



## SONDERDRUCK

Naturally occurring stable calcium isotope ratios  
are a novel biomarker of bone calcium balance  
in chronic kidney disease



# Naturally occurring stable calcium isotope ratios are a novel biomarker of bone calcium balance in chronic kidney disease

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Dysregulated calcium homeostasis is common in chronic kidney disease and causally associated with disorders of bone mineralization. However, radiological measures and biomarkers do not allow accurate evaluation of bone calcium balance. Non-radioactive calcium isotopes, <sup>42</sup>Ca and <sup>44</sup>Ca, are present in our diet and sequestered into body compartments following principles of kinetic isotope fractionation. Isotopically light <sup>42</sup>Ca is preferentially incorporated into bone, while heavier <sup>44</sup>Ca is excreted. The ratio (<sup>44/42</sup>Ca<sub>serum</sub>) increases when bone formation exceeds resorption and vice versa, reflecting bone calcium balance. We measured these calcium isotopes by inductively coupled plasma mass-spectrometry in blood, urine and feces of 42 children with chronic kidney disease and 92 receiving dialysis therapy. We compared the isotope ratios with bone biomarkers and determined total bone mineral content by dual-energy x-ray absorptiometry and peripheral quantitative CT expressed as age-adjusted z-scores. The <sup>44/42</sup>Ca<sub>serum</sub> ratio positively correlated with serum calcium, 25-hydroxyvitamin D and alkaline phosphatases and inversely with serum parathyroid hormone and other bone resorption markers. The <sup>44/42</sup>Ca<sub>serum</sub> ratio positively correlated with age-adjusted z-scores of tibial trabecular bone mineral density and total bone mineral content measured by peripheral quantitative CT, and hip bone mineral density measured by dual-energy X-ray absorptiometry. Significant and independent

predictors of total bone mineral content, measured by, were the <sup>44/42</sup>Ca<sub>serum</sub> ratio and parathyroid hormone. The <sup>44/42</sup>Ca<sub>serum</sub> ratio, repeated after four weeks, highly correlated with baseline values. When adjusted for calcium-containing medications and kidney impairment, the <sup>44/42</sup>Ca<sub>serum</sub> ratio in patients receiving dialysis was 157% lower than that of age-matched children and 29% lower than levels in elderly women with osteoporosis, implying significantly lower bone mineral content. Thus, calcium isotope ratios may provide a novel, sensitive and non-invasive method of assessing bone calcium balance in chronic kidney disease.

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In patients with chronic kidney disease (CKD), mineral bone disorder is common and associated with significant morbidity.<sup>1</sup> Dysregulated calcium (Ca) and phosphate homeostasis leads to abnormalities in bone formation and resorption, and is causally associated with reduced bone mineral density (BMD)<sup>2</sup> and an increased risk of fractures.<sup>3</sup> The growing skeleton of children is uniquely vulnerable: 29% in CKD stage 2 and >90% on dialysis have deficient mineralization,<sup>4</sup> manifesting as bone deformities, and a 2 to 3 fold higher fracture risk compared with healthy peers.<sup>3</sup> The assessment of bone health is a major challenge for physicians.<sup>5,6</sup> Although bone biopsy with histomorphometry is considered “gold standard”

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for assessing bone formation, it is invasive and cannot be easily repeated in children. Bone biomarkers reflect either the bone formation or the bone resorption process but not the net state of bone turnover, nor the bone calcium balance (BCaB).<sup>7</sup> Radiological changes may take months to years to manifest changes in BMD.<sup>7</sup> Dual-energy X-ray absorptiometry (DXA), although widely used, is not a reliable predictor of fracture risk, and is not recommended as a screening tool in patients with CKD.<sup>8</sup> Traditional Ca balance studies administering oral and i.v. Ca isotopes have been performed but are mainly limited to Ca absorption studies.<sup>1,9–12</sup> Radioactive isotopes of Ca have been used in mass balance experiments, but these studies are highly laborious, requiring a “metabolic ward” setting, limiting their routine clinical use. Ca balance has been extensively studied in healthy individuals, but only 2 Ca balance studies have been performed in patients with CKD.<sup>13,14</sup> A biomarker of BCaB that is easily measurable, sensitive, and specific and reflects “real-time” changes in bone mineralization and demineralization is required.

Natural Ca isotopes have been extensively studied in marine geological systems (changes in marine calcification reflect global warming)<sup>15,16</sup> and space travel (to study BCaB in low-gravity environments).<sup>17,18</sup> These naturally occurring stable (i.e., nonradioactive) Ca isotopes are present in our diet and incorporated into different body compartments, and they may provide a novel, noninvasive method of assessing BCaB. Naturally occurring nonradioactive Ca isotopes (<sup>40</sup>Ca, <sup>42</sup>Ca, <sup>43</sup>Ca, <sup>44</sup>Ca, <sup>46</sup>Ca, and <sup>48</sup>Ca)<sup>19–21</sup> are present in our food and water<sup>22</sup> and sequestered in different body compartments following distinct rules of kinetic isotope fractionation, depending on their atomic mass.<sup>15</sup> Isotope fractionation is the physicochemical mechanism for separation of heavier and lighter isotopes in at least 2 body compartments,<sup>21</sup> such that isotopically light Ca is preferentially enriched in the course of chemical transport reactions (e.g., from the diet to the skeleton), whereas the heavy isotope is preferentially excreted in urine and feces due to a Rayleigh distillation behavior.<sup>22–26</sup> As a major consequence, each body compartment is characterized by a unique and individual Ca isotope ratio, which reflects age, sex, and health status, as shown in our previous work.<sup>23,24,27</sup> The ratio of Ca isotopes (e.g., when studying <sup>42</sup>Ca and <sup>44</sup>Ca, the ratio would be expressed as  $\delta^{44/42}\text{Ca}$ ) compared with a known standard is described mathematically in a compartment model<sup>22–24,26,28</sup> that, in turn, is used to quantitatively determine net bone gain or loss of Ca. Thus, when bone formation exceeds bone resorption and the net BCaB is positive, the  $\delta^{44/42}\text{Ca}_{\text{serum}}$  isotope values are higher compared with  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values measured under conditions when bone resorption is the predominant process.

Recently, we studied the naturally occurring Ca isotope ratios in the serum, urine, and feces of healthy individuals and found significantly higher  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values in children compared with young adults.<sup>27</sup> The  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values correlated with height and pubertal stage, reflecting an avid uptake of Ca by the growing skeleton.<sup>27</sup> The  $\delta^{44/42}\text{Ca}_{\text{serum}}$  value correlated positively with biomarkers of bone formation, and inversely with bone

resorption markers, and was a significant and independent predictor of tibial cortical BMD measured on peripheral quantitative computed tomography (pQCT) scan,<sup>27</sup> a reliable measure of bone mineral content.<sup>2,29</sup> Our group and others have also studied the sensitivity of natural Ca isotope measures in animal models<sup>15,20,30,31</sup> and healthy adults,<sup>22,24,25,28,32–37</sup> where  $\delta^{44/42}\text{Ca}$  in serum and urine closely reflected the interventions that alter bone homeostasis, such as complete bed rest,<sup>25</sup> vitamin D supplementation,<sup>35</sup> bisphosphonate treatment,<sup>22</sup> and myeloma-induced bone disease.<sup>37</sup> In a rodent model of 6 animals with CKD, the  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values were lower compared with control animals and correlated with BMD.<sup>31</sup> Although disorders of BCaB are common in CKD,<sup>4,8</sup> the Ca isotope fractionation technique has not been applied in this population so far.

