants in the *PAH* gene (12q22-q24.2) coding for phenylalanine hydroxylase (PAH; EC 1.14.16.1). PAH deficiency is responsible for increased phenylalanine (Phe) concentrations in both blood and brain, leading to progressive encephalopathy with severe intellectual impairment, seizures, hyperactivity, autistic or psychiatric signs in untreated patients [1]. As in many countries, a national newborn screening program for PKU exists since 1972 in France allowing early diagnosis and prompt prescription of Phe-restricted diet. The treatment usually consists in a low natural protein diet excluding most animal protein sources, using medical low-protein foods (for caloric supplies) and Phe-free L-amino acid (AA) supplements (for protein supplies) including vitamins, minerals, and oligo-elements. Tetrahydrobiopterin (BH4)-responsive patients may also be treated with sapropterin alone or combined with a PKU diet. If blood Phe concentrations are stable within the target range (120–360 μmol/L) during childhood, patients with PKU can have normal neurological development and a normal life [2].

High blood Phe concentrations have long been considered as safe in adulthood, but some specific neuro-psychological manifestations may occur in adults who stopped the PKU diet, leading to the concept of "diet for life" [3,4]. The goal is then to maintain low Phe concentrations i.e. < 600 μmol/L in Europe, to prevent potential complications and maybe early neurodegenerative disorders [2]. Despite recommendations, adhesion to a strict low protein diet decreases with age and adults have some difficulties to resume the PKU diet when it has been stopped during childhood or adolescence. Patients have difficulties to manage such a diet in everyday life, due to lack of motivation or dislike of medical food either the taste or the smell of AA supplements [5]. Therefore, many adults do not achieve Phe targets due to an excess of naturel protein intake and/or discontinuation of AA supplements. Total protein intake can also be lower than recommended in adults with PKU and micronutrients' deficiencies are frequent, mainly for iron, zinc, vitamin D3, magnesium, calcium, selenium, iodine, vitamin A and copper [6]. Moreover, bio-availability of AA supplements is lower as compared to AA from natural proteins along with an increased oxidation of free AA [7]. To date, the long-term nutritional consequences of low protein diet have been partially studied in adults with PKU. With respect to bone health, data are lacking about the potential impact of Phe-restricted diet as well as the effects of chronic hyperphenylalaninemia.

Previous studies showed conflicting results about bone status in PKU patients. In a large meta-analysis performed in 2015, including a total of 360 adults and children, Demirdas et al. showed that bone mineral density (BMD), defined as a *Z*-score ≤ -2 (International Society for Clinical Densitometry criteria), was low in approximately 10 % of PKU patients [8]. More recently in 2020, in a systematic review, de Castro et al. analyzed 8 articles assessing the effects of PKU on bone mineral content. These low-sizes studies, which mainly included children or teenagers, reported lower BMD (T-score or *Z*-score values) in PKU patients compared to a control population, but in the 3 studies with *Z*-score measurements, PKU patients did not show a higher frequency of Z-score values under −2.0 [9]. In a larger multicenter European study including 183 PKU adults with a median age of 28 years old, Lubout et al. showed that BMD was significantly lower in PKU patients but remains within a normal range. In this study, only 5,5 % of the PKU patients had Z-score ≤ -2, and the risk for fractures was found to be identical between PKU patients and the general population [10]. Moreover, no risk factors for low BMD have been clearly identified in the literature except for male gender in Lubout's study [10]. Overall, blood Phe concentrations, low vitamin D or PTH concentrations, calcium supplementation, smoking status, alcohol consumption and natural protein or micronutrients intakes were not significantly associated with low BMD [9].

Therefore, some questions remain about the potential long-term consequences of low natural protein diet or associated micronutrients deficiencies on bone health in adults with PKU. The first objective of our study was to assess the prevalence of low bone mineral density (BMD) and bone fractures in a French national cohort of adults with early-treated PKU. The secondary objective was to identify potential risk factors for low BMD, with a focus on nutritional factors.

2. Material and methods

2.1. Inclusion criteria

Data were obtained from the ECOPHEN cohort [11]. This national 5-years prospective cohort (ClinicalTrials.gov Identifier: NCT01619722) included patients over 18 years old with PKU from 17 French centers (Angers, Bordeaux, Brest, Dijon, Grenoble, Lille, Lyon, Marseille, Nancy, Nantes, Paris - Robert Debré, Paris –Necker, Rennes, Rouen, St Etienne, Toulouse, Tours). Inclusion criteria were: PKU patients older than 18 years diagnosed through newborn screening program, written informed consent, social health care affiliation. The exclusion criteria were: a neurological disease which can interfere with neurological disorder seen in PKU, diagnosis of cancer, late PKU diagnosis. The study was funded by the French national hospital program for clinical research (PHRC). The study followed the ethical standards of French committee on human experimentation, and the Helsinki Declaration of 1975, as revised in 2013. The approval was obtained from the relevant committee on human subjects. Informed written consent was obtained from all patients.

