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ORIGINAL ARTICLE

Cross-sectional study of bone metabolism with nutrition in adult classical phenylketonuric patients diagnosed by neonatal screening

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Abstract The mechanism underlying the development of osteopenia or osteoporosis in longstanding phenylketonuria (PKU) remains to be clarified. We investigated the details of bone metabolism in 21 female and 13 male classical PKU patients aged 20–35 years. Vitamin D (VD), parathyroid hormone (PTH), bone turnover markers, and daily nutrient intake were examined. The patients had lower daily energy and protein intake than did the age-matched controls (22 women, 14 men), but their respective fat, VD, and calcium intake did not differ. Serum 1,25-dihydroxy VD and 25-hydroxy VD levels in female and male patient groups were significantly higher and lower than those in respective control groups (females, $P < 0.001$; males, $P < 0.05$ and $P < 0.01$, respectively). Serum intact PTH levels were significantly higher in the female patient group ($P < 0.05$). Urinary calcium levels in the patient groups were significantly higher than those of the control subjects (females, $P < 0.001$; males, $P < 0.05$). Bone resorption markers were significantly higher in patients than in controls, although bone formation markers were not different. Patient serum levels of osteoprotegerin-inhibiting bone resorption were significantly lower (females, $P < 0.001$;

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males, $P < 0.01$). None of the bone parameters correlated significantly with serum phenylalanine or nutrient intake. PKU patients exhibited lower VD status and more rapid bone resorption despite normal calcium–VD intakes.

Keywords Classical phenylketonuria · Osteopenia · Adult · Vitamin D · Bone resorption

Introduction

Phenylketonuria (PKU) is caused by a deficiency of phenylalanine hydroxylase. This disorder is transmitted in a manner of autosomal recessive inheritance [1]. Neurological impairment and mental retardation, as consequences of inappropriately high plasma phenylalanine (Phe) levels, are often severe problems in affected subjects [1–4]. Therefore, PKU children receive phenylalanine-restricted diets almost exclusively to maintain appropriate plasma Phe levels. Nevertheless, high risk for osteopenia or osteoporosis has been shown in long-standing PKU [5–11]. Details of bone metabolism in PKU remain to be clarified, but abnormalities in bone turnover have been reported [6, 9–11]. Recently, Modan-Modes et al. [12] reported that the peak bone mass is lower in adult PKU patients who adhere to phenylalanine-restricted diets, which are rich in energy, protein, and calcium, than in patients not adhering to such a diet [12]. Sufficient knowledge related to this subject is necessary for handling bone diseases in PKU.

This study aimed to obtain fundamental data for establishing an optimal treatment strategy for bone disease in PKU. We investigated the bone metabolism, including vitamin D (VD), parathyroid hormone (PTH), bone turnover markers, and nutritional status, of adult classical PKU patients lacking phenylalanine hydroxylase activity.

Materials and methods

Subjects

We enrolled 34 classical PKU patients (21 women, 13 men) who were 20 to 35 years old. Mass screening at around 5 days of age had revealed hyperphenylalaninemia of more than 20 mg/dl in these patients. Until the age of 15 years, after the diagnosis of classical PKU, they had received phenylalanine-restricted diets with phenylalanine-free milk. Thereafter, the restrictions of phenylalanine varied among the patients. Some patients were almost free of diet restrictions (9 women, 4 men). Others received mildly restricted or less restricted diets. Results show that their serum Phe levels were 10–33 mg/dl (mean ± SD, 22.3 ± 4.5 mg/dl). The patients showed no overt developmental or psychomotor disturbance. None had a history of immobilization or disease-limiting movement.

As controls, we enrolled 36 healthy volunteers (22 women, 14 men) who were 19 to 40 years old. No significant difference was found in age, body mass index, liver function test, or sex hormone levels between female PKU patients and the female controls or between male PKU patients and the male controls (Table 1).

Study design

Quantities of daily energy, protein, fat, calcium, and vitamin D intake were calculated based on a detailed 3-day dietary history. Affected patients or their parents were required to record the amounts of food and drink consumed during the 3 days preceding the clinic visit.

