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Research Article

Calcium isotope ratio in patients with monogenic bone diseases: a prospective, cross-sectional, single-center pilot study

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Abstract

Stable calcium isotope fractions have long been related to the calcium metabolism in living organisms. The blood and urine proportions of calcium isotopes ^{44}Ca and ^{42}Ca ($\delta^{44/42}$Ca) have recently again attracted attention as a potential diagnostic tool in metabolic bone diseases, in particular osteoporosis. The hypothesis is that the lighter isotopes (Ca^{42}) get incorporated into bone more quickly; hence, $\delta^{44/42}$Ca ratios in urine and serum are higher for bone formation and lower for resorption phases. Therefore, $\delta^{44/42}$Ca in blood and urine may serve as an indicator of bone metabolism, potentially reflecting bone density in general. We have conducted clinical characterization by means of laboratory assessment, bone densitometry, HRpQCT, and isotope analysis to test for the hypothesis in patients with monogenic bone diseases. We included 40 adult subjects with hereditary bone diseases, such as early-onset osteoporosis ($n = 7$), osteogenesis imperfecta ($n = 12$), hypophosphatasia ($n = 12$), and X-linked hypophosphatemia (XLH, $n = 9$), and controls ($n = 17$). Regression analyses revealed significant correlations of $\delta^{44/42}$Ca with $Ca/creatinine_{urine}$ ($R^2 = 0.6200$, $p < .0001$), and bone densitometric parameters were significantly correlated with $\delta^{44/42}$Ca (BMD: $\delta^{44/42}Ca_{serum}$ $R^2 = 0.2685$, $p \leq .001$; $\delta^{44/42}Ca_{urine}$ $R^2 = 0.3554$; $p < .0002$). XLH differed significantly from the other diseases and controls by means of higher $\delta^{44/42}Ca_{urine}$. Our results suggest that $\delta^{44/42}$Ca is strongly coupled to urinary calcium excretion in patients with hereditary bone diseases. Significant correlations with BMD suggest an interaction of $\delta^{44/42}$Ca and bone mass though it lacks discriminative power. Further studies are needed to evaluate the utility of $\delta^{44/42}$Ca in clinical practice.

Keywords: calcium isotopes, diagnostic parameter, hereditary bone diseases, bone microarchitecture, HRpQCT

Lay Summary

Calcium plays a fundamental role in bone metabolism, and disturbances in calcium metabolism can contribute to different hereditary bone diseases. In this study, we investigated whether the fraction of stable calcium isotopes ($\delta^{44/42}$Ca) in blood and urine could serve as biomarkers for hereditary bone diseases. By analyzing patients with conditions such as early-onset osteoporosis, hypophosphatasia, osteogenesis imperfecta, and X-linked hypophosphatemia, we identified correlations between calcium isotope ratios, urinary calcium excretion, and BMD. While $\delta^{44/42}$Ca does not strongly differentiate between these diseases, our findings suggest it could provide valuable insights into calcium dynamics and bone turnover. This approach may complement existing diagnostic tools and deepen our understanding of metabolic bone disorders.

Introduction

Since the late 1970s,[1] the fractions of different, stable calcium isotopes have been subject to investigations related to the calcium metabolism in living organisms. The isotopes of interest for bone metabolism are ^{44}Ca and ^{42}Ca, both of which can be measured in urine and serum,[2–4] and their fraction ($\delta^{44/42}$Ca) can be calculated. According to Skulan and De Paolo,[2] the lighter ^{42}Ca isotope is favored during mineralization processes, resulting in a lighter isotopic composition of the bone compared to the nutritional calcium uptake.[2] Correspondingly, in case of accelerated bone resorption, more lighter Ca^{42} may be released from bone into blood and subsequently urine leading to a lowered $\delta^{44/42}$Ca in comparison to a balanced bone metabolism.[2] In cases where osseous calcium is needed, such as for increased bone apposition, a higher Ca^{42} uptake and transition to bone tissue is favored, thus lowering the fractions of Ca^{42} in blood and urine leading to higher $\delta^{44/42}$Ca ratios.[4] Meanwhile, reference values for $\delta^{44/42}$Ca ratios have been established, indicating ranges for balanced bone metabolism. The calcium isotope fractionation $\delta^{44/42}$Ca has been proposed as a potential marker for detecting disturbances in bone metabolism, such as osteoporosis, in a radiation-free, device-independent manner.[3] Eisenhauer et al.[3] even suggest the possibility that $\delta^{44/42}$Ca could replace established diagnostic tools like bone density measurements. However, this perspective represents the opinion of Eisenhauer et al. and has not been substantiated by robust evidence or clinical validation.

In this context, monogenic bone diseases (MGBDs) represent a broad spectrum of very different disorders related

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to calcium, phosphate, and/or bone metabolism causing primary or secondary disturbances in bone mineralization and/or microstructure. These very different bone disorders might be particularly suitable for investigating a new parameter for bone metabolism like $\delta^{44/42}$Ca because they present the extremes of the spectrum of bone disorders. We therefore decided to investigate this parameter in a cohort of patients diagnosed with X-linked hypophosphatasia (XLH), caused by pathogenic variants in the *PHEX* gene, hypophosphatasia (HPP), caused by pathogenic variants in the *ALPL* gene, as well as classical osteogenesis imperfecta (OI)[5] caused by pathogenic variants in the *COL1A1* or *COL1A2* gene, and hereditary early-onset osteoporosis (EOOP) due to pathogenic variants in the *WNT1* or *LRP5* gene.[6]

The objective of this study was to investigate the potential of the calcium isotope fraction in urine and serum in adult patients with MGBDs. Hence, we included patients with brittle bone diseases, namely OI and EOOP, as well as patients with mineralization disorders, namely XLH and HPP. We focused on whether a specific configuration of the proportion of both calcium isotopes in serum or urine can be seen in any of these MGDB and compared to controls. Furthermore, we investigated the correlations of $\delta^{44/42}$Ca with established parameters of calcium and bone metabolism in blood and urine and investigated the correlations of $\delta^{44/42}$Ca with bone density and bone structure measures.

Materials and Methods

Study design

Forty adult patients were included between October 2022 and August 2023 who consulted the specialized outpatient clinics of the Institute of Osteology and Biomechanics (IOBM, University Medical Center Hamburg-Eppendorf). Full bone-specific routine diagnostics was conducted during the patient's consultation. Patients considered for inclusion had to be diagnosed with genetically confirmed EOOP, HPP, OI, or XLH. For EOOP only, monogenic LRP5/LRP6 or Wnt1 variations were included. For OI only, classical OI variations were considered. A control group was created by 17 patients who underwent the same examinations as the study group but without any underlying bone disease. The full patient characterization comprised routine blood and urine sampling including specimens for calcium isotope measurements, bone density measurement by DXA as well as assessment of bone microstructure by HRpQCT. All participants provided written informed consent to the study, which was approved by the local ethics committee (2022-100 817-BO-ff) and carried out in accordance with the Declaration of Helsinki.