We hypothesize that natural Ca isotope fractionation is a sensitive measure of changes in BCaB in children with CKD and on dialysis. We correlated  $\delta^{44/42}\text{Ca}_{\text{serum}}$  and  $\delta^{44/42}\text{Ca}_{\text{urine}}$  with radiological measures of BMD and biomarkers of bone formation and resorption to evaluate the clinical utility of  $\delta^{44/42}\text{Ca}_{\text{serum}}$  and  $\delta^{44/42}\text{Ca}_{\text{urine}}$  as novel biomarkers of BCaB.

## METHODS

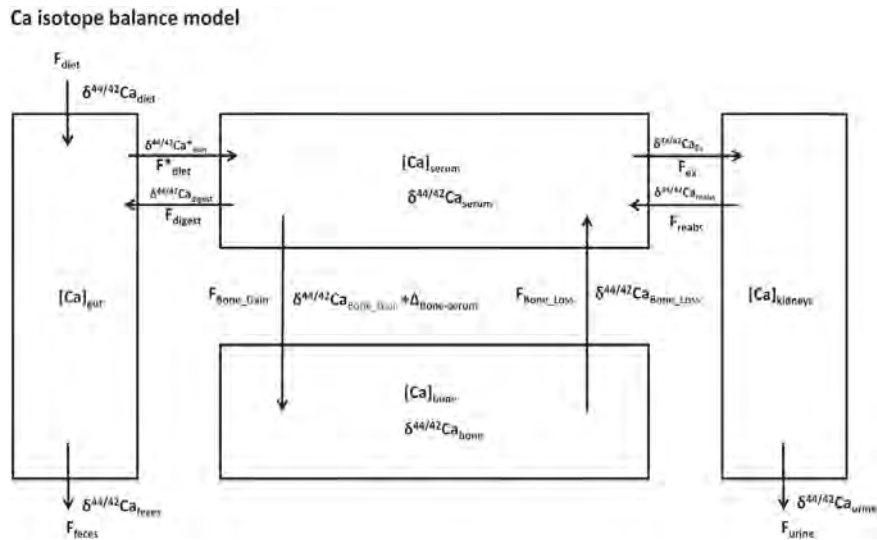
This is a prospective, multicenter study across 5 European pediatric nephrology centers. Children aged <18 years with CKD stages 4–5 (CKD4–5) and those receiving chronic dialysis (hemodialysis [HD] or peritoneal dialysis [PD]) were included. Children with preexisting bone disease (inherited or acquired), those with fractures in the preceding 6 months, those with any acute illness in the preceding 2 weeks, those on glucocorticoid therapy in the preceding year, or those with a lifetime cumulative steroid exposure of >6 months, or those who had received bisphosphonates at any time, were excluded. A total of 134 children (42 in CKD4–5 and 92 on dialysis) were included in the analysis. All participants and/or their caregivers provided informed consent or assent as appropriate. The study is registered on [clinicaltrials.gov](https://clinicaltrials.gov) (NCT03285854) and approved by all local research ethics committees.

## Study measures

Participants were asked to complete a 3-day food diary and bring a 24-hour urine sample and a single fecal sample on the day of the study visit. The study visit included anthropometric measures, fasting blood sampling, DXA, and pQCT. In HD patients, all measurements were taken before a midweek dialysis session. Details of anthropometry, biomarker assays, and radiological measures have been described<sup>27</sup> (full details in [Supplementary Methods](#)).

## Repeatability study

A longitudinal study was conducted in a subset of patients to examine subject variability and test the validity of the Ca isotope technique. In 22 children (7 in CKD5 and 15 on dialysis), we repeated fasting serum and urine tests after a median interval of 24 (interquartile range, 19–33) days. Patients were selected to ensure that there was no change in the CKD status or urine output (24-hour measures recorded) and no change to their Ca-containing medications or vitamin D prescriptions between the 2 assessments. The intrasubject variability in Ca intake was determined from the 3-day food records and was  $\pm 6.3\%$  (interquartile range, 2.1%–9.6%). All biomarkers were measured, but radiological measures were not repeated given the short interval from baseline measures.



**Figure 1 | This mathematical model describes the simplified path of Ca in a healthy person from the diet toward its excretion in urine and feces, and exchange with the Ca reservoir in the bone, via the blood system. The Ca fluxes between compartments and distinct isotopic composition of each compartment are shown.**

**Comparison with healthy controls**

Ca isotope values in CKD and dialysis patients were compared with our recently published data from age-matched healthy children and young adults<sup>27</sup> as well as with data from a study in elderly women with and without osteoporosis.<sup>24</sup> In both studies, healthy participants underwent similar biochemical tests and radiological investigations, including DXA and pQCT.

**Ca isotope measurements**

After Ca extraction and chemical purification (details in [Supplementary Methods](#)), Ca isotope measurements were performed on a multicollector inductively coupled plasma mass spectrometer (Neptune plus; Thermo Fisher Scientific) at the mass spectrometer facilities of the GEOMAR Helmholtz Centre for Ocean Research, Kiel, Germany. The mass spectrometer was equipped with 9 Faraday cups, of which 8 are moveable. The mass spectrometer was set up to measure masses 42, 43, 43.5, and 44 simultaneously. To suppress interfering Ca and Ar hydrides (e.g., 40Ar1H2 on 42Ca), an APEX IR (ESI) sample introduction system was used. All measurements were performed in medium resolution ( $m/\Delta m \sim 4000$ ) on the interference-free plateau of the low mass side of the peaks. This was achieved by choosing an appropriate center cup mass of  $43.687 \pm 0.001$  atomic mass unit (amu) and verified on a daily basis, as previously described.<sup>24</sup> Instrumental fractionation (mass bias) was corrected by applying the standard-sample-bracketing approach. The measurement of a sample was bracketed by measurements of a  $\sim 5$   $\mu\text{g/ml}$  Ca solution prepared from a 10,000  $\mu\text{g/g}$  Ca inductively coupled plasma reference solution (Ca inductively coupled plasma). Every sample was measured at least 4 times during a session, and the mean value was used for further calculations. The Ca isotopic composition is reported as  $\delta^{44/42}\text{Ca}$  in parts per thousand (‰)<sup>38</sup>:

$$\delta^{44/42}\text{Ca} (\text{‰}) = \left[ \frac{({}^{44}\text{Ca}/{}^{42}\text{Ca})_{\text{Sample}}}{({}^{44}\text{Ca}/{}^{42}\text{Ca})_{\text{Reference}}} \right] - 1. \tag{Equation 1}$$