We measured blood levels of intact PTH, 25-hydroxy VD, and 1,25-dihydroxy VD. To evaluate bone remodeling and turnover, we measured the blood and urinary markers for bone formation and resorption [13–15]. Blood bone alkaline phosphatase (BAP) and osteocalcin (OCN) were measured as bone formation markers. As bone resorption markers, the blood pyridinoline cross-linked telopeptide domain of type I collagen (ICTP), urinary deoxypyridinoline (D-Pyr), and urinary *N*-telopeptide of type I collagen (NTx) were measured. Furthermore, blood osteoprotegerin (OPG) was measured because OPG is a major inhibitor of bone resorption [16]. To evaluate calcium (Ca) and phosphorus (P) excretion in urine, urinary Ca/creatinine (Cr) ratio and P/Cr were determined.

Venous blood samples were collected after overnight fasting. They were centrifuged and stored at −80°C until analyses. Urine samples were collected at 9:00–10:00 a.m. and frozen until analyses. This study protocol was approved by the relevant institutional review boards. All patients provided written informed consent before enrollment in the study.

Assays

Serum intact PTH level was determined using a radioimmunoassay (RIA) kit from the Nichols Institute (Quest Diagnostics, Geneva, Switzerland). Serum 1,25-hydroxy VD and 25-hydroxy VD levels were determined using RIA kits from Immunodiagnostic Systems Holdings plc (Boldon, UK) and Diasorin, Inc. (Stillwater, MN, USA), respectively,. Serum OCN levels were determined using an RIA kit (BGP-IRMA; Mitsubishi Kagaku Iatron, Tokyo, Japan). Serum BAP levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Osteo-Links-BAP; Quidel, San Diego, CA, USA). The serum ICTP level was measured using an RIA kit from Orion Diagnostica (Espoo, Finland). Urinary D-Pyr and NTx

were determined using ELISA kits from Quidel Corp. and Inverness Medical Innovation (Chiba, Japan), respectively. An ELISA kit from Biomedia (Vienna, Austria) was used to determine the serum OPG level. Serum estradiol and testosterone levels were determined using enhanced chemiluminescence immunoassay Estradiol-kit and Testosterone-kit (Biocheck, Foster City, CA, USA).

Urinary Ca, P, and Cr levels were determined using routine autoanalyzer methods.

Statistical analyses

Data are presented as mean ± SE. Differences between values of patients and those of controls were estimated using Student's *t* test. The relationship between each pair of parameters was estimated using Pearson's correlation test. All *P* values less than 0.05 were considered statistically significant.

Results

Daily energy and nutrient intakes

Average daily energy and protein intakes in female and male patient groups were significantly lower than those in the respective control groups ($P < 0.01$ and $P < 0.05$, respectively). Average daily fat, Ca, and VD intakes in the patient groups were not different from those in respective control groups (see Table 1). Serum levels of total protein, albumin, and lipids, as well as liver function tests, also did not differ between patient and control groups.

Bone parameters

Intact PTH levels in the 21 female PKU patients were significantly higher than those in the female controls ($P < 0.05$). Their 1,25-dihydroxy VD and 25-hydroxy VD levels were significantly higher and lower, respectively, than those in the female controls ($P < 0.001$) (Table 2).

Regarding serum bone formation markers, no significant differences in serum BAP and OCN levels were found between these two female groups. In contrast, bone resorption markers were significantly higher in female patients ($P < 0.001$). Serum ICTP and urinary D-Pyr and NTx levels in the female PKU patients were significantly higher than those in the female controls ($P < 0.001$). Serum OPG levels in female patients were significantly lower than those in the female controls ($P < 0.001$).Their urinary Ca excretion was significantly higher than that of the female controls ($P < 0.001$), but P excretion was similar to the control level (Table 2).

The 13 male PKU patients showed intact PTH levels similar to those of the male controls. Their 1,25-dihydroxy VD levels were significantly higher than those of the male controls ($P < 0.05$); the 25-hydroxy VD levels were significantly lower than those of the control subjects ($P < 0.01$) (Table 2).

No significant differences were found in serum BAP and OC levels between the male patients and controls. In contrast, all bone resorption markers were significantly higher than those of the respective control subjects ($P < 0.01$). Serum OPG levels in male patients were significantly lower than those in the male controls ($P < 0.01$). Their urinary Ca excretion was significantly higher than that of control subjects ($P < 0.05$), but P excretion was similar to that of control subjects (see Table 2).

Correlations between serum phenylalanine level or daily nutrient intake and bone parameters and those among parameters

In both female and male PKU patient groups, the serum Phe level showed no significant correlation with any bone parameter (Table 3). Moreover, average daily nutrient and energy intakes showed no significant correlation with these parameters (data not shown).