Calcium isotope analysis

Calcium isotope analysis of serum and urine samples was carried out by Osteolabs® GmbH. The unit of the $\delta^{44/42}$Ca values is given in per mil (‰) and is comprised of the isotopic ratio of a sample ($\delta^{44/42}Ca_{sample}$) compared to that of a standard ($\delta^{44/42}Ca_{standard}$). The formula upon which the [‰] value is based is $\delta^{44/42}Ca\ (‰) = [(^{44}Ca/^{42}Ca)_{Sample}/(^{44}Ca/^{42}Ca)_{Reference}] - 1$ according to Eisenhauer et al.[3]

Calcium isotope ratios ($\delta^{44/42}$Ca) were corrected for calcium supplementation, if taken and applicable. According to the manufacturer, this is necessary for patients who supplemented calcium for longer than 400 d, as most calcium supplements are enriched in the heavier Ca^{44} isotope and would hence artificially elevate $\delta^{44/42}$Ca in blood and urine.[7] The laboratory proposed threshold $\delta^{44/42}$Ca to indicate osteoporosis provided by Osteolabs® was −0.85‰ in serum and 0.22‰ in urine samples, respectively.[3] However, a tolerance range of 0.06‰ was applied, resulting in a shift of the diagnostic threshold to −0.91‰ for serum and 0.16‰ for urine, respectively, to account for the external reproducibility, according to the supplemental material of Shroff et al.[7]

Biochemical analysis

Both, blood and urine samples were routinely analyzed in our local laboratory (Institute for Clinical Chemistry and Laboratory Medicine, University Medical Center Hamburg). Calcium, alkaline phosphatase (ALP), creatinine, bone-specific alkaline phosphatase (bALP), gamma-glutamyltransferase (GGT), osteocalcin, P1NP, ß-CTX, PTH, and 25-hydroxyvitamin D_3 ($25(OH)D_3$) were measured in serum samples for routine clinical examination. Urine creatinine, calcium per creatinine (Ca/creatinine), and deoxypyridinoline per creatinine (DPD/crea) levels were also routinely assessed in spot urine at least 2 h after the last uptake of food. Specific test sets and machinery that were used for each specific parameter are listed below: serum parameters of calcium, phosphate, creatinine, GGT, ALP, $25(OH)D_3$, and PTH as well as urine calcium and creatinine were analyzed using a system by Atellica-Solution. Liaison XL, Diasorin was used for serum osteocalcin and b-ALP. Serum P1NP and β-CTX were measured on Cobas E411, Roche Diagnostics. Urine DPD was measured using a set of Immulite-XP, Siemens.

DXA

DXA (Lunar iDXA, GE Healthcare) scans from the lumbar spine (L_1-L_4) and hip (femoral neck and total hip) were performed by trained professionals. BMC, aBMD, *T*-score, and Z-score were calculated by the manufacturer's software. To perform daily calibration scans, the manufacturer's phantom was used according to the recommendations for DXA quality management, including precision checks as per International Society for Clinical Densitometry (ISCD) guidelines.[8]

HRpQCT bone microstructure

HRpQCT (XtremeCT I, Scanco Medical and XtremeCT II, Scanco Medical) was used to examine patients bone microarchitecture and volumetric BMD at the distal radius and distal tibia. The manufacturer's standard in vivo scanning protocols (Xtreme CT I: 59.4 kVp, 900 μA, 100 ms integration time, 82.0 μm voxel size, Xtreme CT II: 68 kVp, 1470 μA, 43 ms integration time, 60.7 μm voxel size) were used.[9] The entire scan area extended over 110 slices (9.02 mm) in XtremeCT 1 and 168 slices (10.2 mm) in XtremeCT 2. Microarchitectural parameters included bone volume to total volume ratio (BV/TV), trabecular number (Tb.N, mm^{-1}), trabecular thickness (Tb.Th, mm), trabecular separation (Tb.Sp, mm), cortical thickness (Ct.Th, mm), cortical pore diameter (Ct.Po.Dm, mm), and cortical porosity (Ct.Po). Volumetric BMD was expressed as total volumetric BMD (Tt.vBMD, $mgHA/cm^3$), cortical vBMD (Ct.vBMD, $mgHA/cm^3$), and trabecular vBMD (Tb.vBMD, $mgHA/cm^3$). Geometric values include total bone area (Tt.Ar, mm^2), trabecular bone area (Tb.Ar, mm^2), cortical bone area (Ct.Ar, mm^2), and cortical perimeter (Ct.Pm, mm). All HRpQCT images were manually evaluated for motion artifacts prior to evaluation and were excluded if motion artifacts of grade 4 or greater were

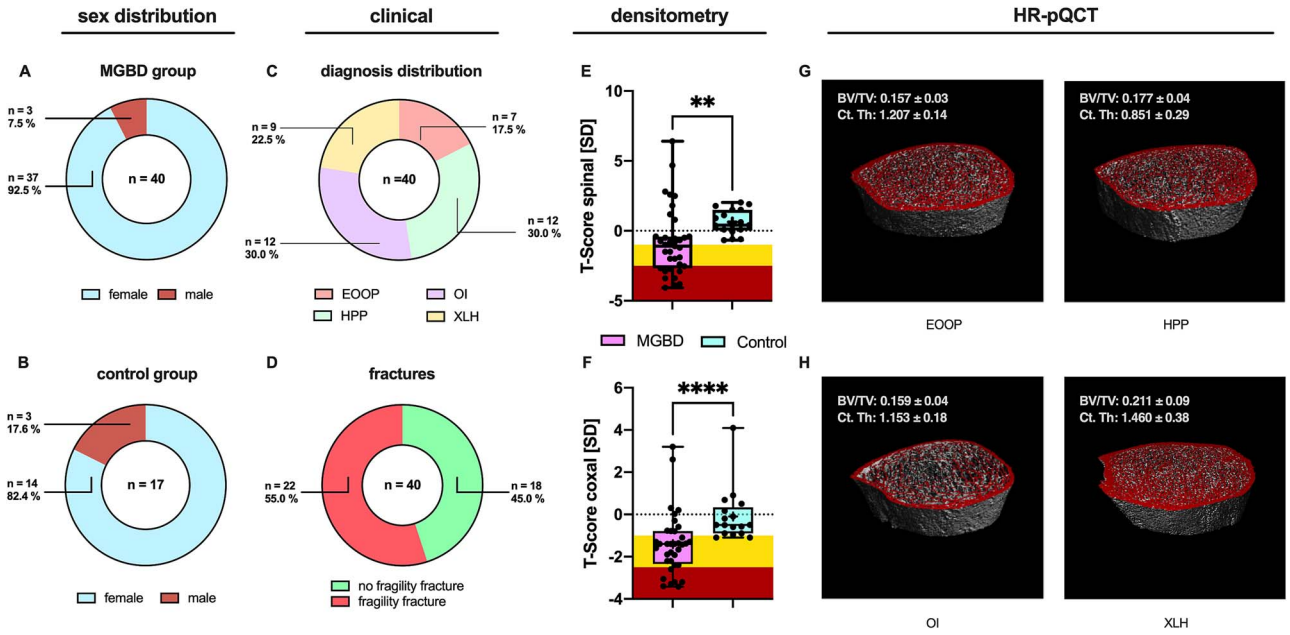

Figure 1. Demographic data and imaging technique examples. The vast majority of MGBD and control patients were female (A, B). The group of patients with MGBD splits into 4 disease groups, consisting of patients diagnosed with EOOP, HPP, OI, and XLH (C). 55% of all MGBD patients experienced fragility fractures (D). The control group exhibits higher *T*-scores in both, the spine and the hip (E, F). Abbreviations: BV/TV, bone volume/total volume; Ct.Th, cortical thickness; EOOP, early onset osteoporosis; HPP, hypophosphatasia; OI, osteogenesis imperfecta; XLH, X-linked hypophosphataemia; SD, standard deviation. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$; $^{****}p < .0001$.

detected.[10,11] By employing the results of Manske et al.[12], all parameters have been recalibrated to match the standards of the second-generation HRpQCT for comparability.

