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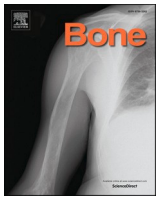

Full Length Article

Calcium isotope composition in serum and urine for the assessment of bone mineral balance (BMB) – The Osteolabs post-market follow-up study

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ABSTRACT

To further explore the clinical applicability of the calcium (Ca) isotope marker (CIM), we determined the $^{44}Ca/^{42}Ca$ isotope ratio in blood serum and urine. This ratio is expressed in the conventional δ-notation (as defined in the text below) specifically as CIM-serum for serum and as CIM-urine for urine. Our study tested the hypothesis that CIM values can differentiate between positive and negative bone mineral balance (BMB) across a diverse clinical population considering variables such as age, gender, and diet. The threshold values (CIM-serum: −0.85 ± 0.06 ‰ and CIM-urine: 0.23 ± 0.06 ‰) established in the OsteoGeo study (NCT02967978, Eisenhauer et al., 2019) were evaluated in 2320 participants as part of a surveillance study referred to as Osteolabs study. The earlier study revealed women with osteoporosis had an average CIM-serum value of −0.91 ± 0.21 ‰ (N = 24) and a CIM-urine value of 0.18 ± 0.33 ‰ (N = 71) that are significantly below the threshold values (p = 0.02 for urine, one-sided Wilcoxon rank test, $p < 0.001$ for serum, one-sided Student's t-test). Diseases affecting BMB such as osteoporosis, acute and chronic kidney disease (CKD), hyperthyroidism, breast cancer, prostate cancer, and myeloma were associated with significantly lower average CIM values, falling below the equilibrium thresholds and indicating negative BMB. In contrast, patients receiving osteoprotective treatments such as denosumab, Romosozumab, bisphosphonates, or hormone replacement therapy for certain diseases, had CIM values above the equilibrium thresholds indicating a positive BMB. Additionally, Ca supplements taken by some of the patients ((N = 22 (serum), N = 49 (urine), median dose: 500 mg) showed a Ca isotope composition approximately 1 ‰ higher than that from a normal diet. Consequently, their CIM values need to be adjusted to account for the amount and duration of supplementation to be comparable to those with a normal diet. Participants taking vitamin D (237 women; 58 men) showed no significant difference from the average values of the study group. Counterintuitively, the possible impact of malnutrition on individual BMB was most pronounced in vegans, who exhibited the highest average CIM-urine values compared to patients on a normal diet ($p < 0.001$, N = 17). The results of this study were consistent with the registered OsteoGeo study (NCT02967978) and other earlier published Ca isotope-based studies on BMB. We confirm that the CIM threshold values determined in the OsteoGeo study are generally valid for this much larger and diverse surveillance study group covering a diverse population encompassing various medical conditions and therapies.

1. Introduction

Calcium is the most abundant mineral in the human body, with the average adult body containing approximately 1 kg of Ca phosphate salts, 99 % of which is stored in the skeleton. Calcium ions (Ca^{2+}) are also present in the blood plasma, as well as in extracellular and intracellular fluids [1]. Homeostatic negative and positive feedback systems tightly regulate the concentration of Ca ions in the blood plasma, maintaining it in a narrow interval from 2.15 to 2.55 mmol/l [2,3]. This concentration is kept constant through a complex interaction involving Ca absorption

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from the intestines, absorption, and resorption of Ca from the bones, as well as reabsorption of Ca from the primary urine in the kidneys.

There are at least three hormones intimately involved in the regulation of the level of Ca in the blood: parathyroid hormone (PTH), calcitonin, and calcitriol (1,25-dihydroxyvitamin D, the active form of vitamin D). This hormonal "feedback loop" is governed by the parathyroid glands and the calcitonin-secreting cells of the thyroid gland by constantly monitoring the blood Ca level. Falling Ca levels trigger the parathyroid glands to release the parathormone (PTH) into the blood, signaling the osteoclast cells to release Ca from the bones. PTH also signals the kidney to increase Ca reabsorption from the primary urine before excretion via secondary urine. Additionally, it stimulates the synthesis of the active form of vitamin D to increase Ca intake from the intestine. Besides PTH, although currently not applied in clinical routine the parathyroid hormone-related protein (PTHrP) has been identified to also play a crucial role in controlling bone Ca release and renal reabsorption [4]. PTHrP regulates Ca levels in the blood by promoting Ca reabsorption in the kidneys and releasing Ca from bones through the same receptors (class B protein-coupled receptors) [5]. However, PTHrP has a broader range of functions mostly related to local tissue-specific roles rather than systemic Ca homeostasis [6].

The disequilibrium between bone formation and bone loss may primarily be age- and hormone-related but also reflects various diseases like osteoporosis, thyroid disease, acute and chronic kidney disease (CKD), cancer metastatic bone formation or resorption, and others. Among them, the most prevalent disease interfering with BMB is osteoporosis. For diagnoses and therapy control, the knowledge of the BMB status is crucial. However, none of the established image-based methods like the dual X-ray absorptiometry (DXA) or peptide-based biomarkers like C-terminal telopeptide of type I collagen (CTX), which reflects bone Ca resorption, or procollagen type I N-terminal propeptide (P1NP), which reflects bone formation, provide sensitive or specific information about the current individual BMB [7,8]. In addition, the measurement of peptide-based biomarkers is also burdened by sample degradation due to factors like temperature, pH, or duration of transport. This degradation can impact the concentration and integrity of the analytes of interest, leading to potential inaccuracies in laboratory results [9].