In the female PKU group, significant correlation was found between two bone resorption markers ($P < 0.01$). Particularly, the correlation between D-Pyr and ITCP was strongest ($r = 0.841$, $P < 0.001$). Here, BAP showed significant correlations with all bone resorption markers ($P < 0.05$ or $P < 0.01$), although OCN was correlated significantly with only one resorption marker, NTx ($P < 0.01$). Urinary Ca excretion was correlated significantly with intact PTH and 1,25-dihydroxy VD levels (Table 3). In contrast, urinary P excretion was not correlated with any other parameter (data not shown).

The male PKU group also showed a significant correlation for each of the two bone resorption markers ($P < 0.01$, $P < 0.001$). Particularly, the correlation between D-Pyr and ITCP was strongest ($r = 0.957$, $P < 0.001$). Actually, BAP, but not OCN, showed significant correlation with D-Pyr and NTx levels (D-Pyr, $P < 0.05$; NTx, $P < 0.01$). The urinary Ca and P excretions were not correlated significantly with any other parameter in this group.

Discussion

Evidence is accumulating that PKU patients are at risk for osteopenia and bone fractures [4–9, 12, 13]. Bone marker abnormalities have been described in recent reports [6, 9–11]. Particularly, increases in bone resorption marker levels

Table 1 Characteristics of phenylketonuric patients and healthy age-matched controls

	Female patients ($n = 21$)	Female controls ($n = 22$)	Male patients ($n = 13$)	Male controls ($n = 14$)
Age (years)	27.1 ± 3.2	27.9 ± 5.1	26.9 ± 3.3	30.3 ± 4.5
Body mass index (kg/m^2)	22.5 ± 1.5	23.7 ± 2.2	24.7 ± 2.4	23.5 ± 2.3
Daily energy intake (kcal/day)	1601 ± 275**	2052 ± 243	2015 ± 215*	2452 ± 243
Daily protein intake (g/day)	66 ± 15**	79 ± 10	72 ± 17*	88 ± 11
Daily natural protein intake (g/day)	40 ± 11***	79 ± 10	47 ± 10***	88 ± 11
Daily fat intake (g/day)	55 ± 21	52 ± 16	59 ± 17	56 ± 16
Daily Ca intake (mg/day)	1212 ± 385	1130 ± 375	1122 ± 386	1055 ± 333
Daily vitamin D intake (IU/day)	127 ± 26	112 ± 23	112 ± 23	112 ± 21
Total protein (g/dl)	7.2 ± 0.3	7.5 ± 0.3	7.3 ± 0.3	7.6 ± 0.3
Albumin (g/dl)	4.3 ± 0.2	4.6 ± 0.2	4.4 ± 0.2	4.7 ± 0.2
Alanine aminotransferase (IU/l)	9 ± 2	11 ± 3	10 ± 2	12 ± 3
Aspartate aminotransferase (IU/l)	18 ± 3	18 ± 4	18 ± 2	18 ± 3
Total cholesterol (mg/dl)	150 ± 13	163 ± 14	154 ± 13	173 ± 17
Triglycerides (mg/dl)	81 ± 20	82 ± 23	85 ± 21	86 ± 20
Low-density lipoprotein cholesterol (mg/dl)	81 ± 8	88 ± 10	81 ± 8	88 ± 10
High-density lipoprotein cholesterol (mg/dl)	50 ± 3	53 ± 4	54 ± 4	56 ± 4
Estradiol (pg/ml)	59 ± 16	55 ± 18	37 ± 6	40 ± 5
Testosterone (ng/dl)	33 ± 12	31 ± 15	555 ± 61	578 ± 77

Data are presented as mean ± SE

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus controls

have been documented in PKU patients [6, 9, 11]. Nevertheless, bone metabolism in PKU has not been studied sufficiently. Accordingly, clinical management to prevent development and progression of bone disease in PKU patients remains to be established.

This study found increased bone resorption markers together with increased urinary Ca excretion in adult PKU patients. However, their blood levels of bone formation markers were similar to those of control subjects. Serum ICTP level, and urinary D-Pyr and NTx levels in patient groups were 1.5 times higher than the respective control levels without increases in the bone formation markers. Such discrepancies between bone resorption and formation marker levels, together with increased urinary Ca, strongly support the inference that PKU patients are at high risk for osteopenia or osteoporosis in later life.