Statistical analysis

Statistical analysis was performed using SPSS software version 28.0.1 (IBM Corp.), JASP software 0.17.2.1 (JASP Team (2023), University of Amsterdam), and GraphPad Prism 9.3.1 (GraphPad Software). Results are presented as absolute values or as means ± standard deviations. All data were tested for normality when required for the specific test. The unpaired 2-tailed Student's *t*-test was used for differences between 2 subgroups for normally distributed data and the Mann-Whitney *U*-test for non-normally distributed data. In group comparison, we stratified the MGBD cohort based on their *T*-score in both hip and spine DXA into 2 groups that scored above or, respectively, equal or below the osteoporotic threshold (*T*-score −2.5 SD), to address the aforementioned (cf. introduction) claim[3] of $\delta^{44/42}$Ca to be able to replace densitometric measures such as DXA. Conversely, we later stratified the MGBD cohort according to their $\delta^{44/42}Ca_{serum}$ and $\delta^{44/42}Ca_{urine}$ using the recommended cutoff by the laboratory as mentioned above. Additionally, we divided the cohort into 2 subgroups with either fractured or nonfractured patients to test if $\delta^{44/42}Ca_{serum}$ and $\delta^{44/42}Ca_{urine}$ differed between both groups. For analysis among multiple MGBD subgroups, we performed an ANCOVA, to test for group differences after including age as a covariant, followed by post hoc analysis (Tukey's). For ANOVA and ANCOVA, Levene's test for equality of variances was conducted prior to further analysis.

Correlation analyses were performed to assess the relationships between calcium isotope ratios and laboratory test results, bone density parameters, and HRpQCT parameters. Using multiple linear regression analysis, we aimed to identify the main predictive factors of $\delta^{44/42}$Ca both in serum and urine, using ca/creatinine$_{urine}$, age, L_1-L_4 BMC, BMI, GFR, BGLAP, vitamin D_3, bAP, P1NP, ß-CTX, and PTH as covariates. The multiple linear regression analysis was carried out to identify the main predictive values of $\delta^{44/42}$Ca using the forward method. Pearson correlation was used for normally distributed data and Spearman correlation for non-normally distributed data. We performed regression analysis with all MGBD patients pooled as well as with each MGBD subgroup. To evaluate the diagnostic potential of $\delta^{44/42}$Ca for XLH, we performed a receiver operating characteristic (ROC) analysis of both $\delta^{44/42}Ca_{serum}$ and $\delta^{44/}42Ca_{urine}$ to assess sensitivity and specificity and compared it to an ROC analysis of L_1-L_4 BMD. All other patients were pooled against XLH. The highest likelihood ratio was selected to choose the respective sensitivity and specificity. Power analyses were performed using G*Power software (G*Power Version 3.1.9.6, Heinrich-Heine University Düsseldorf), utilizing an *F*-test and testing for R^2 deviation from zero for the semilogarithmic model used for urinary calcium per creatinine excretion.

Results

Our study involved a cohort of 40 patients, with 3 males and 37 females and 17 controls. Among the male patients, 2 were diagnosed with OI and one with XLH (Figure 1A). The bone healthy control group comprised 3 males and 14 females (Figure 1B). The MGBD groups included $n_{EOOP} = 7$ patients with EOOP, $n_{HPP} = 12$ patients with HPP, $n_{OI} = 12$ patients with OI, and $n_{XLH} = 9$ patients with XLH (Figure 1E). For 2 of the MGBD patients, no $\delta^{44/42}Ca_{urine}$ was available. None of the control patients had a bone-affecting disease.

Demographic data of the pooled MGBD group and the control group is presented in Figure 1 and Table 1. The mean age of the MGBD group was 47.2 ± 15.4 yr, with the

Table 1. Demographic data of the MGBD and control groups including statistical comparison of the 2 groups.

Parameter	Genetic group ($n = 40$)			Control group ($n = 17$)			Comparison
	Mean	SD	95% CI	Mean	SD	95% CI	p
Demographics							
Age (yr)	47.23	15.43	4.94	49.24	14.27	7.34	.64
Weight (kg)	67.8	16.7	5.6	68.6	14.6	7.5	.66
Height (cm)	161.1	8.5	2.9	171.7	7.6	3.9	<.0001
BMI (kg/m^2)	26.2	6.5	2.2	23.1	3.6	1.9	.09
DXA							
Femoral T-score (SD)	−0.9	1.7	0.6	0.2	1.4	0.7	.003
Femoral Z-score (SD)	−0.3	1.6	0.5	0.7	1.2	0.6	.004
Spinal T-score (SD)	−0.9	2.4	0.8	0.6	0.9	0.5	.001
Spinal Z-score (SD)	−0.2	2.3	0.8	1.2	1.0	0.5	.003
Normal BMD		22.5% (9/40)			100% (17/17)		<.0001
Osteopenia (<−1.0 SD)		37.5% (15/40)			0% (0/17)		.003
Osteoporosis (≤−2.5 SD)		32.5% (13/40)			0% (0/17)		.005
Without DXA		7.5% (3/40)			0% (0/17)		.54
Fragility fractures							
No frag. fracture		70% (28/40)			100% (17/17)		.01
Fragility fracture		30% (12/40)			0% (0/17)		<.0001
Vertebral fractures		50% (6/12)			0% (0)		na
Extravert fractures		50% (6/12)			0% (0)		na
Mechanography							
Grip strength (kg)	22.88	5.65	2.57	30.17	10.59	6.12	.15
CRT maximum force (kN)	1.32	0.13	0.04	1.40	0.18	0.10	.23
CRT time per repetition (s)	2.01	1.20	0.40	1.94	0.58	0.34	.55
Romberg path length EO (mm)	107.60	23.76	12.68	105.50	23.94	18.43	.84
Romberg path length EC (mm)	189.80	49.83	26.55	179.60	58.18	44.70	.65
HRpQCT radius							
BV/TV	0.141	0.067	0.022	0.216	0.044	0.024	<.0001
Tb.vBMD (mgHA/mm^3)	97.6	48.2	15.4	150.9	31.1	16.6	<.0001
Ct.vBMD (mgHA/mm^3)	817.9	112.0	35.9	895.8	56.5	30.1	.01
Tt.vBMD (mgHA/mm^3)	261.8	72.4	23.2	320.1	46.9	25.0	.0003
Ct.Th (mm)	0.805	0.278	0.089	1.036	0.146	0.078	.0002
Tb.N (mm^{-1})	1.018	0.349	0.112	1.410	0.207	0.111	.0001
Tb.Th (mm)	0.223	0.024	0.008	0.225	0.011	0.006	.21
Tb.Sp (mm)	1.169	0.653	0.209	0.680	0.120	0.064	<.0001
HRpQCT tibia							
BV/TV	0.178	0.057	0.019	0.261	0.055	0.029	<.0001
Tb.vBMD (mgHA/mm^3)	115.4	41.6	14.1	176.4	40.7	21.7	<.0001
Ct.vBMD (mgHA/mm^3)	809.2	114.0	38.6	880.2	70.2	37.5	.03
Tt.vBMD (mgHA/mm^3)	230.4	54.2	18.4	303.9	50.0	26.6	<.0001
Ct.Th (mm)	1.140	0.352	0.119	1.433	0.252	0.134	.0006
Tb.N (mm^{-1})	1.050	0.230	0.078	1.353	0.189	0.101	<.0001
Tb.Th (mm)	0.252	0.024	0.008	0.263	0.025	0.014	.11
Tb.Sp (mm)	1.014	0.315	0.106	0.713	0.104	0.055	<.0001

