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Assessment of bone status and bone turnover in pediatric patients with familiar hypomagnesemia with hypercalciuria and nephrocalcinosis

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Familiar hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC) is a rare monogenic tubulopathy. Although some of its features are potentially harmful for skeletal homeostasis, this problem has not been systematically evaluated so far. To evaluate bone mineral density (BMD) in correlation with selected mineral parameters and bone turnover markers (BTMs) to determine the risk of bone mass loss in pediatric patients with FHHNC. The study comprised 28 FHHNC patients aged 4–18 years and 33 healthy, sex - and age matched controls. 6 FHHNC patients showed normal kidney function whereas the remaining 22 presented with CKD grade II- III (median eGFR 73 ml/min/1.73m^2). In both groups, serum levels of calcium (sCa), phosphate (sP), magnesium (sMg), 25(OH)D_3, 1.25 $(OH)_2D_3$, parathormone (PTH) and selected BTMs [BAP, OC, PINP, CTX-I, OPG, SCL, FGF23 and soluble Klotho protein (sKL)] as well as 24-hour urinary calcium excretion (24 h-uCa) were assessed. In addition, BMD of the lumbar spine by DXA method was evaluated. 3 (10.7%) of FHHNC patients showed low BMD (Z-*score* < -2). Although median Z-*score* was lower in FHHNC group in comparison to controls, the difference was not significant. FHHNC patients had significantly higher median PTH, 1.25$(OH)_2D_3$ and 24 h-uCa values as well as lower sMg. Of the BTMs, they had significantly higher FGF23 and CTX-I levels. CTX-I correlated positively with PTH, FGF23 and SCL but negatively with sMg. Moreover, FGF23 and PTH correlated negatively with sKL. Negative correlation between PTH and sMg was noticed. No significant correlations between measured BTMs and eGFR, sCa, sP, 25$(OH)D_3$, 1.25 $(OH)_2D_3$ as well as 24 h uCa were found. None of BTMs significantly correlated with BMD. The results show that pediatric FHHNC patients, regardless of CKD may be at risk for increased bone resorption. Although its pathomechanism is complex, the trigger seems to be Mg depletion, aggravating secondary hyperparathyroidism and leading to the activation of osteolytic processes. However, their clinical significance is unknown, since only minority of patients show osteopenia. Therefore, follow-up of BMD and bone- related laboratory parameters including CTX-I seem to be essential in patients' monitoring, especially in adults with FHHNC.

Keywords FHHNC, Bone mineral density, Bone turnover markers

Abbreviations

1,25$(OH)_2D_3$	Calcitriol
24h-uCa	24-h urine calcium excretion
25$(OH)D_3$	Calcifediol
BAP	Bone specific alkaline phosphatase
BMD	Bone mineral density
BTMs	Bone turnover markers
Ca	Calcium
CKD	Chronic kidney disease
CTX-I	C-terminal telopeptide of type I collagen

DXA	Dual-energy X-ray absorptiometry
eGFR	Estimated glomerular filtration rate
FGF23	Fibroblast growth factor 23
FHHNC	Familiar hypomagnesemia with hypercalciuria and nephrocalcinosis
HC	Hypercalciuria
HTZ	Hydrochlorothiazide
IHC	Idiopathic hipercalciuria
ISCD	International Society for Clinical Densitometry
Mg	Magnesium
NC	Nephrocalcinosis
OC	Osteocalcin
OPG	Osteoprotegerin
PINP	N-terminal propeptide of type I procollagen
POLtube	Polish Registry of Inherited Tubulopathies
PTH	Parathyroid hormone
sCa	Serum level of calcium
SCL	Sclerostin
sKL	Soluble Klotho protein
sMg	Serum magnesium level
sP	Serum level of phosphate
SPH	Secondary hyperparathyroidism
UL	Urolithiasis