**Model for bone Ca absorption and resorption**

We discuss the 4-compartment model proposed by Skulan and De Paolo ([Figure 1](#)) to explain the causal link between measured

changes in the Ca isotope values in the diet ( $\delta^{44/42}\text{Ca}_{\text{diet}}$ ), serum ( $\delta^{44/42}\text{Ca}_{\text{serum}}$ ), urine ( $\delta^{44/42}\text{Ca}_{\text{urine}}$ ), and feces ( $\delta^{44/42}\text{Ca}_{\text{feces}}$ ) with changes in Ca fluxes between body compartments (full details in [Supplementary Methods](#)). In healthy individuals,  $\delta^{44/42}\text{Ca}_{\text{serum}}$  is determined by the diet ( $F_{\text{Diet}}, \delta^{44/42}\text{Ca}_{\text{Diet}}$ ) and the Ca fluxes in (formation) and out (resorption) of the bones. Major factors controlling the  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values are bone resorption/formation processes, expressed by the ratio of  $F_{\text{Bone\_Loss}}$  to  $F_{\text{Bone\_Gain}}$ , such that in healthy individuals:

$$\delta^{44/42}\text{Ca}_{\text{serum}} \approx F_{\text{Bone\_Loss}}/F_{\text{Bone\_Gain}}. \tag{Equation 2}$$

The Ca balance model described above is based on the assumptions that the diet contains Ca from natural biological sources (dairy, vegetables, and meat)<sup>23,39</sup> and that kidney function is normal. To compare the  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values of CKD and dialysis patients with healthy controls, adjustments to the measured values were performed (full details in [Supplementary Methods](#) and [Supplementary Tables S1](#) and [S2](#)).

**RESULTS**

**Baseline characteristics**

The median age of the study population was 11.3 (interquartile range, 6.1–15.7) years. There was no difference in sex, race, or underlying kidney disease between groups, but children on dialysis were older and had lower anthropometric measures ([Table 1](#)). Children on dialysis had a significantly higher Ca intake from medications and total Ca intake from diet and medications compared with those in CKD4–5 ( $P = 0.04$  and  $P = 0.02$ , respectively; [Table 1](#)). A total of 91% of children on dialysis received a vitamin D analog compared with 64% with CKD ( $P = 0.002$ ), but the median vitamin D dosage was comparable between groups. There was a positive association between the cholecalciferol and alfacalcidol dose and  $\delta^{44/42}\text{Ca}_{\text{serum}}$  in CKD and dialysis patients ([Supplementary Figure S1](#)). Unlike age-matched healthy children in whom there was a strong correlation between

**Table 1 | Demographics of the study population**

Variable	CKD (n = 42)	Dialysis (n = 92)	P value
Age, yr	10.0 (4.9 to 14.0)	11.8 (5.8 to 15.1)	0.04
Female sex, n (%)	26 (61.9)	53 (57.6)	0.19
Tanner stage			
Males (1–3/4/5)	6:5:5	15:15:9	0.27
Females (1–3/4/5)	11:8:7	22:14:17	0.54
Race (White/Asian/Black/mixed or other)	30:8:3:1	66:13:9:4	0.81
Underlying kidney disease (dysplasia/glomerulonephritis/cystic kidney disease/others or unknown)	28:6:4:4	51:19:13:9	0.63
Type of dialysis (PD/HD)	—	37:55	—
Time in CKD (eGFR, <30 ml/min per 1.73 m <sup>2</sup> ) yr	4.1 (0.9 to 8.7)	3.8 (0.6 to 9.2)	0.77
Time on dialysis, yr	—	2.3 (0.9–5.4)	—
Anthropometry			
Height SDS	–1.1 (–2.6 to –0.09)	–2.0 (–3.7 to –1.1)	0.02
Weight SDS	–0.43 (–0.67 to 0.93)	–1.7 (–3.1 to 0.8)	0.04
Body mass index SDS	0.46 (–0.67 to 0.93)	–0.12 (–0.98 to 0.75)	0.04
Ca intake from diet and medications			
Ca intake from diet, mg/d <sup>a</sup>	536 (354 to 708)	550 (400 to 769)	0.09
Ca intake from medications, mg/d <sup>b</sup>	475 (300 to 1125)	900 (319 to 1256)	0.04
Total Ca intake from diet and medications, mg/d	1015 (883 to 1382)	1304 (863 to 1949)	0.02
RNI for total Ca intake from diet and medications, %	97 (87 to 143)	127 (78 to 183)	0.003
Native vitamin D (cholecalciferol)			
n (%)	30 (71.4)	74 (80.4)	0.25
Dosage, IU/d	2400 (0 to 8000)	3200 (0 to 12,000)	0.09
Active vitamin D (alfacalcidol or calcitriol)			
n (%) <sup>c</sup>	27 (64)	84 (91)	0.002
Dosage, µg/d	0.28 (0.2 to 1.35)	0.30 (0.2 to 1.55)	0.61
Other medications			
Cinacalcet	0	0	—
Growth hormone	1	2	—

CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; HD, hemodialysis; PD, peritoneal dialysis; RNI, reference nutrient intake; SDS, SD score.

<sup>a</sup>Diet diaries available in n = 27 CKD and n = 42 patients on dialysis.

<sup>b</sup>Medications include calcium-based phosphate binders (n = 22 CKD, and n = 77 dialysis) and calcium supplements (n = 2 CKD, and n = 7 dialysis).

<sup>c</sup>Calcitriol, n = 3 (all dialysis); rest all on alfacalcidol.

Data are presented as median (interquartile range), unless otherwise indicated.

$\delta^{44/42}\text{Ca}_{\text{serum}}$  and skeletal growth (height and Tanner stage),<sup>27</sup> no correlation was seen in CKD4–5 and dialysis patients. A total of 37 (40%) of the dialysis cohort were anuric.

**Serum Ca isotope fractions and biomarkers of mineral bone disease**

Children with CKD had a lower serum ionized Ca, phosphate, magnesium, and parathyroid hormone (PTH) compared with those on dialysis (Supplementary Table S3). Correlations of  $\delta^{44/42}\text{Ca}_{\text{serum}}$  with biomarkers are shown in Figure 2. Serum ionized Ca levels showed a positive correlation with  $\delta^{44/42}\text{Ca}_{\text{serum}}$  in CKD and dialysis patients ( $P < 0.0001$  [ $R^2 = 0.45$ ] and  $P = 0.03$  [ $R^2 = 0.13$ ], respectively). Serum alkaline phosphatase (ALP) also showed a positive linear correlation with  $\delta^{44/42}\text{Ca}_{\text{serum}}$  in dialysis ( $P < 0.001$ ,  $R^2 = 0.38$ ) but not in CKD patients ( $P = 0.44$ ). Similarly, serum 25-hydroxyvitamin D concentrations positively correlated with  $\delta^{44/42}\text{Ca}_{\text{serum}}$  in dialysis patients only ( $P = 0.04$ ,  $R^2 = 0.17$ ). PTH inversely correlated with  $\delta^{44/42}\text{Ca}_{\text{serum}}$  in dialysis patients ( $P = 0.013$ ,  $R^2 = 0.37$ ), with a trend toward significance in CKD patients ( $P = 0.06$ ,  $R^2 = 0.18$ ). Similarly, biomarkers of bone resorption tartrate-resistant acid phosphatase 5b and C-terminal telopeptide of type I collagen showed an inverse correlation with  $\delta^{44/42}\text{Ca}_{\text{serum}}$  ( $P = 0.004$  [ $R^2 = 0.31$ ] and