Abbreviations: Ct.Th, cortical thickness; Ct.vBMD, cortical volumetric BMD; CRT, chair rising test; EO, eyes open; EC, eyes closed; na, not assessed; Tb.BV/TV, trabecular bone volume/total volume; Tb.vBMD, trabecular volumetric BMD; Tt.vBMD, total volumetric BMD; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation.

control group's mean age being 49.2 ± 14.3 yr ($p = .6478$). Notably, the mean age of the HPP group was significantly higher than every other group ($p = .0002$). In terms of fracture incidence, 55% of MGBD patients had experienced fragility fractures (Figure 1D). T-scores in the hip and spine DXA differed significantly ($p_{spine} = .0114$; $p_{hip} < .0001$) between the mean of MGBD and the control group (MGBD: mean T-score$_{hip}$: −0.9 ± 1.7; mean T-score$_{spine}$: −0.8 ± 2.4; control: mean T-score$_{hip}$: −0.1 ± 1.2 mean T-score$_{spine}$: 0.6 ± 0.9) (Figure 1E, F). For none of the patients, calcium supplement correction was needed.

Correlation analysis of serum and urine parameters and calcium isotope measures

Performing a linear correlation analysis of regular serum and urine parameters with $\delta^{44/42}$Ca, a significant correlation between $\delta^{44/42}$Ca and Ca/creatinine$_{urine}$ was measured both in $\delta^{44/42}$Ca$_{serum}$ ($R^2 = 0.2622$, $p = .0020$) and $\delta^{44/42}$Ca$_{urine}$ ($R^2 = 0.5337$, $p < .0001$). All linear correlations are given in Table S1. A better fit of the Ca/creatinine$_{urine}$ curves was derived using a semilogarithmic regression, as evidenced both visually and mathematically ($\delta^{44/42}$Ca$_{serum}$: $R^2 = 0.3579$, $p = .0002$; $\delta^{44/42}$Ca$_{urine}$: $R^2 = 0.6200$, $p < .0001$, Figure 2A). Power calculations revealed a power of 0.99 of the semilogarithmic model. A weak association between GFR and $\delta^{44/42}$Ca$_{serum}$ was observed with no correlation detected for $\delta^{44/42}$Ca$_{urine}$. No significant correlations were detected for bone turnover parameters (DPD/crea$_{urine}$, P1NP, or β-CTX; Figure 2C). Table S1 displays all significant correlations of $\delta^{44/42}$Ca in serum and urine specimens of MGBD patients with laboratory values per disease.

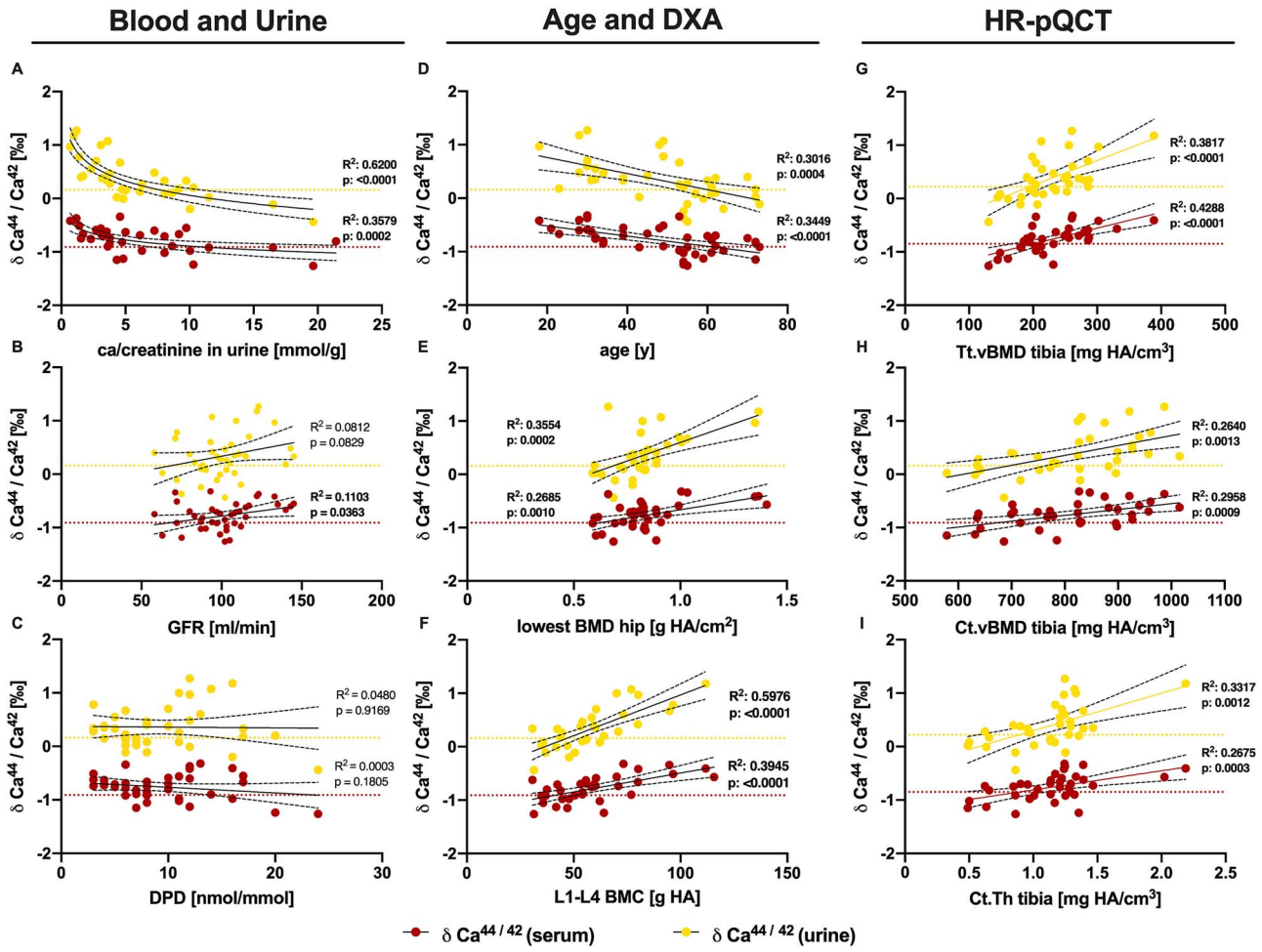

Figure 2. Correlation analysis of serum analysis, DXA, and HRpQCT with $\delta^{44/42}$Ca. Correlation analysis revealed the best fit to $\delta^{44/42}$Ca and Ca/creatinine$_{urine}$ when using a logarithmic model (A). Only a weak correlation with GFR and $\delta^{44/}$42Ca$_{serum}$ was detected (B), and DPD/creatinine$_{urine}$ does not correlate with both $\delta^{44/42}$Ca ratios (C). Age, lowest hip BMD, and L_1-L_4 BMC correlated significantly with both $\delta^{44/42}$Ca$_{serum}$ and $\delta^{44/42}$Ca$_{urine}$ (D-F). HRpQCT imaging revealed significant correlations of $\delta^{44/42}$Ca with Tt.vBMD$_{tibia}$, Ct.vBMD$_{tibia}$, and Ct.Th$_{tibia}$ (G-I). Abbreviations: Ct.vBMD, cortical volumetric BMD; Ct.Th, cortical thickness; DPD, deoxypyridinoline/creatinine in urine; Tt.vBMD, total volumetric BMD.