In preliminary evaluations, we measured the lumbar spine bone mineral density (BMD) at L1–L4 levels using dual-energy X-ray absorptiometry (QDR-4500; Hologic, Waltham, MA, USA) in 10 of the 21 female patients and 7 of the 13 male patients. The BMD values were, respectively, 0.823–0.993 g/cm^2 (mean ± SD, 0.856 ± 0.041 g/cm^2) and 0.936–1.152 g/cm^2 (1.042 ± 0.064 g/cm^2). The values were 81–97% (91% ± 3%) and 90–101% (94% ± 2%) of the mean of age-matched and sex-matched healthy controls. The SD scores were −1.7 to −0.1 (−0.7 ± 0.3) and −1.3 to +0.2 (−0.4 ± 0.2), respectively. The patients tended to have lower BMD at the lumbar spine. The results were consistent with those described in other reports [5–8, 11, 12].

In our PKU patients, serum 25-hydroxy VD levels were decreased, although serum 1,25-dihydroxy VD levels were increased. We inferred that the 1,25-hydroxy VD level was increased compensatively via an action of the PTH to activate 1α-hydroxylase [17, 18]. A significant increase in serum iPTH levels was found only in female patients, not in male patients.

We were unable to detect differences in VD and Ca intake between PKU patients and controls. The control serum Phe level, which is determined mainly by the intake, was also not correlated with any bone metabolism parameter in the patients. It would therefore be impossible to explain the lower VD status according to Ca, VD, or Phe intake. The underlying mechanism for their lower vitamin D status remains unclear.

Modan-Modes et al. [12] reported that the peak bone mass is decreased in adult PKU patients who adhere to phenylalanine-restricted diets, which are rich in VD, protein, and minerals involving Ca, compared to those not adhering to such diets. Results of this study also support the contention that sufficient nutrient intake might not necessarily result in normal mineral accumulation in bones of PKU patients.

Bone remodeling is regulated by cross-talk reactions between osteoblasts and osteoclasts [19, 20]. Earlier reports

Table 2 Bone markers of control and patient groups

	Female patients ($n = 21$)	Female controls ($n = 22$)	Male patients ($n = 13$)	Male controls ($n = 14$)
Intact PTH (pg/ml)	37.5 ± 2.4*	32.3 ± 3.5	36.5 ± 3.8	32.7 ± 3.7
1,25 $(OH)_2$ vitamin D (pg/ml)	58.4 ± 2.7***	41.6 ± 3.1	50.6 ± 2.0*	39.9 ± 2.7
25 (OH) vitamin D (ng/ml)	18.7 ± 1.3***	27.6 ± 2.1	22.2 ± 1.7**	30.0 ± 2.6
Bone alkaline phosphatase (U/l)	22.7 ± 2.2	21.7 ± 2.5	28.5 ± 2.7	25.4 ± 2.7
Osteocalcin (ng/ml)	5.6 ± 0.7	5.9 ± 0.5	5.4 ± 1.0	5.5 ± 0.6
U-D-Pyr (nmol/mmol creatinine)	7.3 ± 0.5***	4.9 ± 0.4	5.2 ± 0.5**	3.8 ± 0.6
U-NTx (nmol/mmol creatinine)	47.8 ± 6.1***	31.7 ± 5.1	54.7 ± 12.1**	38.3 ± 10.5
ICTP (ng/ml)	4.6 ± 0.2***	3.0 ± 0.2	4.3 ± 0.3**	3.0 ± 0.2
Osteoprotegerin (pmol/l)	3.3 ± 0.3***	4.7 ± 0.4	3.1 ± 0.2**	4.3 ± 0.2
U-Ca/Cr (mmol/mmol creatinine)	0.46 ± 0.08***	0.33 ± 0.08	0.42 ± 0.10*	0.34 ± 0.07
U-P/Cr (mmol/mmol creatinine)	1.33 ± 0.23	1.23 ± 0.28	1.41 ± 0.22	1.35 ± 0.26

Data are presented as mean ± SE

Intact PTH, intact parathyroid hormone; U-D-Pyr, urinary deoxypyridinoline; U-NTx, urinary *N*-telopeptides of type collagen; ICTP, pyridinoline cross-linked telopeptide domain of type I collagen

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus controls

Table 3 Correlation among bone parameters, including vitamin D and PTH, in 21 female patients (bold letters) and 13 male patients (plain letters)