Legal authorizations of the study were obtained in 2011 (CPP Ouest-I Tours n°2011-R11, 24/05/2011; ANSM n°B110423–80, 26/04/2011). The study started on 15/03/2012 and ended 06/07/2020.

The following classification of phenylketonuria was used: classical PKU, on non-classical (mild) PKU or mild hyperphenylalaninemia if plasma Phe level at neonatal screening was above 1200 μmol/L, between 600 and 1200 μmol/L or less than 600 μmol/L respectively [12].

2.2. Data collection

Data were extracted from the inclusion visit of the ECOPHEN study. We collected age, sex, birth year, body mass index, bone fractures, history of Phe restricted diet as well as biological parameters (Supplemental Table S1). Dietary intakes were recorded by a 3-day nutritional assessment with centralized calculation of caloric, total protein, natural protein, protein from AA supplements and calcium intakes. Natural proteins were animal or vegetal proteins provided by foods. Total protein intake included both natural proteins and proteins provided by AA supplements. Calcium intake included dietary calcium and that provided by AA supplements and drug supplements. Low natural protein diet was defined as natural protein intake below 0.6 g/kg/day, with or without AA supplements consumption.

BMD was determined using Hologic DISCOVERY Dual energy X-ray absorptiometry (DXA) system, with T-score and *Z*-score determinations.

The International Society for Clinical Densitometry (ISCD) criteria were used to define a "low-BMD" in young adults as Z-score ≤ -2, for vertebral and/or femoral skeletal site [13].

The facture rate was determined by the percentage of patients who had at least one bone fracture. Fracture histories were collected during interviews with patients and/or extracted from clinical records in each study centers.

2.3. Statistical analysis

Statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL) v21. The level of significance was 2 sided and set at 5 % ($p < 0.05$). To test the normal distribution of quantitative variables, a Kolmogorov – Smirnov test was used ($p > 0.05$). On all the tested characteristics, only a few had a normal distribution: total proteins, proteins' intakes from AA supplements, calcemia, phosphoremia, selenium, vitamin B9, phenylalanine concentrations and phenylalanine/tyrosine ratio.

Quantitative variables were expressed as mean and standard error (SE). Categorical variables were expressed as number (n) and percentage (%). When the distribution was not normal, quantitative variables were expressed as median, and minimum-maximum (min-max).

Fisher's exacts test were used to compare categorical variables. For quantitative variables, means were compared by ANOVA in case of normal distribution and Wilcoxon-Mann- Whitney test was used to compare the median of variables which did not follow a normal distribution.

Univariate association analysis was conducted to compare classical PKU versus non-classical patients, patients with normal or low-BMD, and patients who stopped their low-Phe diet in childhood with whom never stopped. Multivariate analyses were performed using a binary logistic regression model (entry method) with variables with $p < 0.05$ in univariate analysis and/or relevant baseline characteristics, including tests for interaction between variables. Standardized variables were used to introduce continuous variable in this model.

3. Results

3.1. Cohort description

The ECOPHEN cohort included 191 patients. The present study excluded patients with mild persistent hyperphenylalanemia and 7 patients without any BMD data, and 4 patients with exclusion criteria. Thus, the study included the 171 remaining patients, 147 with classical PKU and 24 with non-classical PKU (see Flow chart in supplemental Fig. 1). Cohort characteristics are presented in Table 1. Two thirds of the patients were women. No women were postmenopausal. The median age of the cohort was 27 years-old and the median body mass index (BMI) was 24 kg/m^2. Non-classical PKU patients were significantly younger than classical PKU patients, and tended to have a higher BMI. As expected, plasma phenylalanine concentrations were significantly higher and plasma tyrosine concentrations were significantly lower in classical PKU patients as compared to the non-classical ones.

Regarding diet characteristics, about half of patients were considered to be under low natural protein diet (natural protein intake <0.6 g/kg/day), including one third of the patients who never stopped such diet. Half of patients took AA supplements. There was no significant difference in frequency of low natural protein diet between classical and non-classical PKU groups (Supplemental data, Table S2). No patients took GMP in this study as GMP products were not yet available in France at the time of our study.