Correlation analysis of DXA parameters and calcium isotope measures

Analysis of DXA and $\delta^{44/42}$Ca parameters revealed multiple significant correlations with each other (Table 2). All these correlations were positive and showed favorable R^2 values in $\delta^{44/42}$Ca$_{urine}$ compared to $\delta^{44/42}$Ca$_{serum}$. Interestingly, XLH appeared at the upper end of the cohort in BMD, BMC, and *T*-score exhibiting high normal bone mass values. To exclude XLH as a confounding factor, we ran the same correlation analysis without XLH patients (Table 2). When excluding XLH from the analysis, R^2 values lowered on average by 64.48% ± 11.65% and *p*-values left significant in 8 out of 12 correlations (Figure S1). In summary, excluding XLH from the analysis did not change the direction or strength of the associations observed in the regression analyses. However, the overall quality of the relationships was affected, with *p*-values increasing and statistical significance weakening (Table 2).

Correlation analysis of HRpQCT bone parameters and $\delta^{44/42}$Ca

In HRpQCT, Tt.vBMD$_{tibia}$, Ct.vBMD$_{tibia}$, and Ct.Th$_{tibia}$ were significantly correlated with $\delta^{44/42}$Ca (cf. Table S1 and Figure 2G-I) with an effect size $R^2 > 0.25$. Additionally, BV/TV$_{radius}$, Tt.BMD$_{radius}$, Tb.vBMD$_{radius}$, Tb.N$_{radius}$, BV/TV$_{tibia}$, Tb.vBMD$_{tibia}$, and Tb.Th$_{tibia}$ showed significant but weaker ($R^2 < 0.25$) positive correlations. A full overview of all significant correlation of HRpQCT parameters with $\delta^{44/42}$Ca parameters of the MGBD group and each subgroup can be found in Table S1.

Group comparisons

The complete MGBD patient cohort was stratified into 2 subgroups: one exhibiting osteoporotic BMD as per WHO standards,[13] and another surpassing this established threshold (Figure 2A-D). There were no significant differences in $\delta^{44/42}$Ca$_{serum}$ or $\delta^{44/42}$Ca$_{urine}$ when comparing patients stratified by *T*-score. Subsequently, the MGBD cohort was stratified by $\delta^{44/42}$Ca$_{serum}$ and $\delta^{44/42}$Ca$_{urine}$ thresholds ($\delta^{44/42}$Ca$_{serum}$: −0.91‰; $\delta^{44/42}$Ca$_{urine}$: 0.16‰), respectively (Figure 2E-H). In $\delta^{44/42}$Ca$_{serum}$, subjects above the threshold significantly higher *T*-scores were seen than in subjects below this threshold. This was true in both *T*-score spine ($p = .0115$, Figure 3E) and *T*-score hip ($p = .0231$, Figure 3G). The same pattern, but with a greater effect, was observed for $\delta^{44/42}$Ca$_{urine}$, where subjects above the threshold in *T*-score spine ($p = .0026$, Figure 3F) and hip ($p = .0036$, Figure 3H) had significantly higher *T*-scores than the ones below the threshold.

Table 2. Correlation analysis of DXA and $\delta^{44/42}$Ca.

	DXA	With XLH	Without XLH
$\delta^{44/42}Ca_{serum}$	L1-L4 BMD	$R^2 = 0.4007$; $p \leq .0001$	$R^2 = 0.2084$; $p = .0286$
	L1-L4 BMC	$R^2 = 0.3945$; $p \leq .0001$	$R^2 = 0.2452$; $p = .0101$
	L1-L4 *T*-score	$R^2 = 0.3188$; $p = .0004$	$R^2 = 0.0911$; $p = .1260$
	Lowest hip BMD	$R^2 = 0.2685$; $p \leq .0001$	$R^2 = 0.1992$; $p = .0196$
	Lowest hip BMC	$R^2 = 0.3132$; $p = .0006$	$R^2 = 0.0731$; $p = .1911$
	Lowest hip *T*-score	$R^2 = 0.2356$; $p = .0027$	$R^2 = 0.0749$; $p = .1588$
$\delta^{44/42}Ca_{urine}$	L1-L4 BMD	$R^2 = 0.5974$; $p \leq .0001$	$R^2 = 0.1632$; $p = .0622$
	L1-L4 BMC	$R^2 = 0.5976$; $p \leq .0001$	$R^2 = 0.2430$; $p = .0123$
	L1-L4 *T*-score	$R^2 = 0.5405$; $p \leq .0001$	$R^2 = 0.1496$; $p = .0509$
	Lowest hip BMD	$R^2 = 0.3554$; $p \leq .0001$	$R^2 = 0.2900$; $p = .0045$
	Lowest hip BMC	$R^2 = 0.4832$; $p \leq .0001$	$R^2 = 0.1325$; $p = .0804$
	Lowest hip *T*-score	$R^2 = 0.2825$; $p = .0012$	$R^2 = 0.0864$; $p = .1367$

Abbreviation: XLH, X-linked hypophosphatemia.