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Familiar hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC) is a rare autosomal recessive renal tubular disease caused by mutations in the *CLDN16* (OMIM # 248250; FHHNC type 1) or *CLDN19* (OMIM # 248250; FHHNC type 2) genes which encode the tight junction proteins, claudin-16 and – 19, respectively[1]. As they are involved in the paracellular reabsorption of magnesium (Mg) and calcium (Ca) in the thick ascending limb of Henle's loop, the primary defect leads to the renal loss of both cations[2]. As a consequence, patients develop hypomagnesemia, medullary nephrocalcinosis (NC) and progressive chronic kidney disease (CKD). In FHHNC type 2, a coexistence of ocular abnormalities is also observed[1,3]. The exact incidence of FHHNC is not known but its prevalence is estimated < 1/1,000,000 individuals[1]. Of note, according to the Polish Registry of Inherited Tubulopathies (POLtube), FHHNC type 1 is the second common monogenic tubulopathy diagnosed in Poland and more than 40 patients, children and adults have been registered so far (unpublished data).

Hypercalciuria (HC) and secondary hyperparathyroidism (SPH) are the hallmarks of FHHNC[3–6]. Although these features suggest a disturbed calcium homeostasis, to the best of our knowledge, their impact on bone mineralization in the course of FHHNC has never been evaluated. Therefore, in this cross-sectional study we aimed to evaluate bone mineral density (BMD) in correlation with selected mineral parameters and bone turnover markers (BTMs) to determine the risk of bone mass loss in pediatric patients with this disease.

Materials and methods

Twenty-eight patients (15 males, 13 females) from 22 families aged 4–18 years (median 10.0 years) with FHHNC type 1 confirmed by molecular analysis and registered in the POLtube registry were enrolled. At diagnosis, all of them presented with typical clinical symptoms of FHHNC, including hypomagnesemia (serum Mg < 0.7 mmol/l), hypercalciuria (24 h-urinary Ca > 4 mg (0.1 mmol)/kg) and NC stage 2b or 3 according to *Hoyer's* ultrasound grading system[7]. Since diagnosis, all patients received oral magnesium supplementation (10–20 mg Mg^{2+}/kg/24 h). The majority of them were treated with hydrochlorothiazide (HTZ) (0.5–1.5 mg/kg) to reduce calciuria and those with severe SPH received 1-hydroxycholecalciferol at a dose of 0.25–0.5 µg. 6/28 (21.4%) patients showed normal kidney function (eGFR > 90 ml/min/1.73m^2) whereas the remaining 22 (78.6%) presented with CKD grade II- III (median eGFR 73; range 37–88 ml/min/1.73m^2).

Thirty-three (15 males, 18 females) sex - and age (6–16 years; median 8.0 years) matched apparently healthy children and adolescents evaluated in our center for minor, non- organic bladder dysfunctions served as controls. The baseline characteristics of FHHNC patients and controls are shown in Table 1.

In all FHHNC patients and controls dual - energy X-ray absorptiometry (DXA) of the lumbar spine (L2-L4) using HOLOGIC Wi Bone Densitometer (Bedford, USA) was performed. In 17 FHHNC patients and controls total body less head (TBLH) DXA was also assessed.

	FHHNC ($n=28$)	Controls ($n=33$)
Boys/girls (n)	15/13	15/18
Age in years; median (range)	10 (4–18)	8 (6–16)
eGFR (ml/min/1.73m^2); median (range)	76 (37–107)	153 (107–177)
eGFR < 90 ml/min/1.73m2 (n)	22/28	0
Short stature (n)	2/28	0
Nephrocalcinosis in stage 2b/3 (n)	28/28	0
Type of mutation		
Homozygous Leu151Phe	13	-
Compound heterozygous Ser110Arg-Trp234Cys	4	
Compound heterozygous Leu145Pro-Leu151Phe	4	
Homozygous Ser205Phe	2	
Compound heterozygous Ser120Arg-Tyr288Stop	1	
Compound heterozygous Trp234Cys-Leu151Phe	2	
Compound heterozygous Gly239Arg-Gly245Asp	1	
Compound heterozygous Leu151Phe- Ser110Arg	1	

Table 1. Baseline characteristic of patients with FHHNC and controls.