Three patients took sapropterin, two of them associated with a low natural protein diet. Three patients had calcium supplementation, three in vitamin D, and seven in other micronutriments: 3 patients took iron, 4 selenium, 2 vitamin B9, 1 vitamine B12, 1 zinc.

There was no significant difference between classical and non-classical PKU for calcium and protein intakes, either total, natural or from AA supplements, and regarding biological data or most micronutrients concentrations. The only statistically significant difference concerned zinc concentrations, which were lower in non-classical PKU than in classical PKU. However, about 2/3 of the two groups (67.1 %) had vitamin D levels below the recommended threshold of 75 nmol/L (Supplemental data, Table S2).

3.2. Bone status comparison

Among the 171 patients with available BMD data, 149 had recorded *Z*-scores. The median femoral and vertebral Z-score were both −0.6. 5.4 % of patients had low femoral Z-score and 8.7 % low vertebral *Z*-score (Z-score ≤ −2). 11.4 % of patients were considered as having low-BMD (femoral and/or vertebral Z-score ≤ −2). The fracture rate was 34.5 %. All these data showed no significant difference between classical and non-classical PKU.

The comparison of patients with low BMD and normal BMD is shown in Table 2. Gender was not associated with low BMD. Nevertheless, females had a significantly lower value of vertebral Z-score (median of −1.2 (min-max, −2.6 – +2.1) vs − 0.4 (min-max, −2.7 – +3.0) in males), $p = 0.006$), with no difference between gender concerning femoral Z-score (data not shown).

Table 1
Comparison of general and bone characteristics between classical and non classical PKU patients.

	All patients		Classical PKU		Non classical PKU		p
	N	Median (min-max) or n (%)	N	Median (min-max) or n (%)	N	Median (min-max) or n (%)	
Global characteristics							
Age at inclusion (years)	171	27.0 (18.1–45.0)	147	27.4 (18.1–45.0)	24	24.2 (18.1–36.7)	0.047
Birth year > 1990	62	36.3 %	48	32.7 %	14	58.3 %	0,021
Sex (F) %	171	67.3 %	147	67.3 %	24	66.7 %	1.000
BMI (kg/m^2)	171	24.0 (16.7–43.4)	147	23.7 (16.7–43.4)	24	25.9 (18.6–36.2)	0.098
Phenylalanine* (μmol/L)	169	1142.4 (34.6)	145	1201.4 (36.8)	24	786.3 (63.5)	<0.001
Tyrosine (μmol/L)	169	38.0 (11–93)	145	37.0 (11–79)	24	49.5 (24–93)	<0.001
Phenylalanine/Tyrosine ratio*	169	31.5 (1.23)	145	33.8 (1.32)	24	17.4 (1.68)	<0.001
Bone characteristics							
Femoral *Z*-score	149	−0.6 (−3.0 – +2.1)	130	−0.7 (−3.0 – +2.1)	19	−0.1 (−2.7 – +1.2)	0.320
Femoral Z-score < −2	149	8 (5.4 %)	130	7 (5.4 %)	19	1 (5.3 %)	1.000
Vertebral Z-score	150	−0.6 (−2.7 – +3.0)	131	−0.7 (−2.7 – +3.0)	19	−0.4 (−2.1 – +1.1)	0.256
Vertebral Z-score < −2	150	13 (8.7 %)	131	12 (9.2 %)	19	1 (5.3 %)	1.000
Femoral or Vertebral Z-score < −2	149	17 (11.4 %)	130	16 (12.3 %)	19	1 (5.3 %)	0.698
Fracture rate	171	34.5 %	147	49 (33.3 %)	24	10 (41.7 %)	0.489

BMI, body mass index; F, female; N, number of patients studied.
Quantitative variables are expressed as median and (min-max) and categorical variables were expressed as number (n) and percentage (%). Fisher's exacts test were used for categorical variables. ANOVA was performed for normally distributed quantitative variables, Wilcoxon-Mann- Whitney test if not.
* Normally distributed variable, expressed as mean (SEM).

Table 2
Comparison between low and normal BMD groups.