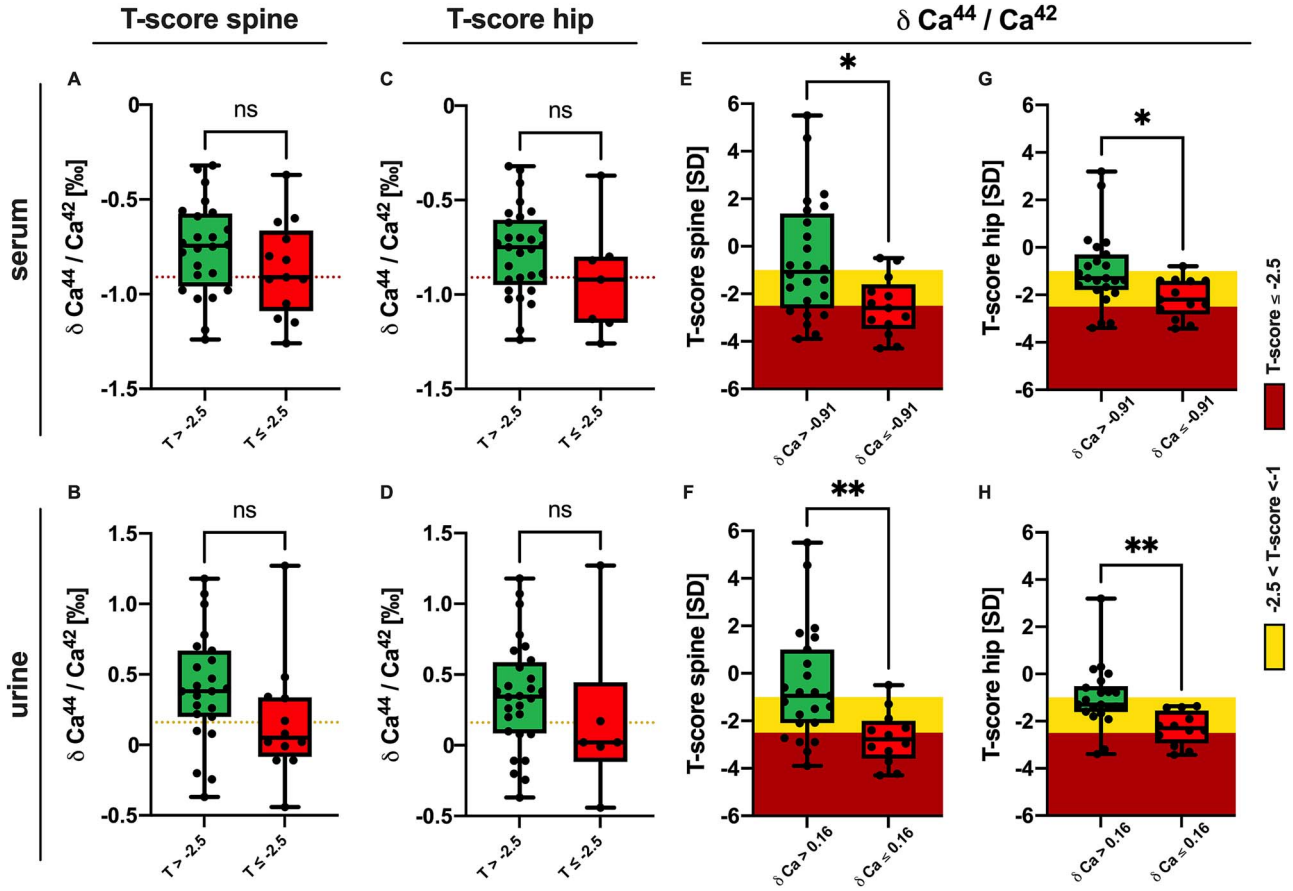

Figure 3. Comparative analysis of a $\delta^{44/42}$Ca based cutoff with established *T*-score-based thresholds. No significant differences were observed in $\delta^{44/42}Ca_{serum}$ (A, B) or $\delta^{44/42}Ca_{urine}$ (C, D) levels when stratifying by *T*-score. Stratifying the cohort using respective thresholds for $\delta^{44/42}Ca_{serum}$ and $\delta^{44/42}Ca_{urine}$ (serum: −0.91‰; urine: 0.16‰), in both spine (E, F) and hip *T*-score (G, H) $\delta^{44/42}$Ca was significantly higher in the group above the threshold. Abbreviations: ns, not significant; SD, standard deviation. $^*p < .05$; $^{**}p < .01$; $^{***}p < .001$; $^{****}p < .0001$.

When dividing the cohort into a group with and without fractures, $\delta^{44/42}Ca_{serum}$ did not differ significantly between the groups ($p = .0639$). However, $\delta^{44/42}Ca_{urine}$ was significantly higher in the group without fractures compared to the fractured group ($p = .0487$).

Comparison of MGBD subgroups

To compare the MGBD subgroups, we used the ANCOVA to account for significant age differences detected comparing the HPP group to all other subgroups. Age had a significant effect on $\delta^{44/42}$Ca levels ($\delta^{44/42}Ca_{serum}$: $\eta^2 = 0.1412$; $p = .0018$; $\delta^{44/42}Ca_{urine}$: $\eta^2 = 0.2497$; $p < .001$).

For $\delta^{44/42}Ca_{serum}$, no significant differences were measured between any MGBD and controls when comparing the mean per group (Figure 4A) yet a visual impression of elevated levels in XLH. For $\delta^{44/42}Ca_{urine}$, significant differences were measured when comparing XLH to EOOP, OI, and control (Figure 4B). Subsequently, we tested whether XLH solely stood out in $\delta^{44/42}$Ca or also in other tested parameters. No differences were seen in $Ca/creatinine_{urine}$ (the strongest