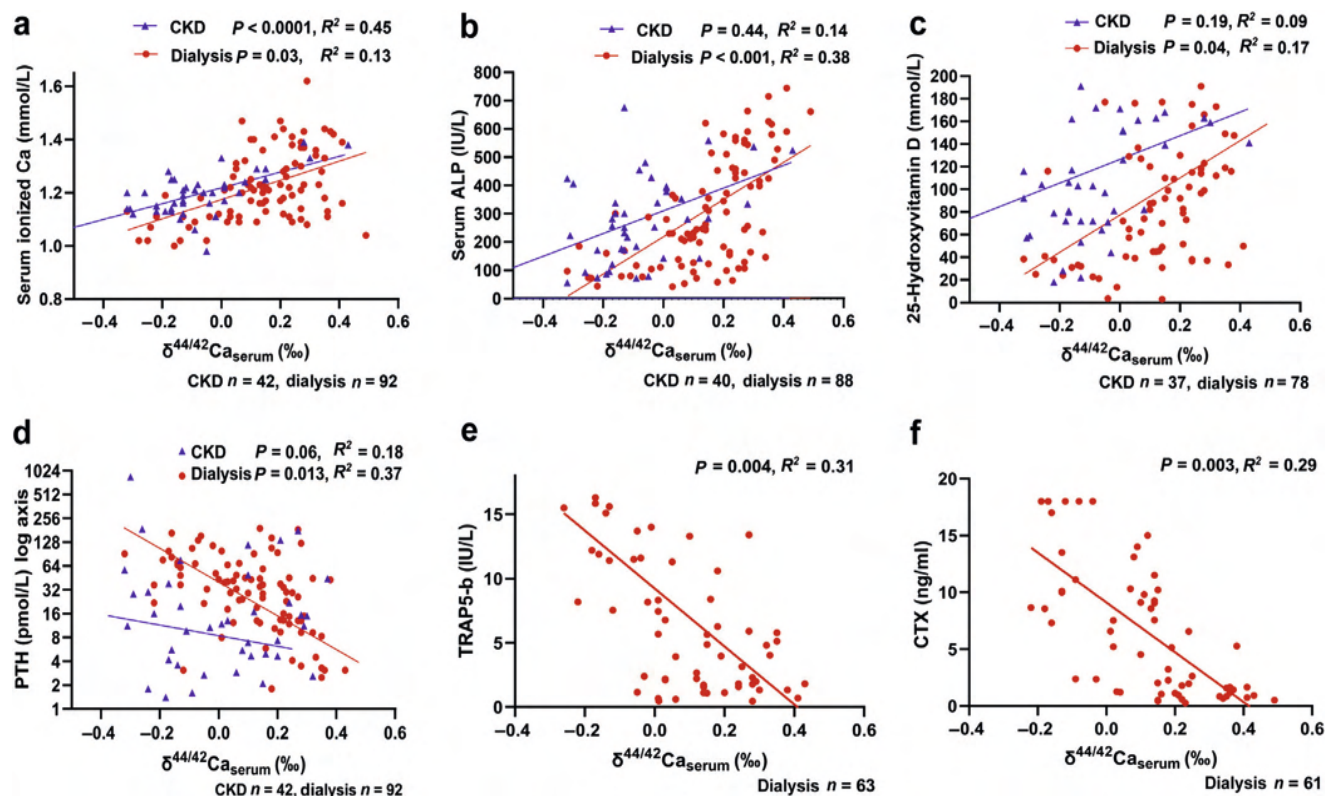
$P = 0.003$  [ $R^2 = 0.29$ ]) in dialysis patients. There were no correlations between  $\delta^{44/42}\text{Ca}_{\text{urine}}$  and any of the biomarkers in CKD and dialysis patients.

**Ca isotope fractions and radiological measures**

There was no significant difference in DXA or pQCT measures between children with CKD4–5 and those on dialysis (Supplementary Table S3). In dialysis patients, both the DXA hip and lumbar spine z-scores correlated positively with  $\delta^{44/42}\text{Ca}_{\text{serum}}$  ( $P = 0.002$  [ $R^2 = 0.41$ ] and  $P = 0.002$  [ $R^2 = 0.24$ ], respectively; Figure 3). In dialysis patients, both the tibial trabecular volumetric BMD z-score and the total bone mineral content z-score (calculated from the trabecular and cortical BMD measured by pQCT) correlated positively with  $\delta^{44/42}\text{Ca}_{\text{serum}}$  ( $P = 0.001$ ,  $R^2 = 0.37$  [Figure 3c] and  $P < 0.0001$ ,  $R^2 = 0.52$  [Figure 3d]), implying increasing bone mineral content as bone Ca content increases. In patients with CKD4–5, no correlations were seen between  $\delta^{44/42}\text{Ca}_{\text{serum}}$  and any radiological measures. Furthermore,  $\delta^{44/42}\text{Ca}_{\text{urine}}$  did not correlate with any of the radiological measures in CKD4–5 or dialysis patients.

On univariable analysis, significant correlations with the total bone mineral content z-score (in the dialysis cohort) were  $\delta^{44/42}\text{Ca}_{\text{serum}}$ , ionized Ca, ALP, PTH, cross-linked





**Figure 2 | Correlation of measured  $\delta^{44/42}\text{Ca}_{\text{serum}}$  (‰) with (a) serum ionized calcium, (b) alkaline phosphatase (ALP), (c) 25-hydroxyvitamin D, (d) parathyroid hormone (PTH), (e) tartrate-resistant acid phosphatase 5-b (TRAP5-b), and (f) cross-linked telopeptides of type 1 collagen (CTX). Chronic kidney disease (CKD) patients are shown in blue triangles, and dialysis patients are shown in red circles, with the number of patients included for each analysis indicated on the graphs.**

C-telopeptide of type I collagen (CTX), and DXA lumbar spine z-score (Supplementary Table S4). After adjustment for age, sex, and Tanner stage, all variables with  $P < 0.2$  on univariable analysis were included in a stepwise multivariable regression model. Significant and independent predictors of the total bone mineral content z-score were  $\delta^{44/42}\text{Ca}_{\text{serum}}$  ( $\beta = 0.68$ ,  $P = 0.006$ ) and PTH ( $\beta = -0.39$ ,  $P = 0.04$ ), together predicting 67% of the variability in bone mineral content, with the strongest correlation seen with  $\delta^{44/42}\text{Ca}_{\text{serum}}$ . There were no significant correlations between  $\delta^{44/42}\text{Ca}_{\text{serum}}$  and any DXA measurements in CKD or dialysis cohorts.

### Effect of dialysis

Children on HD and PD had comparable  $\delta^{44/42}\text{Ca}_{\text{serum}}$  and  $\delta^{44/42}\text{Ca}_{\text{urine}}$  values ( $P = 0.12$  and  $P = 0.69$ , respectively; Supplementary Figure S2A). There was no difference in  $\delta^{44/42}\text{Ca}_{\text{serum}}$

$\text{Ca}_{\text{serum}}$  values between dialysis patients who were anuric compared with those with a urine output ( $P = 0.11$ ; Supplementary Figure S2B).