	Normal BMD		Low BMD		p
	N	Median (min-max) or n (%)	N	Median (min-max) or n (%)	
Global characteristics					
Age at inclusion (years)	132	27.1 (18.2–45.0)	17	21.8 (18.1–39.9)	0.059
Birth year > 1990	132	46 (34.8)	17	10 (58.8)	0,066
Sex (F)	132	88 (66.7)	17	11 (64.7)	1.00
BMI (kg/m2)	132	24.4 (17.3–43.3)	17	20.4 (16.7–33.6)	0.002
Phenylalanine* (μmol/L)	131	1138.6 (39.4)	17	1012.5 (102.5)	0.276
Tyrosine (μmol/L)	131	38.0 (11.0–93.0)	17	37.0 (22.0–58.0)	0.574
Phenylalanine/Tyrosine* (ratio)	131	31.4 (1.44)	17	27.9 (2.66)	0.401
Bone characteristics					
Fracture rate (≥ 1 fracture)	132	45 (34.1 %)	17	8 (47.1 %)	0.296
Diet characteristics					
Total Energy Intakes (kcal)	116	1912.1 (923.2–3771.0)	14	1753.6 (1328.1–2861.0)	0.663
Total protein intakes* (g/kg/day)	116	0.9 (0.03)	14	1.1 (0.1)	0.015
AA supplements	130	68 (52.3 %)	17	12 (70.6 %)	0.199
Hypoprotidic food	132	36 (27.3 %)	17	5 (29.4 %)	1.00
Stopping diet age (years)	85	11.0 (2.0–32.0)	6	7.0 (4.0–14.0)	0.128
Undergoing low natural protein diet	132	76 (57.6 %)	17	14 (82.4 %)	0.07
Never stopped diet	132	42 (31.8 %)	17	10 (58.8 %)	0.029
Natural protein intakes (g/kg/day)	116	0.62 (0.1–1.59)	14	0.58 (0.16–1.33)	0.804
Protein intakes from AA Supplements* (g/kg/day)	53	0.53 (0.04)	9	0.80 (0.11)	0.010
Protein intakes > 1 g/kg/day	116	41 (35.3 %)	14	9 (64.3 %)	0.044
Calcium intakes (mg)	116	873.0 (219.0–2345.5)	14	916.1 (390.2–2060.6)	0.539
Calcium Phosphate metabolism data					
25 OH Vitamin D (nmol/L)	127	62.0 (10.0–144.0)	16	62.5 (19.0–150.0)	0.573
Vitamin D < 30 nmol/L	127	10 (7.9 %)	16	1 (5.9 %)	1.00
Vitamin D < 75 nmol/L	127	86 (67.7 %)	16	11 (68.8 %)	1.00
Calcemia* (mmol/L)	131	2.38 (0.08)	17	2.42 (0.02)	0.095
Phosphoremia* (mmol/L)	121	0.97 (0.02)	17	1.05 (0.04)	0.079
PTH (ng/L)	119	33.0 (6.0–125.1)	17	35.0 (10.0–54.0)	0.937
Micronutrients status					
Copper (μg/L)	129	993.0 (530.0–2347.0)	16	1062.5 (700.0–2090.0)	0.182
Copper < 880 μg/L	129	49 (38.0 %)	16	3 (18.8 %)	0.171
Zinc (μmol/L)	128	12.2 (4.2–18.1)	17	13.5 (10.1–23.3)	0.149
Zinc < 7.80 μmol/L	128	5 (3.9 %)	17	0 (0 %)	1.000
Selenium* (μg/L)	111	76.4 (1.52)	15	67.8 (5.13)	0.072
Selenium < 70 μg/L	126	43 (34.1 %)	15	9 (60.0 %)	0.086
Vitamin B9* (nmol/L)	126	27.1 (1.28)	16	30.9 (4.78)	0.334
Vitamin B9 < 5 nmol/L	126	1 (0.8 %)	16	0 (0 %)	1.00
Vitamin B12 (pmol/L)	127	274.0 (74.0–987.0)	17	237 (103–941)	0.494
Vitamin B12 < 140 pmol/L	127	6 (4.7 %)	17	3 (17.6 %)	0.074
Vitamin A (μmol/L)	124	2.14 (1.29–4.24)	16	2.7 (1.1–3.8)	0.058
Vitamin A < 1.40 μmol/L	124	5 (4.0 %)	16	2 (12.5 %)	0.183
Vitamin E (μmol/L)	22	26.0 (17.0–43.0)	3	20.0 (20−33)	0.603
Vitamin E < 20 μmol/L	22	3 (13.6 %)	14	0 (0 %)	1.00

BMI, body mass index; F, female; N, number of patients studied.
Quantitative variables are expressed as median and (min-max) and categorical variables were expressed as number (n) and percentage (%). Fisher's exacts test were used for categorical variables. ANOVA was performed for normally distributed quantitative variables, Wilcoxon-Mann- Whitney test if not.
* Normally distributed variable, expressed as mean (SEM).