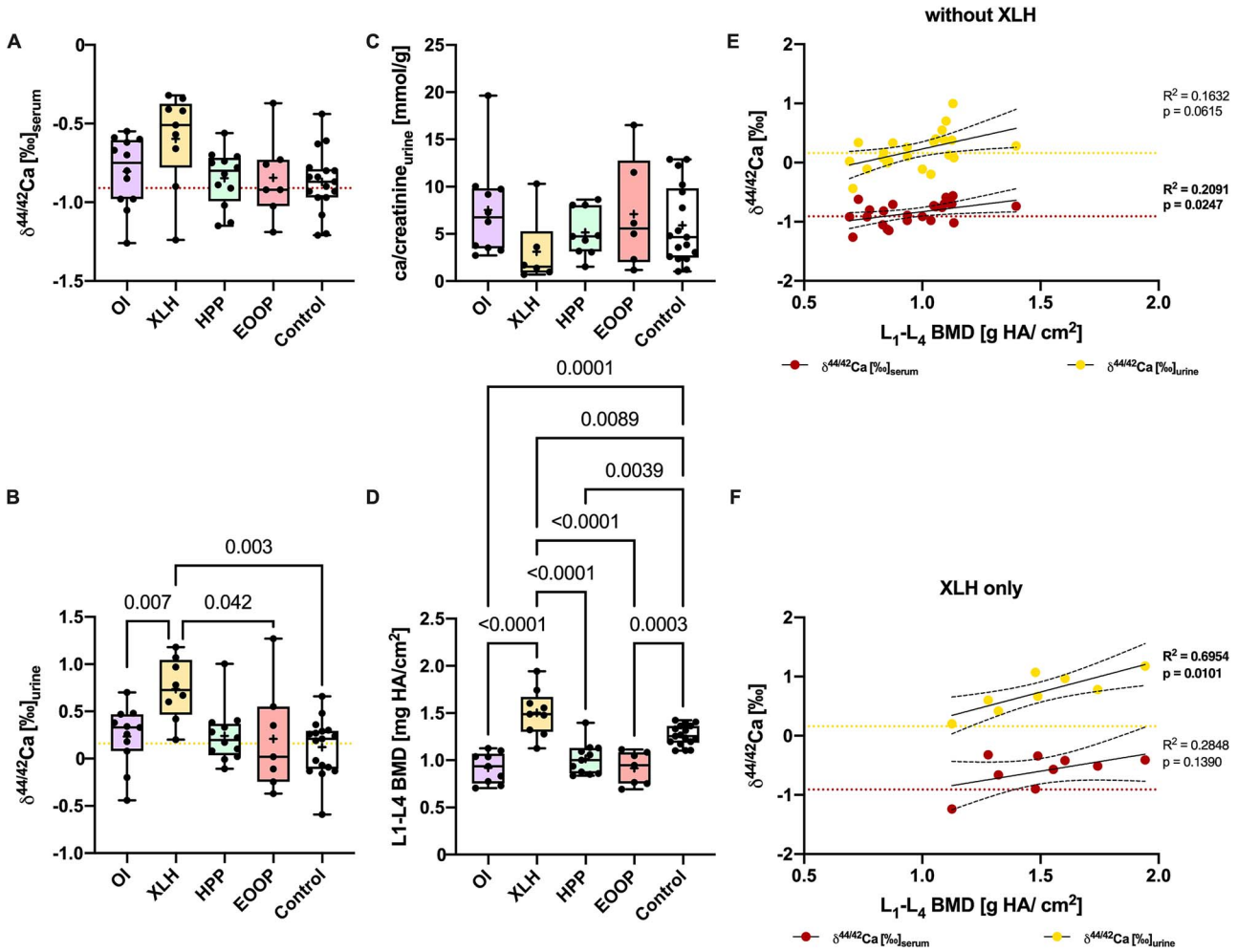

Figure 4. ANCOVA of $\delta^{44/42}$Ca and 2-way ANOVA for Ca/creatinine$_{urine}$ and L_1-L_4 with correlation analyses in the XLH subgroup. Using ANCOVA, we compared each MGBD subgroup and the control group in terms of their $\delta^{44/42}$Ca, with correction for age. While no differences were visible in $\delta^{44/42}$Ca$_{serum}$ (A), XLH was higher than OI, EOOP and the control group in $\delta^{44/42}$Ca$_{urine}$ (B). In L_1-L_4 BMD (D), XLH and controls differed significantly from all other groups, including each other. In Ca/creatinine$_{urine}$, XLH was trending towards lower values (C). Correlating spinal BMD with $\delta^{44/42}$Ca, results remained significant in serum after exclusion of XLH (E). In the XLH subgroup alone, the correlation of spinal BMD with $\delta^{44/42}$Ca was significant in urine (F). Abbreviations: ANCOVA, analysis of covariance; Tb.Sp, trabecular separation; XLH, X-linked hypophosphataemia, $*p < .05$; $**p < .01$; $***p < .001$; $****p < .0001$.

correlation), while XLH was significantly higher in L_1-L_4 BMD than every other group (Figure 4C, D). Furthermore, regarding L1-L4 BMD, all groups differed from control by exhibiting a lower BMD except XLH being significantly higher (Figure 4D).

Since $\delta^{44/42}$Ca$_{serum}$ in XLH was significantly different from other groups (Figure 4B) and appeared with higher BMD (Figure 4D), we examined whether or not XLH affected the correlations of densitometric parameters with $\delta^{44/42}$Ca. In a pooled group with all MGBD patients except XLH, L_1-L_4 BMD was only significantly correlated with $\delta^{44/42}$Ca in serum ($\delta^{44/42}$Ca$_{serum}$: $R^2 = 0.2091$, $p = .0247$; $\delta^{44/42}$Ca$_{urine}$: $R^2 = 0.1632$, $p = .0615$) (Figure 4E), while the analysis of the same parameters in the XLH subgroup was only significant in urine $\delta^{44/42}$Ca ($\delta^{44/42}$Ca$_{serum}$: $R^2 = 0.2848$, $p = .1390$; $\delta^{44/42}$Ca$_{urine}$: $R^2 = 0.6954$, $p = .0101$) (Figure 4F). All other correlations are listed in Table S1.

XLH sensitivity and specificity

ROC analysis revealed a sensitivity of 77.78% (95% CI: 45.26-96.05%) for $\delta^{44/42}$Ca$_{serum}$ at a diagnostic cutoff value of >-0.665 with specificity of 77.42% (95% CI: 60.19-88.60%). The area under the curve[14] for $\delta^{44/42}$Ca$_{serum}$ was 0.7706. For $\delta^{44/42}$Ca$_{urine}$, we chose a cutoff value of >0.575 was chosen, yielding a sensitivity of 75.00% (95% CI: 40.93-95.56%) and specificity of 90.00% (95% CI: 74.38-96.54%) with an AUC = 0.8583. In comparison, for L_1-L_4 BMD, a cutoff value of >1.119 was calculated, with a sensitivity of 100% (95% CI: 70.09-100.00%) and a specificity of 85.19% (95% CI: 67.52-94.08%). The AUC of BMD was 0.9774.

Parameter interaction in multiple linear regression

For $\delta^{44/42}$Ca$_{serum}$, the model consisted of the following, significant parameters: L_1-L_4 BMC, P1NP, and vitamin D_3, which explained 54.9% of the variance ($R^2 = 0.5489$; adjusted $R^2 = 0.4947$; root mean squared error (RMSE) = 0.164; Durbin-Watson Statistic = 1.5921 at $p = .2348$). The associated beta-coefficients are 0.7334 for L_1-L_4 BMC, -0.3176 for P1NP, and -0.2928 for vitamin D_3. For $\delta^{44/42}$Ca$_{urine}$, including L_1-L_4 BMC, ca/creatinine$_{urine}$ and age the model explained 79.9% of the variance ($R^2 = 0.7991$; adjusted $R^2 = 0.7739$; RMSE = 0.186; Durbin-Watson Statistic = 2.210

at $p = .6786$). For $\delta^{44/42}Ca_{urine}$, the beta-coefficients were 0.4528 for L_1-L_4 BMC, −0.3897 for ca/creatinine$_{urine}$, and −0.2800 for age. In both models, L_1-L_4 BMC has the highest normalized beta-value meaning the standardized slope, close to ca/creatinine$_{urine}$ in $\delta^{44/42}Ca_{urine}$.

Discussion

The analysis of calcium isotopes in blood and urine and calculating its ratio $\delta^{44/42}$Ca has recently gained interest as potentially helpful in osteoporosis diagnostics. By representing a measure for the balance of bone turnover, the $\delta^{44/42}$Ca ratio might indicate osteoporosis in patients with values below a specific threshold.[3] Consequently, this study aimed to investigate the diagnostic potential of the $\delta^{44/42}$Ca ratio in adult patients with MGBDs, namely EOOP, HPP, OI, and XLH as they represent the extremes of the spectrum of bone disorders. Specifically, we have examined whether patients with increased fracture susceptibility (milder in EOOP, more pronounced in OI) or osteomalacia (milder in HPP, more pronounced in XLH) do differ from each other or control regarding calcium isotope excretion via urine ($\delta^{44/42}Ca_{Urine}$) or serum isotope ratio ($\delta^{44/42}Ca_{Serum}$). In addition, we analyzed the correlation of the calcium isotope ratios to established laboratory and technical parameters for the diagnostic and monitoring of bone diseases.

In laboratory analysis, the strongest correlation for $\delta^{44/42}$Ca was found with the normalized urinary calcium excretion in spot urine, consistent with the literature.[15] Thus, $\delta^{44/42}$Ca may indicate a generally increased calcium excretion, which can be found in states of increased bone resorption and has earlier been associated with lower BMD.[16] Kidney function assessed by GFR was only weakly correlated with $\delta^{44/42}$Ca in this cohort of individuals with unaffected kidney function. This is of importance since kidney function is known to influence the calcium balance and the largest study of $\delta^{44/42}$Ca today was conducted in kidney-diseased children.[7] Well-established bone turnover markers[17] mirroring the bone resorption (DPD/crea$_{urine}$, β-CTX) and formation (bALP, osteocalcin, P1NP), as well as the ratio of P1NP and β-CTX did not correlate with $\delta^{44/42}$Ca, thus $\delta^{44/42}$Ca seems to reflect a different aspect of bone turnover/metabolism in adult patients with MGBD and healthy controls.