To assess Ca fluxes during dialysis and their effect on isotope composition, in a subgroup of 10 children on HD, paired serum and dialysate  $\delta^{44/42}\text{Ca}$  ratios at baseline, 1 hour, and 4 hours into the dialysis session were measured (Supplementary Figure S3). The  $\delta^{44/42}\text{Ca}_{\text{serum}}$  increased by 0.1 to 0.2 ‰ from baseline to the end of the HD session ( $P = 0.01$ ), indicating some exchange of Ca from the dialysate into blood; this was independent of the Ca content of the dialysis

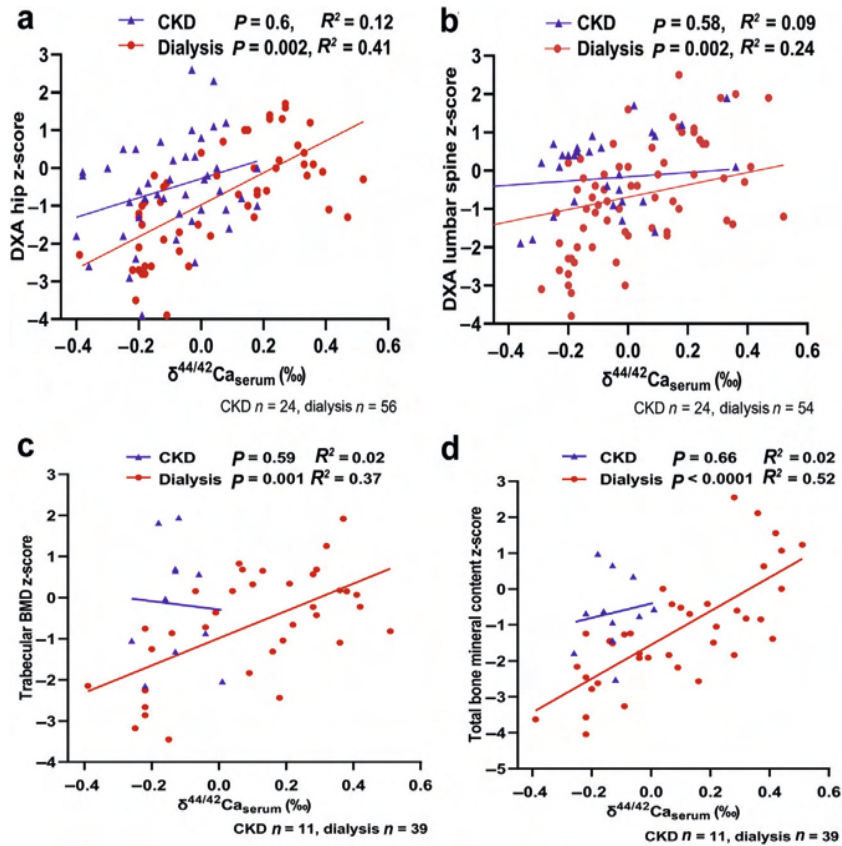
fluid. Despite the Ca flux from the dialysis fluid to the blood, no change was seen in  $\delta^{44/42}\text{Ca}$  in the effluent dialysate ( $P = 0.07$ ), although patient numbers are small. Similarly, in 12 children on PD, we measured paired serum and dialysate  $\delta^{44/42}\text{Ca}$  ratios at the start and end of the overnight continuous cycling peritoneal dialysis treatment. Again, there was no difference in  $\delta^{44/42}\text{Ca}_{\text{serum}}$  ( $P = 0.08$ ) or in  $\delta^{44/42}\text{Ca}$  in the effluent dialysate ( $P = 0.38$ ).

### Repeatability study

The  $\delta^{44/42}\text{Ca}_{\text{serum}}$  and  $\delta^{44/42}\text{Ca}_{\text{urine}}$  values were highly comparable between baseline and repeated testing at 1 month:  $\delta^{44/42}\text{Ca}_{\text{serum}}$ , 0.11 (−0.08 to 0.21)‰ at baseline versus 0.12 (−0.10 to 0.20)‰ at 1 month ( $R^2 = 0.89$ ,  $P < 0.0001$ ); and  $\delta^{44/42}\text{Ca}_{\text{urine}}$ , 0.17 (−0.28 to 1.03)‰ at baseline versus 0.21 (−0.31 to 1.11)‰ at 1 month ( $R^2 = 0.77$ ,  $P = 0.006$ ). The  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values at repeated testing correlated with serum ionized Ca ( $R^2 = 0.34$ ,  $P = 0.03$ ) and serum ALP ( $R^2 = 0.29$ ,  $P = 0.04$ ), and inversely with PTH ( $R^2 = 0.29$ ,  $P = 0.01$ ) and tartrate-resistant acid phosphatase 5b ( $R^2 = 0.43$ ,  $P = 0.01$ ; in dialysis patients only).

### Comparing Ca isotope values of CKD and dialysis patients with age-matched healthy controls

After adjustment for the isotopic composition of Ca-containing medications and kidney impairment, the  $\delta^{44/42}\text{Ca}_{\text{serum}}$  levels were significantly higher in healthy



**Figure 3 | Correlation of measured  $\delta^{44/42}\text{Ca}_{\text{serum}} (\text{‰})$  with bone densitometry measures.** Top panel: dual-energy X-ray absorptiometry (DXA) scans of the (a) hip and (b) lumbar spine (expressed as z-scores for age and sex). Bottom panel: peripheral quantitative computed tomography scan of the tibia for (c) trabecular density z-score and (d) bone mineral content. Linear regression analyses show significant positive correlations with  $\delta^{44/42}\text{Ca}_{\text{serum}}$ , confirming an increase in bone mineral content. BMD, bone mineral density; CKD, chronic kidney disease.

children when compared with the combined CKD and dialysis cohort ( $P < 0.0001$ ), with lowest levels in dialysis patients (Figures 4 and 5). The  $\delta^{44/42}\text{Ca}_{\text{urine}}$  values were significantly lower in children with CKD and on dialysis compared with healthy children ( $P < 0.0001$ ), with lowest  $\delta^{44/42}\text{Ca}_{\text{urine}}$  levels in dialysis patients ( $P = 0.006$  compared with CKD).

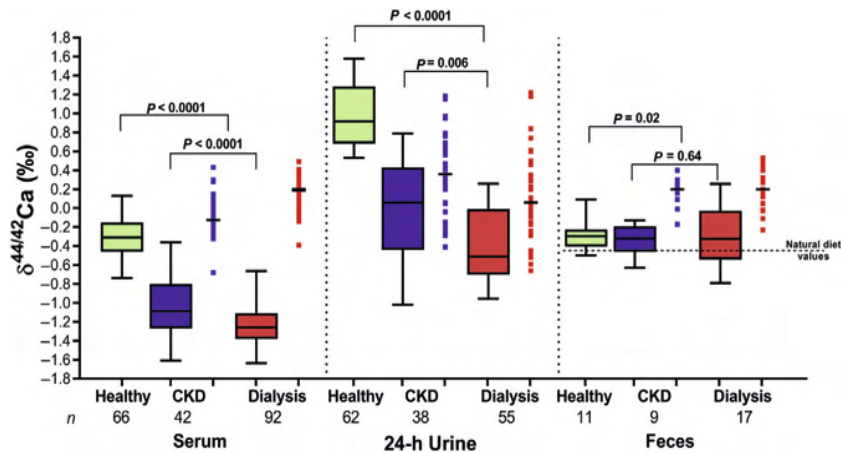
In all groups, there was a strong positive correlation between  $\delta^{44/42}\text{Ca}_{\text{serum}}$  and  $\delta^{44/42}\text{Ca}_{\text{urine}}$  (for CKD,  $R^2 = 0.47$ ; for dialysis,  $R^2 = 0.42$ ; Figure 5a and b, showing values adjusted for medications and for medications and estimated glomerular filtration rate, respectively), comparable to that seen in healthy children ( $R^2 = 0.55$ ).<sup>27</sup> There was no difference in the adjusted values for  $\delta^{44/42}\text{Ca}_{\text{feces}}$  in CKD and dialysis cohorts ( $P = 0.64$ ).