According to recommendations of International Society for Clinical Densitometry (ISCD), low BMD was defined when Z-*score* BMD was lower or equal to -2.0 standard deviation for age and sex and osteoporosis if clinically significant fracture history was additionally present[8,9]. In all participants selected parameters of mineral metabolism and BMTs were assessed. They comprised fasting serum levels of calcium (sCa), phosphate (sP), magnesium (sMg), calcifediol (25 (OH)D_3), calcitriol (1,25 $(OH)_2D_3$), bone specific alkaline phosphatase (BAP) as well as plasma parathyroid hormone (PTH), osteocalcin (OC), N-terminal propeptide of type I procollagen (PINP), C-terminal telopeptide of type I collagen (CTX-I), osteoprotegerin (OPG), sclerostin (SCL), fibroblast growth factor 23 (FGF23) and soluble Klotho protein (sKL) levels. 24-h urine collections were performed to assess calcium excretion (24 h-uCa). In addition, serum creatinine concentration was measured and estimated glomerular filtration rate (eGFR) was calculated.

Plasma concentrations of BMTs were measured using specific enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's instructions: OC (Immunodiagnostic Systems, Great Britain), PINP (Cloud-Clone Corp., USA), CTX-I (Immunodiagnostic Systems, Great Britain), OPG (BioVendor, Czech Republic), SCL (TECO medical Group, Switzerland), FGF23 (Immunotopics, Inc., USA) and sKL (IBL International, USA). The remaining biochemical parameters were assessed by certified laboratory techniques in our hospital laboratory. In children and adolescents, eGFR was calculated by the *Schwartz*- formula[10].

The statistical analysis was performed using STATISTICA 13.5 software (StatSoft, Polska). As the majority of results were not normally distributed (verify by Shapiro-Wilk test), the specific differences and correlations were tested using non-parametric *Mann-Whitney*- and *Spearman* tests, respectively. P values ≤ 0.05 were considered significant.

Ethic approval and consent to participate

All methods were performed in accordance with the relevant guidelines and regulations.

The study was approved by Ethics Committee of the Medical University of Lublin (KE-0254/74/2017). Informed consent was obtained from all pediatric participants' legal representatives and participants > 16 years of age.

Results

None of the FHHNC patients fulfilled the criteria of osteoporosis and 3 (10.7%) of them showed low BMD of the lumbar spine. Although median value of BMD Z-*score* of the L2-L4 was lower in FHHNC group in comparison to controls [-0.25 (-2.6–0.8) vs. 0.1 (-1.9-0.9), respectively], the difference was not significant. There was no statistically significant difference between the L2-L4 and TBLH BMD Z- *score* values. 2/28 (7.1%) FHHNC patients showed short stature (height < 5th percentile for age and sex).

In comparison with controls, FHHNC patients had significantly higher median plasma PTH (184 vs. 29.8 pg/ml, $p < 0.001$) and serum 1.25$(OH)_2D_3$ (62.5 vs. 53.7 pg/l, $p < 0.05$) levels as well as significantly lower sMg (0.62 vs. 0.9 mmol/l, $p < 0.001$) concentrations. They also showed significantly higher 24 h-uCa (6.0 vs. 2.2 mg/kg, $p < 0.001$). Serum concentrations of Ca, P and 25$(OH)D_3$ did not significantly differ between both groups (Table 2).

Furthermore, FHHNC patients had significantly higher median plasma FGF23 and CTX-I levels (103 vs. 78.3 pg/ml, $p < 0.01$ and 4.27 vs. 2.03 ng/ml, $p < 0.001$, respectively). The remaining BTMs did not show significant differences between both groups (Table 2).

In FHHNC patients, plasma CTX-I positively correlated with levels of PTH ($r = 0.33$, $p < 0.05$), FGF23 ($r = 0.33$, $p < 0.05$) and SCL ($r = 0.38$, $p < 0.05$), but negatively with sMg ($r = -0.47$, $p < 0.05$). Moreover, FGF23 and PTH levels correlated negatively with sKL ($r = -0.28$, $p < 0.05$ and $r = -0.29$, $p < 0.05$, respectively). A negative relationship between PTH and Mg levels was also found ($r = -0.72$, $p < 0.05$). No significant correlations between