	Phe	BAP	OCN	Intact PTH	1,25 $(OH)_2$ VD	25 (OH) VD	U-Ca/Cr	U-P/Cr	U-D-Pyr	U-NTx	ICTP
Phe	/	−0.068	−0.225	0.326	0.391	−0.262	0.227	0.131	−0.127	−0.200	0.111
BAP	0.240	/	0.331	0.155	0.303	0.138	0.332	0.296	0.612**	0.607**	0.511*
OCN	−00.502	0.210	/	0.244	0.239	0.217	0.414*	0.319	0.309	0.634**	0.374
Intact PTH	00.326	−0.166	−0.534	/	0.333	−0.090	0.443*	−0.178	0.329	−0.007	0.167
1,25 $(OH)_2$ VD	0.564	0.275	−0.138	−0.152	/	0.250	0.528**	−0.111	0.252	0.187	0.269
25 (OH) VD	−0.565	−0.612*	−0.047	0.231	−0.392	/	0.308	−0.091	−0.054	0.091	−0.145
U-Ca/Cr	0.237	0.289	0.195	−0.196	0.506	−0.184	/	−0.279	0.206	0.164	0.102
U-P/Cr	−0.299	0.105	0.007	−0.157	−0.399	0.166		/	0.100	0.233	0.149
U-D-Pyr	−0.089	0.653*	0.537	−0.652*	0.260	−0.471	0.351	0.277	/	0.679**	0.841***
U-NTx	−0.228	0.743**	0.381	−0.619*	0.157	−0.519	0.250	0.312	0.826**	/	0.650**
ICTP	0.082	0.729*	0.459	−0.469	0.350	−0.621*	0.395	0.333	0.957***	0.795**	/

Presented data are *r* values

Phe, phenylalanine; BAP, bone alkaline phosphatase; OCN, osteocalcin; Intact PTH, intact parathyroid hormone; D-Pyr, urinary deoxypyridinoline; U-NTx, urinary N-telopeptides of type I collagen; ICTP, pyridinoline cross-linked telopeptide domain of type I collagen

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

have described that many biological substances participate in cross-talk reactions [19–22]. Particularly, an effect of OPG inhibiting the signal transduction between osteoblasts and osteoclasts, leading to suppressed bone resorption, has been documented [16, 22]. Serum OPG levels in the PKU patients were significantly lower than those in healthy controls. Accordingly, the predominance of bone resorption over bone formation in our patients was explained, at least in part, by the lowered OPG levels.

Yannicelli et al. [23] reported that phenylalanine per se affected the bone mineral status in animal models. In our patients, the serum phenylalanine level showed no significant correlation with any bone marker level. It therefore appears unlikely that serum phenylalanine level represents a major determinant factor of bone status in PKU patients.

The bone metabolism in our patients might be attributable to their dietary pattern and cholesterol metabolism. For many patients, the natural protein content was assumed to be lowered, in various degrees, in the diets. They ingested low-phenylalanine proteins and specialized milk, in which some proportion of natural amino acids was replaced by phenylalanine-free amino acids. It has been

suggested that cholesterol production is often reduced in PKU [24, 25]. In fact, the serum cholesterol level was not increased, even in our patients taking plenty of lipids. Considering that vitamin D production is linked to cholesterol production, the lowered vitamin D status in our patients is likely to be associated with such limited cholesterol production. Future studies will be undertaken to investigate the bone metabolism of PKU in terms of dietary patterns and cholesterol metabolism.

The contribution of oxidative stress to bone diseases has been demonstrated [21, 22]. Enhancement of oxidative stress in PKU has been reported recently [26–28]. Therefore, it seems plausible that the promoted bone resorption in PKU is also, in part, attributable to the enhanced oxidative stress. This issue must be addressed in future studies.

Overall, the data gained from this study suggest a decreased vitamin D status and the predominance of bone resorption over bone formation in adult classical PKU patients. These findings appeared to be more prominent for female patients than for male patients. First, we expected that estrogen-inhibiting OPG production was deeply associated with osteopenia in the female group. However, sex hormone levels did not differ between this group and the control group. Many factors other than sex hormones, such as exercise, lifestyle, and dietary patterns, might influence bone metabolism in PKU patients, particularly female patients [29–31].

Additional investigations must be undertaken to detect the determinant factors of bone metabolism status in PKU.

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