## DISCUSSION

In this proof-of-concept study, we have shown that the ratio of naturally occurring Ca isotopes in serum ( $\delta^{44/42}\text{Ca}_{\text{serum}}$ ) is a significant and independent predictor of BCaB and may be a novel biomarker of BCaB in patients on dialysis. The  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values correlated with a wide range of currently available measures of bone mineral status, including bone densitometry and biomarkers of bone formation and resorption used in routine clinical and research practice, but were the strongest predictor of total

bone mineral content. Children with CKD and on dialysis had  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values that were 157% (95% confidence interval, 108%–248%) lower than age-matched children<sup>27</sup> (Figures 4 and 5b) and 29% (95% confidence interval, 20%–35%) lower than levels reported even in elderly osteoporotic women, implying a significant loss of bone mineral content.

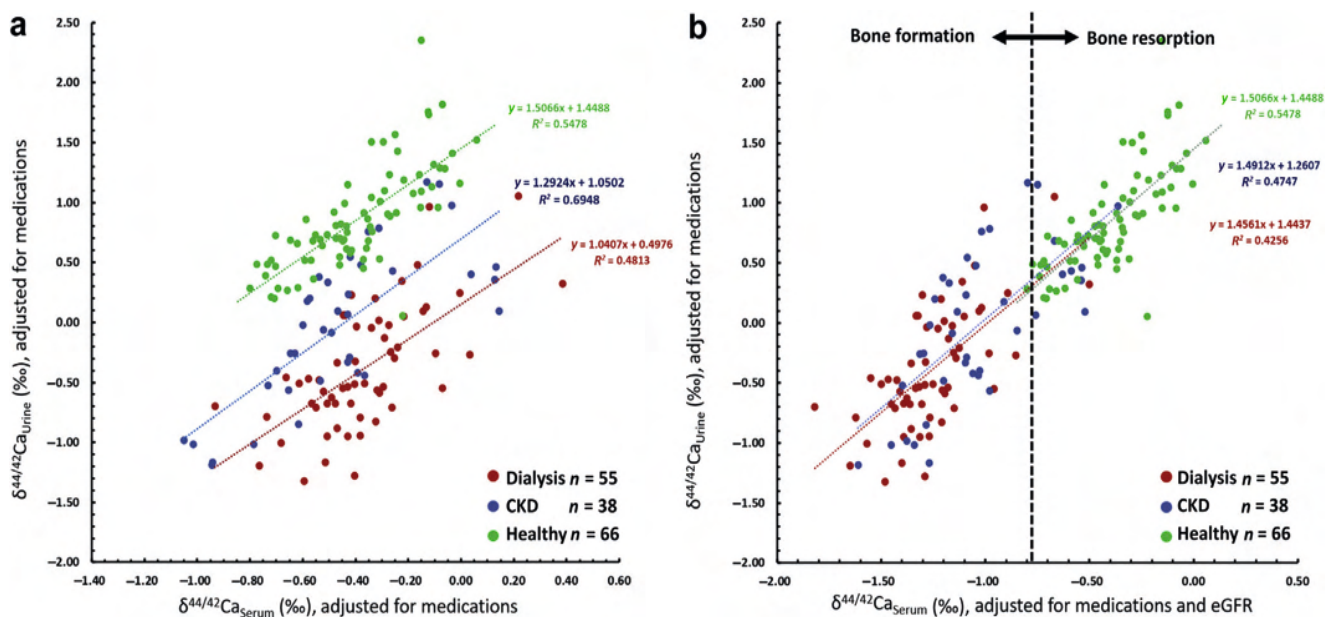
Mineral bone disorder of CKD causes abnormalities in BCaB, particularly in the growing skeleton.<sup>40</sup> The  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values of our CKD and dialysis patients indicate significant reduction in BCaB compared with healthy age-matched children (Figure 5b). As shown in our earlier work, the growing skeleton of healthy children avidly absorbs Ca with the highest  $\delta^{44/42}\text{Ca}$  serum values and an  $F_{\text{Bone\_Loss}}/F_{\text{Bone\_Gain}}$  ratio of  $\sim 0.4$  (Figure 6), implying that 2.5 times more Ca is accumulated in the bones than lost by bone resorption.<sup>27</sup> Bone mineral accretion continues into the 30s, when peak bone mass (the maximum amount of bone acquired at the end of skeletal development) is reached,<sup>41</sup> and the  $F_{\text{Bone\_Loss}}/F_{\text{Bone\_Gain}}$  ratio is  $\sim 1$ .<sup>27</sup> With increasing age, there is progressive bone resorption leading to a net loss of Ca.<sup>24</sup> The CKD cohort with an average age of 10 years showed a bone resorption/formation ratio comparable to that of healthy 30-year-old adults ( $F_{\text{Bone\_Loss}}/F_{\text{Bone\_Gain}} \sim 1$ ; Figure 6). Children on dialysis had an  $F_{\text{Bone\_Loss}}/F_{\text{Bone\_Gain}}$  ratio below  $\sim 10$ ,



**Figure 4 |** Ca isotope ratios expressed as  $\delta^{44/42}\text{Ca}$  (‰) in serum, 24-hour urine and feces in healthy children (green bars), children with chronic kidney disease (CKD) stages 4–5 (blue bars), and children on dialysis (red bars). The small blue and red bars show the measured values (unadjusted data) in CKD and dialysis patients, respectively. Large boxes show data adjusted for variations in calcium intake from medications (for  $\delta^{44/42}\text{Ca}_{\text{urine}}$  and  $\delta^{44/42}\text{Ca}_{\text{feces}}$  values), and adjustments for both medications and kidney impairment for  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values. Large boxes mark the 25<sup>th</sup> and 75<sup>th</sup> quartiles, the horizontal line marks the median, and whiskers mark the 1% to 99% limits of the data. Between-group differences are compared by analysis of variance. The horizontal dotted line marks the Ca isotope value of the normal diet in healthy individuals.