Parameter	FHHNC	Controls	*p* value
	Median (range)		
FGF23 (pg/ml)	103.0 (35.6-414.7)	78.3 (31.1-158.1)	<0.01
sKL (pg/ml)	1653.8 (748.3-4387.4)	1951.8 (523.0-5092.9)	0.4
OPG (pmol/l)	4.39 (2.57–12.3)	4.36 (2.12–21.2)	0.49
OC (ng/ml)	128.7 (88.0-1722.6)	106.7 (44.0-302.0)	0.21
PINP (pg/ml)	46170.2 (38201.0-221557.0)	47137.1 (20196.8-208478.0)	0.98
CTX-I (ng/ml)	4.27 (1.29–9.50)	2.03 (0.75–3.9)	<0.001
SCL (ng/ml)	0.81 (0.34–1.80)	0.78 (0.51–1.3)	0.31
25-OHD_3 (ng/ml)	23 (8.4–67.0)	23.6 (12.2–53.2)	0.96
1,25-$(OH)_2D_3$ (pg/ml)	62.5 (11.8–100)	53.7 (32.0-94.7)	<0.05
sCa (mmol/l)	2.50 (1.7–2.80)	2.53 (2.1–2.7)	0.87
sMg (mmol/l)	0.62 (0.36–0.80)	0.9 (0.77–1.1)	<0.001
sP (mmol/l)	1.48 (0.81–2.1)	1.52 (1.17–1.74)	0.46
BAP (U/l)	74 (27.7–103.0)	64.5 (34.6–97.9)	0.28
PTH (pg/ml)	184 (63.0-1167.0)	29.8 (12.5–59.1)	<0.001
24 h-uCa (mg/kg)	6.0 (1.30–14.5)	2.2 (1.1–3.7)	<0.001
BMD Z-*score* (L2-L4)	-0.25 (-2.6–0.8)	0.10 (-1.9-0.9)	0.8
BMD Z-*score* (TBLH)*	-0.29 (-2.7-0.7)	0.12 (-1,8-0.97)	0.7

Table 2. The serum/plasma levels of assessed parameters in FHHNC- and control group. *Assessed only in 17 patients and controls.

	CTX-I (ng/ml)	SCL (ng/ml)	FGF23 (pg/ml)	sKL (pg/ml)	PTH (pg/ml)	sMg (mmol/l)
CTX-I (ng/ml)	-	r = 0.38 p < 0.05	r = 0.33 p < 0.05	NS	r = 0.33 p < 0.05	r = -0.47 p < 0.05
SCL(ng/ml)	r = 0.38 p < 0.05	-	NS	NS	NS	NS
FGF23 (pg/ml)	r = 0.33 p < 0.05	NS	-	r = -0,28 p < 0.05	r = 0.33 p < 0.05	NS
sKL (pg/ml)	NS	NS	r = -0.28 p < 0.05	-	r = -0.29 p < 0.05	NS
PTH (pg/ml)	r = 0.33 p < 0.05	NS	r = 0.33 p < 0.05	r = -0,29 p < 0.05	-	r = -0.72 p < 0.05
sMg (mmol/l)	r = -0.47 p < 0.05	NS	NS	NS	r = -0.72 p < 0.05	-

Table 3. Detected significant correlations between assessed parameters in FHHNC group.

measured BTMs and eGFR, sCa, sP, 25(OH)D_3, 1.25 $(OH)_2D_3$ as well as 24 h uCa were found (Table 3). None of the BTMs correlated with BMD Z-score values.