DXA measurements of the entire MGBD cohort correlated with $\delta^{44/42}$Ca parameters, with stronger associations observed in urine sampling compared to serum sampling. However, the correlation between $\delta^{44/42}$Ca and densitometric parameters became significantly weaker after the exclusion of XLH patients from the analysis. Thus, the XLH subgroup predominantly drove the effect size of the correlations in the pooled cohort by higher BMD and $\delta^{44/42}$Ca values. However, the characteristics of the interaction of $\delta^{44/42}$Ca and densitometric measures remained the same. Thus, $\delta^{44/42}$Ca may not directly reflect BMD but rather a metabolic state evident in XLH patients, which is linked to higher BMD and $\delta^{44/42}$Ca values. HRpQCT and $\delta^{44/42}$Ca parameters had significant but only weak effect sizes in correlations in both, density and structure, indicating limited interaction of $\delta^{44/42}$Ca levels and bone structure or 3D density.

To further understand the interaction of $\delta^{44/42}$Ca and BMD parameters, a stratification for osteoporotic T-scores[13] was performed but did not indicate significant differences in $\delta^{44/42}$Ca. This indicates that adult MGBD patients with low BMD do not necessarily exhibit a low $\delta^{44/42}$Ca. However, stratification for the $\delta^{44/42}$Ca thresholds did reveal significantly lower T-scores in the group of low $\delta^{44/42}$Ca. Interestingly, this effect was especially accentuated in the spot urine sampling. This may be plausible since increasing serum calcium levels are physiologically addressed by an increased renal calcium excretion to maintain constant serum calcium levels.[18] Additionally, testing $\delta^{44/42}$Ca to be indicative of fractures, our results point towards differences in the $\delta^{44/42}$Ca, specifically in urine sampling.

In $\delta^{44/42}Ca_{serum}$, after adjusting for age, no significant differences were seen among adult MGBD patients. In urine, only XLH patients exhibited increased $\delta^{44/42}$Ca. This higher $\delta^{44/42}$Ca suggests increased calcium incorporation (low ^{42}Ca) due to the calcium need ("hungry bone") known for malacia conditions.[5] Together with lower calcium excretion, the spinal BMD was significantly higher in the XLH cohort, compared to all other groups, suggesting an increase in bone mass (more malacic bone) in these patients. All included XLH patients experienced naturally severe renal phosphate wasting, causing dysfunctional bone structures[19] and matrix formation[20,21] potentially leading to increased total volume of mineralized and unmineralized (osteoid) bone tissue.[22] XLH is characterized by higher osteoid quantities,[14] a feature shared with growing bone during adolescence. Thus, high $\delta^{44/42}$Ca values in both conditions might align with increased bone formation.[3]

Interestingly, the HPP cohort also known to present at least in part as a mineralization disorder with increased osteoid volume did not exhibit significantly increased $\delta^{44/42}$Ca ratios, even when adjusted for its significantly higher age. However, the increased osteoid volume here results from reduced enzymatic activity rather than low serum levels of electrolytes such as phosphate or calcium, which may explain the difference in $\delta^{44/42}$Ca. Consequently, while electrolyte metabolism remains unaffected, the subsequent mineralization process is disrupted despite adequate calcium and phosphate serum levels.

MGBD that are primarily described by a fracture susceptibility due to low bone mass or decreased bone quality, such as EOOP and OI, do not exhibit a state of osteomalacia or "hungry bone", which may be a reason why these groups do not exceed or fall below the thresholds.

With respect to the diagnostic potential of the calcium isotope ratio $\delta^{44/42}$Ca, it is important to note that significant correlation to BMD assessed by DXA and bone microstructure assessed by HRpQCT could be found. In addition, patients stratified by $\delta^{44/42}$Ca thresholds did exhibit significantly different BMD values. However, no significant differences in $\delta^{44/42}$Ca were found between patients stratified by BMD within the osteoporotic range and those above. Moreover, in the cohort of adult MGBD patients, $\delta^{44/42}$Ca ratios below the threshold indicating disturbed bone balance were only found in 11 out of 40 patients in serum and 12 out of 38 patients in urine. In contrast, in the healthy control group 7 out of 17 individuals showed $\delta^{44/42}$Ca below the threshold in serum and 7 out of 17 individuals in urine. Thus, only about one-third of the MGBD patients showed $\delta^{44/42}$Ca values below the threshold, while half of the healthy control individuals also showed $\delta^{44/42}$Ca values below the threshold.

Since interactions of the different, measured parameters are multiple, we have performed multiple linear regression. Interestingly, our results point to the L1-L4 BMC and calcium excretion to be the strongest parts of the regression. This

indicates BMC to have a main influence on the $\delta^{44/42}$Ca values with positive slope and calcium excretion counteracting the effect of BMC with a negative slope. Yet, our data does not indicate clear advantages or enhanced diagnostic accuracy of $\delta^{44/42}$Ca compared to existing methods in adults with MGBD.

This study has limitations, such as a rather small cohort for each disease group. But given all MGBD being rare diseases, subgroup size is already reasonable for such, and results achieve statistical significance for the studied parameters.

Conclusion

In conclusion, most significant correlations for calcium isotope ratios $\delta^{44/42}$Ca were detected with calcium per creatinine$_{urine}$ also reflecting the potential of renal calcium excretion in spot urine as a parameter for monitoring bone metabolism. In addition, significant correlations for $\delta^{44/42}$Ca were observed with BMD, particularly spinal BMD from urine sampling, as well as with certain HRpQCT parameters, most notably the total mineral content at the tibia. However, the results of this study do not support $\delta^{44/42}$Ca as a potential diagnostic tool for MGBD. Among all MGBD groups, XLH stood out with the most significant results regarding increased $\delta^{44/42}$Ca and BMD, likely due to the increased calcium demand ("hungry bone") associated with this pronounced malacic condition. The data presented here call for further investigation into calcium isotopes to clarify the metabolic processes reflected by $\delta^{44/42}$Ca and assess its utility in monitoring metabolic bone diseases. Particularly, larger and more uniform cohorts are needed to investigate this parameter of interest.

Author contributions

Robert Munziger: Methodology, Formal analysis, Investigation, Writing—Original Draft Felix N. Schmidt: Conceptualization, Methodology, Formal analysis, Investigation, Writing—Original Draft, Writing—Review & Editing, Supervision, Project administration. Mikolaj Bartosik: Methodology, Formal analysis, Writing—Review & Editing. Florian Barvencik: Writing—Review & Editing. Ralf Oheim: Writing—Review & Editing, Supervision, Project administration. Michael Amling: Conceptualization, Writing—Review & Editing, Supervision, Project administration.

Robert Munzinger (Data curation, Investigation, Methodology, Validation, Visualization), Felix N. von Brackel (Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Visualization), Mikolaj Bartosik (Data curation, Formal analysis, Methodology), Michael Amling (Conceptualization, Project administration, Supervision, Validation), and Ralf Oheim (Project administration, Supervision)

Robert Munzinger and Felix N. von Brackel contributed equally to this manuscript and share therefore first authorship.

Supplementary material

Supplementary material is available at *JBMR Plus* online.

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Conflicts of interest

R.M., FvB., M.B., and M.A. have no conflict of interest. F.B. has received speakers' fees from Alexion, UCB, Diasorin and has received institutional research grants from UCB and Alexion. R.O. has served as a speaker and advisory board member for Kyowa Kirin, Inozyme, Ipsen, Pharmacosmos, Mereo, and UCB and has received an institutional research grant from Kyowa Kirin and UCB.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request. Data access will be provided in accordance with applicable local guidelines, laws, and the ethical approval obtained for this study.

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