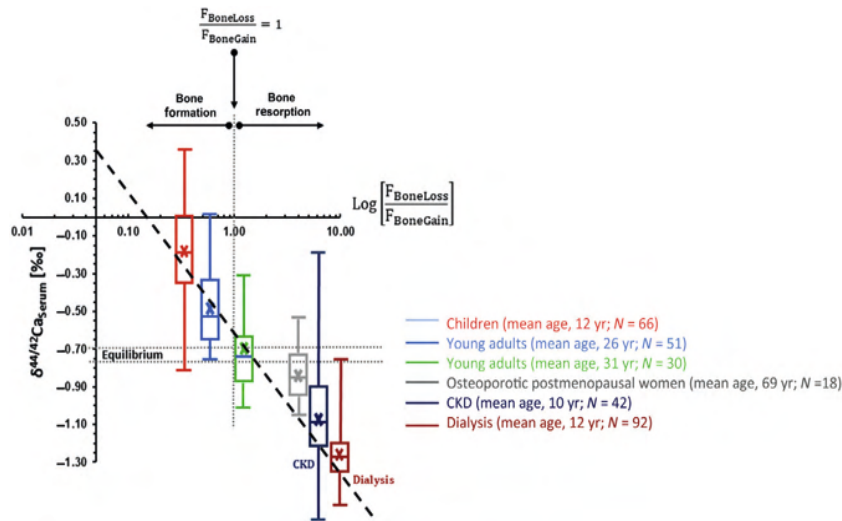
showing a more profound loss of bone mineral content than even elderly osteoporotic women.<sup>24,27</sup> The skeletal loss and gain of Ca are in equilibrium ( $F_{\text{Bone\_Loss}}/F_{\text{Bone\_Gain}} \sim 1$ ) at a  $\delta^{44/42}\text{Ca}_{\text{serum}}$  value of about  $-0.76$  ‰.<sup>24</sup> As shown in Figure 5, almost all the healthy children showed  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values above this threshold, whereas most of the CKD group and almost all the dialysis patients showed  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values below the threshold. Our Ca isotope data corroborate with

bone histology findings of mineralization defects even in early stages of CKD, with 29% of children in CKD stage 2 and 79% in CKD stage 4–5 having reduced bone mineralization.<sup>4</sup> Repeated measurements of  $\delta^{44/42}\text{Ca}_{\text{serum}}$  and  $\delta^{44/42}\text{Ca}_{\text{urine}}$  in a subset of children in whom kidney function (including urine output and dialysis prescription) was unchanged, and diet and medications were kept constant, revealed a high degree of correlation between measures at different time



**Figure 5 |** The correlation between medication-adjusted (a) and medication- and renal function-adjusted (b)  $\delta^{44/42}\text{Ca}_{\text{serum}}$  and  $\delta^{44/42}\text{Ca}_{\text{urine}}$  (‰) values in healthy children ( $n = 66$ ), chronic kidney disease (CKD) patients ( $n = 38$ ), and patients on dialysis ( $n = 55$  with urine output). A strong correlation is seen between  $\delta^{44/42}\text{Ca}_{\text{serum}}$  and  $\delta^{44/42}\text{Ca}_{\text{urine}}$  values in healthy children and children with CKD, indicating their close interdependency based on the Ca balance model (Figure 1). A similar but weaker correlation was seen between  $\delta^{44/42}\text{Ca}_{\text{serum}}$  and  $\delta^{44/42}\text{Ca}_{\text{urine}}$  in children on dialysis. The vertical dotted line at  $\delta^{44/42}\text{Ca}_{\text{serum}}$  value of  $-0.76$  ‰ marks the bone formation/bone resorption threshold level and the transition from net bone Ca gain and net bone Ca loss. It can be seen that most CKD and almost all dialysis data plot below the threshold value, indicating a net bone Ca loss, whereas all of the healthy children’s data are above the threshold value, indicating net bone Ca accrual and skeletal mineralization.





**Figure 6 |** This figure shows the  $\delta^{44/42}\text{Ca}_{\text{serum}}$  (‰) values of people of different ages and health status as a function of their bone Ca loss and gain ratios calculated from a model presented in our earlier work.<sup>24,27</sup>  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values and  $F_{\text{Bone\_Loss}}/F_{\text{Bone\_Gain}}$  ratios are inversely related with highest  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values ( $F_{\text{Bone\_Loss}}/F_{\text{Bone\_Gain}}$  ratio < 1) in healthy young children and lowest  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values in elderly people with osteoporosis and chronic kidney disease (CKD) and dialysis patients ( $F_{\text{Bone\_Loss}}/F_{\text{Bone\_Gain}}$  ratio > 1). Horizontal dotted lines mark a threshold for the transition from net gain of Ca (indicating mineralization and bone mass accumulation) to a net loss of Ca (indicating bone demineralization and osteoporosis) between -0.7 to 0.76 ‰. Children with CKD have a significantly higher  $F_{\text{Bone\_Loss}}/F_{\text{Bone\_Gain}}$  ratio compared with healthy age-matched children, with values that are below the threshold at which bone formation and resorption are in equilibrium; these values would usually be seen in individuals who have osteoporosis. In dialysis patients, the  $F_{\text{Bone\_Loss}}/F_{\text{Bone\_Gain}}$  ratio is even higher than in elderly osteoporotic individuals, indicating that bone resorption is about 10-fold higher than bone formation. The diagonal dotted line connects the median  $\delta^{44/42}\text{Ca}_{\text{serum}}$  value of each group of patients.

points; this preliminary step provides some validation of the isotope fractionation technique.

Comparing our novel biomarker  $\delta^{44/42}\text{Ca}_{\text{serum}}$  with a wide range of measures of BCaB, including biomarkers of bone formation and resorption as well as bone densitometry, revealed  $\delta^{44/42}\text{Ca}_{\text{serum}}$  as the strongest independent predictor for total bone mineral content measured by pQCT. We used pQCT as the best available measure of bone mineral content to compare with isotope data. Unlike DXA, pQCT, clearly distinguishing trabecular and cortical bone, has been used to show progressive decrease in bone mineral content with disease severity in CKD patients<sup>29</sup> and predicts fracture risk.<sup>2</sup> Importantly, several limitations of pQCT must be recognized that limit its application (it provides an image of a single bone compared with the whole skeleton, is not widely available, is highly operator dependent, and lacks robust reference data),<sup>6</sup> thereby stressing the need for a more robust marker of bone Ca content. Second, traditional biomarkers only qualitatively reflect either bone formation or bone resorption, but do not indicate the changes in bone Ca content.<sup>5,7</sup> In current clinical practice, DXA is our only tool for estimating BCaB. However, serum Ca accounts for <0.1% of total body Ca, and due to tight negative feedback control, cannot reflect the total body Ca.<sup>1</sup> Although bone histology is considered the “gold standard,” this is difficult to perform and cannot be easily repeated. The sensitivity of DXA in predicting changes in BMD is poor: radiology tests capture only a single area of the skeleton and can take months to years to manifest a change.<sup>6,42</sup> As change in BCaB is a dynamic process, BMD measurement by radiography or histology cannot provide

information on short-term changes in Ca status, such as with pharmacologic interventions. Thus, Ca isotope measures provide an urgent unmet need in the diagnosis and management of mineral bone disorder of CKD. In future, the predictive value of bone turnover markers coupled with Ca isotope diagnostics to determine the BCaB marker  $\delta^{44/42}\text{Ca}_{\text{serum}}$  may be considered together for a comprehensive assessment of bone health in patients with CKD. Although Ca isotope diagnostics are currently a research tool, the laboratory measurement of Ca isotopes is constantly being refined to improve the technique, increase throughput, and thereby reduce the costs. Our laboratory has one multicollector inductively coupled plasma mass spectrometer machine solely devoted to fully automated Ca isotope measurements on a 24/7 basis, yielding a sample throughput in the order of about 150 samples per week at a cost of about €25 per sample.