Discussion

Although many of the symptoms of FHHNC may be potentially harmful for skeletal homeostasis, to the best of our knowledge, this a first study focused on bone metabolism in FHHNC patients. As mentioned, one of them is persistent hypercalciuria observed since infancy[3–6]. Together with hypocitraturia is among the main causes of the development of NC and urolithiasis (UL) in FHHNC, however the latter is less common[1,3,6,11]. Several studies showed that increased urinary Ca excretion, mainly idiopathic one (IHC) may be a risk factor for development of osteoporosis in adult patients with calcium UL[12–14]. This association was also reported in pediatric patients as almost one third of children with UL and IHC have been diagnosed with decreased BMD[15–17]. However, in these studies, bone loss was recognized already at Z-*score* <-1 and not <-2 as currently defined, making the number of osteopenic patients rather overestimated. In our study, despite of HC being present in all patients, only 10.7% of them showed BMD Z-*score* below − 2. Although median value Z-*score* in this group was lower in comparison to controls, i.e. -0.25 vs. 0.1, respectively, the difference was not significant. Similarly, to studies in adults with calcium UL, in our patients we did not find significant correlations between 24 h-uCa and BMD Z-*score* values[18,19]. The reason is unclear, but it was speculated that osteopenia in patients with calcium UL may not only be caused by urinary calcium loss but also may be triggered by additional factors associated with IHC as diet, inflammatory cytokines or polymorphism of selected genes[20,21]. Obviously, HC in FHHNC and IHC are pathogenetically distinct entities making simple comparison oversimplified. We can't also exclude, that our results might be influenced by HTZ treatment, effective in reduction of calciuria in some FHHNC patients[4,6].

Another characteristic feature of FHHNC is SHP. It occurs already in patients with normal kidney function and is usually overexpressed in relation to the stage of CKD[3–6]. Its exact pathomechanism is not fully understood but it seems to be caused by HC and moderated by hypomagnesemia[22–24]. The negative correlation between plasma PTH and sMg levels in our study seems to confirm this hypothesis. As a result, SHP enhances tubular Ca and Mg reabsorption as well as may stimulate bone Ca resorption[22–24]. Although the clinical significance of the latter on skeletal system is unknown, the comorbidity of an extreme SHP and a high- grade bilateral slipped capital

femoral epiphysis in an adolescent boy with FHHNC was reported[25]. Other skeletal anomalies rarely observed in patients with FHHNC as chondrocalcinosis or amylogenesis imperfecta are unlikely related to SHP but rather to *CLDN* mutations or hypomagnesemia per se[26,27]. Since 50–60% of total body Mg resides in the skeleton, its deficiency could potentially have negative impact on bone status. In fact, experimental studies indicate possible bone mass loss in hypomagnesemia by affecting osteoblastic bone formation and mineralization[28]. In humans, these observations were confirmed only indirectly showing correlation between a low-Mg diet and decreased BMD, mostly in premenopausal women as well as a positive effect of Mg supplementation on osteoporosis treatment[28,29].

Although assessment of BMD using DXA is still the gold standard test for the diagnosis of osteoporosis it does not reflect the dynamics of bone metabolism. Especially in pediatric population, a clinical value of BMD Z-*score* may be limited due to technical reasons, as well as only partial correlation with a fracture risk[8,30]. Therefore, an assessment of circulating bone derived products become increasingly used as an additional non-invasive diagnostic method. In particular, BTMs provide information about the metabolic activity of the entire skeleton, including bone formation and resorption[31,32]. In our study we found significantly higher plasma level of CTX-I in FHHNC group compared to controls. This fragment of type I collagen is considered as one of the most specific markers of bone resorption[33,34]. Because of its characteristics, together with PINP it forms a pair of parameters of opposing processes of bone turnover, recommended for assessing the prediction of bone fractures and monitoring the osteoporosis treatment[35]. In osteoporotic women CTX-I seems to be even better predictor of fractures than the assessment of BMD[36]. Although some studies on postmenopausal women or adults with diabetes mellitus type 2 (DM2) showed significant negative correlation between plasma level of CTX-I and BMD we did not observe it in our study[37,38]. However, we found that CTX-I positively correlated with plasma PTH, FGF23 and SCL as well as negatively with sMg concentrations. It may indicate a possible impact of SHP and hypomagnesemia on bone resorption. This is somehow consistent with some experimental studies on Mg-deficient rats and mice, in which an increase in osteoclast number and their activity were observed. It was hypothesized that increased osteoclastic bone resorption may be caused by the local stimulation of inflammatory cytokines by Mg depletion[28]. Relation of CTX-I to SCL- suppressor of osteoblasts activity found in our study may also indicate additive inhibition of bone formation[39].