In the low BMD group, patients had a lower BMI and were younger than in the normal BMD group, although this result did not reach statistical significance. The three patients who took sapropterin all belonged to the normal BMD group.

The fracture rate, phenylalanine and tyrosine concentrations were not different between low or normal BMD patients. Patients with low BMD showed better adherence to the low natural protein diet, as almost twice as many patients never stopped their diet compared to the normal BMD group (58.8 % vs 31.8 %, $p = 0.029$), (Table 2). They also had significantly higher intakes in total protein and protein from AA supplements, with a higher proportion of patients who consumed more than 1 g/kg/day of total protein.

Concerning plasma micronutrients concentrations, there was no difference between the two groups, especially for vitamin D status or calcium intakes. Interestingly, selenium deficiency was twice as frequent in the low-BMD group as in normal group (60 % vs 34.1 %), and B12 deficiency was four times more frequent (17.6 % versus 4.7 %), although these results were above significant statistical threshold (Table 2).

In multivariate analysis (binary logistic regression) adjusted on age, sex, BMI, "born after 1990" and "never stop diet" variables, low BMD remained significantly associated with "never stop diet" variable with a significant interaction between "born after 1990" and "never stop diet" variables: patients born after 1990 and who never stopped diet had a 4.7 times risk to have low BMD in this model ($p = 0.005$).

3.3. Comparison of patients who stopped PKU diet vs patients who never stopped

In the cohort, 54 patients never stopped their low natural protein diet (31.6 %). Among the 117 patients who stopped diet, the median age of diet interruption was 10 years-old (min-max, 2–32) and 55/117 patients (47 %) stopped definitively the diet. Compared to patients who ever stopped their diet, the patients who never stopped the diet were significantly younger and thinner, and more frequently born after 1990. They had 2.6 times more risk of reaching the low-BMD status (19.2 %). Their fracture rate was also significantly higher (46.3 % vs 29.1 %, $p = 0.0037$) (Table 3).

Regarding diet characteristics, there was no difference in caloric or total protein intakes between the two groups. In the group who ever stopped the diet, some patients could have restarted a low natural protein diet later. Nevertheless, these patients took less AA supplements (39.7 % vs 71.7 %, $p < 0.001$) and consumed significantly more natural proteins, than patients who never stopped the diet (Table 3).

Our analysis did not show any difference in all micronutrients' concentrations tested except for vitamin B12: patients who never stopped their diet were more likely to be deficient in vitamin B12

Table 3
Comparison of patients who stopped their PKU diet during childhood and patients who never stopped the diet.