Our data support and extend work from other groups showing that  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values reflect changes in BCaB in healthy individuals. The National Aeronautics and Space Administration has conducted experiments to mimic reduction in BCaB in an antigravity environment, where healthy adult volunteers showed a significant decrease in  $\delta^{44/42}\text{Ca}$  in serum and urine starting by day 10 of complete bed rest, correlating with markers of bone resorption.<sup>26,34,36</sup> In contrast, osteoclast inhibition with the bisphosphonate alendronate lead to an increase in  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values.<sup>22</sup> In a group of otherwise healthy young men with vitamin D deficiency, 3 weeks of vitamin D supplementation increased  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values.<sup>35</sup> In patients with multiple myeloma, a malignancy that causes an activation of osteoclasts, resulting

in bone loss, patients with active disease had significantly lower  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values compared with those with non-active disease.<sup>37</sup> Ca isotope ratios in blood and urine are exquisitely sensitive to interventions that affect bone formation and resorption processes, consistently manifesting changes within days and providing a “real-time” picture of the bone Ca fluxes in the whole skeleton. Hence, as illustrated in the above study in patients with multiple myeloma,<sup>37</sup> future applications of this work could include serial measurements of  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values to monitor changes in response to alterations in diet or medications that can affect BCaB, such as antiresorptive therapies, phosphate binders (calcium based vs. calcium free), and vitamin D analogs.

We studied the effect of PD and HD on  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values in a subgroup of children. In children on HD,  $\delta^{44/42}$

$\text{Ca}_{\text{serum}}$  increased from baseline to the end of an HD session, whereas no change was seen in  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values after an overnight session of cycling PD (Supplementary Figure S3). Only 0.5 g (i.e., <0.1% of total body Ca) is present in the circulation, and this is exchanged rapidly with the considerably larger Ca reservoir of 1200 to 1500 g in the skeleton, such that Ca has a short residence time in the blood in the order of only about 1 hour (details in Supplementary Methods: the concept of Ca residence time in human blood). Thus, deviations in Ca concentration as well as Ca isotope composition following a dialysis session are rapidly and completely returned to normal within a few hours. The baseline Ca isotope levels (measured at 12 or 48 hours after the previous dialysis session in PD and HD patients, respectively) were comparable across HD and PD cohorts (Supplementary Figure S3). Thus,  $\delta^{44/42}\text{Ca}_{\text{serum}}$  is determined mainly by the interaction with bone Ca fluxes, reflecting the ratio of bone Ca absorption and resorption.

This proof-of-concept study is the first to investigate the use stable Ca isotopes in adults or children with kidney dysfunction, but limitations of our work as well as the outline of future studies to promote our understanding of the utility of isotopes in medicine must be noted. We were unable to evaluate the effect of growth on changes in Ca isotope ratios as this was a cross-sectional study design. The CKD cohort was small and heterogeneous, perhaps explaining the lack of

correlations between  $\delta^{44/42}\text{Ca}_{\text{serum}}$  and some biomarkers and radiological measures, prompting the need for further study with larger patient numbers. Although controlled diets or weighed food records are the most rigorous analysis of dietary Ca intake, in routine clinical practice, analysis of 3-day food diaries<sup>43</sup> offers the most accurate assessment. The overall uncertainty of the reported data may be larger because of systematic uncertainties introduced by the corrections for different diets and kidney dysfunction. Ca absorption could not be studied; nevertheless, individual patient data of  $\delta^{44/42}$

$\text{Ca}_{\text{serum}}$  already account for their absorbed dietary Ca. We measured DXA hip and lumbar spine rather than “total body less head” DXA scans, in keeping with standard clinical practice and latest guidelines,<sup>44</sup> but acknowledge that variations due to skeletal heterogeneity should be considered. We

measured total ALP and not bone-specific ALP, but bone-specific ALP represents 80% to 90% of total circulating ALP in growing children with normal liver function,<sup>45</sup> and guidelines suggest that total ALP can be routinely used, except in patients with liver disease.<sup>5</sup> A crucial question in dialysis patients is to determine the fate of the absorbed Ca: whether it is in the bones or ectopically deposited in extrasosseous tissues, like blood vessels; further studies to measure the Ca isotopic composition in bone and arterial biopsy samples collected from the same individual are under way. Unfortunately, phosphorus has only 1 stable isotope, and so the isotope fractionation technique that requires at least 2 isotopes cannot be applied to study phosphorus levels.

In summary, this proof-of-concept study suggests that the naturally occurring stable Ca isotope ratio in serum is a significant and independent predictor of bone Ca content in children with CKD and on dialysis and is more sensitive and accurate than routinely used measures of BMD, such as DXA or bone biomarkers. The clinical utility of Ca isotopes as a biomarker of BCaB may also be applicable in other patient groups who have diseases that affect bone health (such as adults with CKD or on dialysis, chronic childhood diseases, and inherited bone diseases) or receive medications, like steroids, chemotherapy, and antiresorptive treatments, that affect the bone. Future studies that perform serial measurements of Ca isotope ratios may aid prognostication of fracture risk in patients with CKD as well as other disease cohorts.

#### DISCLOSURE

AE and AK are cofounders of Osteolabs GmbH. All the other authors declared no competing interests.

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#### SUPPLEMENTARY MATERIAL

Supplementary File (Word)

Supplementary Methods.

**Table S1.** Calcium isotope composition of calcium-based phosphate binders and calcium supplements.

**Table S2. (A)** Adjustment of measured values for the calcium (Ca) isotopic composition of medications. **(B)** Correction of  $\delta^{44/42}\text{Ca}_{\text{serum}}$

values (medication adjusted) for the influence of renal impairment (glomerular filtration rate [GFR] adjusted).

**Table S3.** Bone biomarkers, calcium (Ca) isotope ratios, and radiological evaluation.

**Table S4.** Multivariable regression analysis for predictors of the total bone mineral content z-score.

**Figure S1.** The measured  $\delta^{44/42}\text{Ca}_{\text{serum}}$  values showed a positive correlation with the cholecalciferol and vitamin D analog (alfacalcidol) dosage.

**Figure S2.** (A) The measured (unadjusted)  $\delta^{44/42}\text{Ca}_{\text{serum}}$  and  $\delta^{44/42}\text{Ca}_{\text{urine}}$  were comparable in patients on hemodialysis (HD) and peritoneal dialysis (PD). (B) There was no difference in measured (unadjusted)  $\delta^{44/42}\text{Ca}_{\text{serum}}$  in dialysis patients with or without a urine output, suggesting negligible amounts of calcium (Ca) isotope fractionation by the kidneys in dialysis patients.

**Figure S3.** Changes in the measured (unadjusted)  $\delta^{44/42}\text{Ca}$  values in serum and dialysate during a dialysis session in hemodialysis (HD) (A,B) and peritoneal dialysis (PD) (C,D) patients. Among children on HD, 7 were dialyzed against fluid containing 1.25 mmol/L calcium (Ca) and 3 against 1.75 mmol/L Ca. All children were dialyzed with PD fluid containing 1.25 mmol/L of Ca.

**Figure S4.** The  $\Delta_{\text{urine-serum}}$  shows a positive nonlinear correlation with their corresponding estimated glomerular filtration rate (eGFR) values.

**Figure S5.** A schematic diagram adapted from the calcium (Ca) balance model in Figure 1 for chronic kidney disease (CKD) and dialysis patients, showing the complex processes involved when Ca homeostasis is affected in renal impairment. For CKD patients, the compartment model for healthy individuals (Figure 1) can still be applied, with levels closely correlating with the estimated glomerular filtration rate. However, for dialysis patients, including those who are anuric, the kidney compartment is replaced by a dialysis compartment. Extrasosseous calcification is shown as a potential compartment that can account for Ca isotope fractionation.

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