In FHHNC group we found significantly higher plasma FGF23 when compared to controls. This osteocyte/osteoblast derived phosphaturic factor and calcitriol inhibitor plays a pivotal role in mineral and bone metabolism[40,41]. Among others, it seems to be involved in the osteo- differentiation process, including the proliferation of premature osteoblasts and inhibition of the mineralization of mesenchymal cells[42–44]. It was reported that high plasma FGF23 levels may impair BMD in animal models as well as in patients on hemodialysis[45–47]. In addition, the relationship between high plasma FGF23 accompanied by low sKL levels and fracture risk in patients with DM2 and CKD was also found[48]. It is well known that plasma FGF23 levels constantly increase as eGFR decreases in the course of CKD, mainly as a compensatory response to phosphate retention[49]. However, elevated FGF23 level in early CKD without hyperphosphatemia like in our patients, cannot be simply explain in this way. It is proposed that the main stimulus could be CKD-related low tubular Klotho expression in the distal tubule, causing FGF23 resistance[50]. Recently, in non-dialysis CKD patients, the positive association of plasma FGF23 level with urinary fractional Mg excretion (FEMg) independently of eGFR, PTH or vitamin D, was found[51]. Similarly to our results, there was no significant relation between plasma FGF23 and sMg levels. Unfortunately, we did not measure FEMg in this study but its increased values are the hallmark of FHHNC and they are event present in the absence of hypomagnesemia[6]. The possible link between FGF23 and renal Mg handling was first noted in animal model a decade ago as an increased plasma FGF23 level in Mg-deficient rats was found[52]. Subsequently, reduction of serum FGF23 in CKD mice treated with Mg supplement was observed[53]. Although the mechanism of this relationship is not clear, it was speculated that FGF23-Klotho signaling may control TRPM6- a Mg^{2+} -permeable cation channel expression in the distal convoluted tubulus[51]. This mechanism may also hypothetically take place in FHHNC or in other hypomagnesemic conditions. Although median plasma sKL level in our FHHNC patients did not significantly differ from that in controls, it nicely correlated with plasma FGF23 level. This finding, and a positive correlation of FGF23 with plasma PTH concentration appear to reflect compensatory mechanisms in early CKD[54].

We acknowledge that our study has some limitations that may have affected the final results. First, the study group was relatively small, but this was due to the very low prevalence of FHHNC. Second, for ethical reasons we could not discontinue patients' treatment, including Mg supplementation, HTZ or vitamin D. However, as the comparison with the control group showed, its effects were partial, as the median values of sMg, 24 h-uCa or PTH were far different from the normal ranges. Although the study was cross-sectional, all patients were uniformly assessed per protocol in one center, which eliminates possible variations in values due to different laboratory methods applied.

In conclusion, the results of our study show that pediatric FHHNC patients, regardless of CKD may be at risk for increased bone resorption. Although its pathomechanism is complex, the trigger seems to be Mg depletion, aggravating SHP and leading to the activation of osteolytic processes. Therefore, the adequate Mg supplementation may be crucial in maintaining bone health in this specific group of patients. As in our previous study[6], 60% of FHHNC patients showed hypocitraturia, we propose to use magnesium citrate preparations at the maximum tolerated dose, usually 10–20 mg Mg^{2+}/kg/24 h, divided in 3 doses to keep the serum magnesium > 0,7 mmol/l. However, it may be difficult to achieve in some of them due to excessive urinary Mg loss and intestinal intolerance of magnesium preparations. Considering the risk of osteopenia, we suggest to follow-up BMD and bone- related laboratory parameters including CTX-I, also and perhaps especially in adults with FHHNC.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions
B.B. prepared study protocol, analyzed and interpreted the data and wrote the manuscript, A.W.P. prepared study protocol, collected study group, data samples, analyzed patients data, P.S. prepared study protocol, collected study and control groups, data samples, analyzed and interpreted data and wrote the manuscript, M.Z., B.P., M.S., A.M., A.R.K., M.T., D.O.N., K.Z., J.Z. collected study group and patient data, M.K. genetic analysis.

Declarations

Competing interests
The authors declare no competing interests.

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