	Stopped diet		Never stopped diet		p
	N	Median (min-max) or n (%)	N	Median (min-max) or n (%)	
Global characteristics					
Age at inclusion (years)	117	28.0 (18.1–45.0)	54	22.3 (18.1–43.1)	<0.001
Birth year > 1990	117	31 (2.6 %)	54	31 (5.7 %)	<0.001
Sex (F) %	117	70.1 %	54	64.8 %	0.293
BMI (kg/m^2)	117	24.5 (16,7–43.4)	54	22.5 (17.2–34.5)	0.006
Phenylalanine* (μmol/L)	115	1174.7 (43.1)	54	1073.8 (57.1)	0.175
Tyrosine (μmol/L)	115	40.0 (16.0–93.0)	54	33.5 (11.0–92.0)	<0.001
Phenylalanine/Tyrosine* (ratio)	115	30.1 (1.39)	54	34.5 (4.47)	0.092
Bone characteristics					
Femoral *Z*-score	97	−0.6 (−2.7–1.5)	52	−0.8 (−3.0–2.1)	0.422
Femoral Z-score < −2	97	4 (4.1 %)	52	4 (7.7 %)	0.451
Vertebral Z-score	97	−0.6 (−2.3–3.0)	53	−0.8 (−2.7–1.8)	0.152
Vertebral Z-score < −2	97	6 (6.2 %)	53	7 (13.3 %)	0.223
Femoral or Vertebral Z-score < −2	97	7 (7.2 %)	52	10 (19.2 %)	0.034
Fracture rate (≥ 1 fracture)	117	34 (29.1 %)	54	25 (46.3 %)	0.037
Diet characteristics					
Total Energy Intakes (kcal)	107	1917.5 (923.3–3665.5)	44	1934.4 (1939.4–3771.0)	0.889
Total protein intakes* (g/kg/day)	107	0.9 (0.04)	44	0.9 (0.05)	0.665
AA supplements	116	46 (39.7 %)	53	38 (71.7 %)	<0.001
Hypoprotidic food	117	25 (21.4 %)	54	18 (33.3 %)	0.128
Natural protein intakes (g/kg/day)	107	0.70 (0.1–1.63)	44	0.53 (0.16–1.44)	0.034
Protein from AA Supplements* (g/kg/day)	40	0.57 (0.05)	26	0.54 (0.06)	0.745
Protein intakes > 1 g/kg/day	107	45 (42.1 %)	44	18 (41.0 %)	1000
Calcium intakes (mg)	107	807.8 (236.1–2345.5)	44	893.9 (218.9–4640.4)	0.356
Calcium Phosphate metabolism data					
25 OH Vitamin D (nmol/L)	115	59.0 (10.0–144.0)	49	63.0 (19.0–150.0)	0.119
Vitamin D < 30 nmol/L	115	11 (9.6 %)	49	3 (6.1 %)	0.558
Vitamin D < 75 nmol/L	115	79 (68.9 %)	49	31 (63.2 %)	0.586
Calcemia* (mmol/L)	115	2.38 (0.01)	54	2.39 (0.01)	0.437
Phosphoremia* (mmol/L)	109	1.0 (0.02)	53	1.0 (0.03)	0.273
PTH (ng/L)	106	32.0 (10.0–125.1)	50	31.0 (6.0–84.0)	0.182
Micronutrients status					
Copper (μg/L)	115	1022.0 (530.0–2347.0)	52	925.0 (570.0–2090.0)	0.474
Copper < 880 μg/L	115	36 (31.3 %)	52	22 (42.3 %)	0.219
Zinc (μmol/L)	115	12,3 (4.2–17.4)		12.4 (7.5–23.3)	0.970
Zinc < 7.80 μmol/L	115	2 (1.7 %)	52	3 (5.8 %)	0.175
Selenium* (μg/L)	112	76.1 (1.51)	50	73.5 (2.76)	0,353
Selenium < 70 μg/L	112	35 (31.2 %)	50	23 (46.0 %)	0.078
Vitamin B9* (nmol/L)	117	26.7 (1.41)	47	25.9 (1.78)	0.737
Vitamin B9 < 5 nmol/L	117	0 (0 %)	47	1 (2.2 %)	0.287
Vitamin B12 (pmol/L)	114	290.5 (89.0–987.0)	50	237.5 (74.0–941 0.0)	0.014
Vitamin B12 < 140 pmol/L	114	3 (2.6 %)	50	8 (16.0 %)	0.004
Vitamin A (μmol/L)	114	2.1 (0.6–4.2)	47	2.2 (1.2–3.9)	0.245
Vitamin A < 1.40 μmol/L	114	5 (4.4 %)	47	3 (6.4 %)	0.693
Vitamin E (μmol/L)	18	25.0 (17.0–39.0)	7	28.0 (17.0–43.0)	0.458
Vitamin E < 20 μmol/L	18	2 (11.1 %)	7	1 (14.3 %)	1000

BMI, body mass index; F, female; N, number of patients studied.
Quantitative variables are expressed as median and (min-max) and categorical variables were expressed as number (n) and percentage (%). Fisher's exacts test were used for categorical variables. ANOVA was performed for normally distributed quantitative variables, Wilcoxon-Mann- Whitney test if not.
* Normally distributed variable, expressed as mean (SEM).

(Table 3).

In multivariate analysis (binary logistic regression) adjusted on age, sex, BMI, low BMD, "never stop diet" variable remained significantly associated with low BMD with a significant interaction between low BMD and age (OR 1.9; $p = 0.007$).

4. Discussion

The present work is one of the largest studies focusing on bone health in adults with early-treated PKU. To our knowledge, this is also the first multicenter cohort designed to prospectively study bone health along with a large collection of dietary and nutritional data. As previously described in the literature, most adults with PKU in our cohort have an overall normal median BMD. Indeed, median femoral and vertebral *Z*-scores were close to those previously reported in adult PKU patients [8,10]. Nevertheless, a small subgroup of patients had a low-BMD defined by Z-score ≤ -2.

If we compare our results with Lubout's study including 183 adults, the frequency of low BMD was not different in the vertebral site (8.7 % vs 5.5 % for Lubout, $p = 0.99$) but was significantly increased for the femoral site (5.4 % vs 1.6 % for Lubout, $p = 0.01$, compared by Fisher exact test). The population of Lubout's study was fairly similar in terms of age and BMI: the median age was 27 years old in our study vs 28 in Lubout's, whereas the median BMI was 24.0 kg/m^2 in our study vs 24.9 kg/m^2 in Lubout's [10]. Demirdas et al. reported low BMD frequency at 10 % in 2015 in a meta-analysis which included 360 child and adult patients [8]. The facture rate of 34.5 %, is also higher in our study than in Lubout's study (16.4 %) but remaining lower than in general English population (estimated at 38.2 %) [10].

Our study suggest for the first time the association between low BMD and prolonged low natural protein diet over time, specifically in younger adults who never stopped the PKU diet since childhood. Total protein intakes were significantly higher in low BMD group but associated with parameters reflecting a greater adherence to low protein diet. Indeed, in the low BMD group, patients followed more frequently a low protein diet and consumed more protein from AA supplements. Total protein intake in the low BMD group exceeded those of the normal BMD group probably due to the higher AA supplementation.

Confirmatively, patients who never stopped diet had a significantly

higher risk to have low BMD than patients who stopped diet (19.2 % vs 7.2 % respectively). These patients were also younger and more frequently born after 1990 in comparison to patients who stopped diet. These findings are consistent with the evolution of diet recommendations in PKU, which led to the concept of lifelong diet with lower Phe target in adults (< 600 μmol/L) since 2017 in Europe. and 2018 in France [2,14].

The formation of the peak bone mass at adolescence is critical to prevent bone fragility at adulthood. Low protein intake, as demonstrated in malnutrition, could be detrimental for skeletal growth by lowering the production of IGF-1 and by inducing a resistance to the anabolic action of IGF-1, which IGF-1 plays a key role in calcium-phosphate metabolism during growth [15]. We could speculate that PKU patients who never stopped the low natural protein diet would constitute a lower peak bone mass leading to bone fragility at adulthood, whereas patients who stopped their diet before puberty, with a median age of interruption at 10 years old in our study, would constitute rather a normal bone mass.

In the present study, we did not find any significant association between fractures and low BMD but patients who never stopped low protein diet had a significant higher fracture rate than patients who stopped diet. The association of low BMD with prolonged low-Phe diet in PKU, despite higher intakes of total protein and protein from AA supplements, suggests that the nature of protein intake (natural proteins vs free AA) may influence bone metabolism in PKU patients. Protein can be classified as “fast” and “slow” protein based on their AA absorption. Natural protein such as casein are considered as “slow” protein source, whereas free AA are considered as “fast” protein source. Free AA do not require digestion and are directly absorbed by the small intestine. Therefore, plasma AA levels rise quickly after ingestion but also fall faster compared to whole protein source intake such as casein [16]. Dangin et al. compared protein retention in healthy subjects after a meal of 30 g of casein vs 30 g of free AA. They showed, using leucine balance, that slow protein source (casein) leads to a better post prandial protein utilization of free AA [7]. Fast assimilation was also associated with higher oxidation rates [16]. Moreover, the timing and portion size of the AA supplement consumption may also play a role in protein metabolism. Mönch et al. compared AA blood levels and nitrogen excretion after consumption of the same amount of free AA in one or three doses spread over the day in PKU patients. They showed that urinary nitrogen excretion was greater with one compared to three portions of free AA, suggesting an increased catabolism in the first case [17]. Thus, several arguments suggest that free AA consumption leads to higher oxidation rates, lower protein retention, and less bioavailability for protein synthesis than natural protein, which could influence bone protein framework. Therefore, current guidelines recommend increasing protein requirements of individuals with PKU by 20–40 % compared the general population, notably by increasing free AA supplements intake [2]. To date, few data support the efficacy of such recommendations to compensate low bio-availability of free AA [18]. It is also hypothesized that free AA supplements are responsible of a metabolic acidosis, buffered by the excretion of bone calcium, and could decrease bone mineralization [19,20]. In our study, the higher total protein intake (+ 19 %) in the low BMD group compared to normal BMD group seemed insufficient to maintain bone quality. Nevertheless, no data are available to check the sustainability of such increased protein intake over time.

The comparison between low BMD with normal BMD patients also showed an association with lower BMI as in Lubout’s study [10]. Low BMI is a classical bone risk factor identified in healthy young adults, women being the most studied population [21].

Regarding micronutrients, we highlighted in our study that the risk of low BMD was not correlated to calcium intake nor to vitamin D deficiency at the time of the study. However, we showed a trend for lower selenium concentrations in low-BMD PKU patients. Selenium status was positively associated with BMD in European elderly healthy men [22]. Experimental data show that growth retardation induced by selenium deficiency is associated with impaired bone metabolism and osteopenia in second-generation selenium-deficient rats [23]. Data are poor about selenium supplementation and bone health in humans. A large double-blinded, placebo-controlled trial on post-menopausal women did not show any difference on terminal cross-linking telopeptide of type I collagen (CTX-1) after 26 weeks of selenium supplementation [24], CTX-1 being a bone resorption biomarker recommended by the International Osteoporosis Foundation (IOF) and International Federation of Clinical Chemistry (IFCC) for use in fracture risk prediction and monitoring of osteoporosis [25].

Moreover, patients who never stopped their diet, who had more frequently a low BMD, were more prone to vitamin B12 deficiency. The Framingham Osteoporosis study found a positive correlation between vitamin B12 concentrations and hip BMD in men, vertebral BMD in women [26]. However, most longitudinal studies found no association between vitamin B12 and BMD, and were conducted on older patients than in our cohort [27].

Even if no clear association with micronutrients concentrations has been shown in our study and literature, we cannot exclude a combined effect of several micronutrients insufficiencies/deficiencies on bone health on the long term.

Our study has strengths and limitations. Our cohort is one of the largest studies on bone health in young adults with PKU, and the first with a prospective design and such a large collection of dietary and nutritional data. One of the limitations is that collected data did not include some established osteoporosis risk factors such as smoking status, alcohol consumption and patients’ level of physical activity. Declarative nutritional data can also be impaired by a bias of social approval. We can note exclude a misclassification bias for the PKU groups (classical vs non classical) that could mask a difference on BMD between the two groups. As phenylalanine was only measured once at inclusion without retrospective data, it was not possible to analyze the potential link between current and past metabolic balance and bone density in this study. The compliance to the diet and the protein intakes during the past could not be analyzed and can limit the conclusion between past diet and low BMD. We also had no information on the mechanism of the fractures or their temporality, which could help to distinguish traumatic vs osteoporotic fractures. Finally, we had a lack of statistical power in the low-BMD group due to the small number of patients.

5. Conclusions

Our study identified a subgroup of PKU adult patients with low BMD and showed that prolonged low natural protein diet, recommended to prevent neuro-psychological complications of PKU, was associated with low BMD. The nature of protein intakes could be involved in bone fragility in PKU patients due to low bioavailability of free AA as compared to natural proteins. The 5 years longitudinal follow-up of the ECOPHEN cohort with repeated BMD measurement and biological data will investigate this hypothesis.

Our work emphasizes the need of regular BMD follow up and monitoring of dietary intakes and micronutrients supplementations, mainly in adults who never stopped their diet.

Further studies, including lifelong data collected in large registries, are needed to better understand the mechanism of low BMD in PKU patients and more generally in low natural protein diets used in the treatment of inborn metabolic diseases.

Ethical statements

ECOPHEN cohort ClinicalTrials.gov Identifier: NCT01619722

The study followed the ethical standards of French committee on human experimentation, and the Helsinki Declaration of 1975, as revised in 2013. The approval was obtained from the relevant committee on human subjects. Informed written consent was obtained from all

patients.

Legal authorizations of the study were obtained in 2011 (CPP Ouest-I Tours n°2011-R11, 24/05/2011; ANSM n°B110423–80, 26/04/2011).

Data statement

The data presented in this study are available upon reasonable request from the corresponding author.

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CRediT authorship contribution statement

Elisa Dybal: Writing – original draft, Visualization, Formal analysis, Data curation. **François Maillot:** Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **François Feillet:** Writing – review & editing, Resources, Investigation. **Alain Fouilhoux:** Writing – review & editing, Resources, Investigation. **Leonardo Astudillo:** Writing – review & editing, Resources, Investigation. **Christian Lavigne:** Writing – review & editing, Resources, Investigation. **Jean-Baptiste Arnoux:** Writing – review & editing, Resources, Investigation. **Sylvie Odent:** Writing – review & editing, Resources, Investigation. **Claire Gay:** Writing – review & editing, Resources, Investigation. **Manuel Schiff:** Writing – review & editing, Resources, Investigation. **Karin Mazodier:** Writing – review & editing, Resources, Investigation. **Alice Kuster:** Writing – review & editing, Resources, Investigation. **Vincent Rigalleau:** Writing – review & editing, Resources, Investigation. **Christel Thauvin-Robinet:** Writing – review & editing, Resources, Investigation. **Vanessa Leguy-Seguin:** Writing – review & editing, Resources, Investigation. **Claire Douillard:** Writing – review & editing, Resources, Investigation. **Sybil Charrière:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors report no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgme.2025.109044.

Data availability

Data will be made available on request.

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