As a common tool for diagnosing osteoporosis, unfortunately, the sensitivity of DXA is only about 61 %, while its specificity is around 85 % for diagnosing osteoporosis. The relatively low sensitivity indicates that some individuals with osteoporosis may not be detected based solely on DXA results. Therefore, currently additional clinical tools and risk assessment models, such as the FRAX tool, are often used alongside DXA to provide a more comprehensive evaluation of fracture risk and bone health [10,11].

Innovative technologies for the early diagnosis of osteoporosis are highly desirable to improve the current healthcare situation for osteoporosis. Earlier publications already demonstrated that BMB can be best estimated by the novel non-radioactive and minimal-invasive CIM values measured in serum and urine [12–16]. The CIM values reflect the BMB of the whole skeleton related to the patient's age, gender, and health status [15,17,18]. In particular, the BMB status is determined by comparing the measured CIM value with an empirically determined threshold value of -0.85 ± 0.06 ‰ ($N = 80$, 2 SD) in serum and of 0.23 ± 0.06 ‰ in urine (N = 80; 2 SD) [12], which marks the equilibrium between Ca mineralization and demineralization in the bones. When Ca absorption and bone formation exceed bone resorption, BMB is positive and both CIM values of CIM-serum and CIM-urine are higher than the respective threshold value [12]. On the other hand, in situations where bone resorption is the predominant process, BMB is negative and both CIM values will decrease below the threshold values.

CIM threshold values were determined by an ROC analysis in the frame of the OsteoGeo study [12]. For this study, post-menopausal women aged 50–75 years who had at least one risk factor for osteoporosis by age 60 years or at least 2 risk factors by age 50 years, were invited to participate in the study. Women with a known fracture within the previous 3 months, those with renal failure, cancer, and hyperparathyroidism or on sex hormone treatment were excluded as well as women with vitamin D deficiency (defined as 25-hydroxy vitamin D level < 25 nmol/l).

The obtained results are following theoretical assumptions and Ca compartment modeling [13]. Comparison of the CIM method to DXA as the "gold" standard in the OsteoGeo and other follow-up clinical studies as well as to biochemical bone turnover markers like CTX, NTX, and P1NP have shown that the CIM is generally the best predictor for bone Ca loss and osteoporosis with a sensitivity of higher than 94 % for CIM-serum and 79 % for CIM-urine. Specificity is about 55 % for serum and 79 % for urine predicting about twice as many patients to suffer from Ca loss and a higher risk of osteoporosis and possible fractures when compared to DXA, suggesting that CIM indicates low BMB in 'real time' and far earlier than radiological measures [12]. The latter may in particular reflect the observation that most bone fractures occur in women with normal BMB or osteopenia rather than osteoporosis [19].

About two years after the end of the OsteoGeo study, a yet unpublished follow-up study was conducted using structured telephone interviews to further verify the predictive power of the Ca biomarker in diagnosing Ca loss, and assessing the higher risk for osteoporosis and fractures relative to DXA. The interviews reported 12 fractures occurring within two years post-study. Three of these fractures were non-adequate and related to high-trauma accidents, while the remaining nine were attributed to low-trauma osteoporosis-adequate fractures. Among the nine osteoporosis-related fractures, only three had DXA T-score values below -2.5. The other six fractures showed T-scores indicating osteopenia or were considered healthy. In contrast, all nine osteoporosis-adequate fractures were associated with CIM values below the threshold for both serum and urine. Although the interview data is not statistically significant, the trend suggests the Ca biomarker's predictive power. Until now, only a few studies with restricted numbers of participants have shown clinical CIM applications [12,14,15]. For example, for the first time, CIM values showed that prostate cancer (PCa) patients receiving androgen deprivation therapies (ADT) had low BMB as early as 12 weeks of treatment. Within this time, bone status changed from CIM values reflecting normal and healthy values to values comparable to post-menopausal osteoporotic women. The authors further suggested verifying Ca isotopes as a sensitive tool to predict fracture risk, and if CIM can be implemented in clinical practice to monitor bone health in PCa patients receiving ADT [14]. In another study, Shroff et al. (2022) concluded that CIM may provide a novel, sensitive, and non-invasive method of assessing BMB in CKD [15]. Furthermore, Gallant and Zheng (2022) suggested that Ca isotope may have the potential to be used as a new standard for BMB in the future [20]. All previous CIM studies have typically involved fewer than 100 participants. Despite the small sample sizes, results consistently showed that CIM values above the threshold are statistically significantly linked to positive bone health, and values below the threshold are associated with negative bone health (for statistical details see [13–15]). CIM values significantly correlate with bone markers such as P1NP, indicating Ca gain and bone mineralization, as well as with markers like CTX, indicating Ca loss and bone demineralization [12–15]. The high statistical significance observed even with small participant numbers is most likely based on CIM's large effect size which is expressed by a high Cohen's d value of 1.11, in the OsteoGeo study. Consequently, CIM studies can achieve statistically significant results with smaller participant groups, making it a cost-effective and efficient option compared to other markers that require larger sample sizes [21,22].

Another notable benefit of using stable Ca isotopes in blood and urine is sample stability, particularly when compared to peptide-based biomarker diagnostics. Since stable Ca isotope ratios are measured using inorganic mass spectrometry, there is reduced sensitivity to and potential for modification from factors such as organic breakdown during sample preparation and storage. This ensures greater reliability and consistency in the diagnostic results. To further demonstrate that

the CIM approach for early detection of BMB disturbances leading to osteoporosis is also valid for the general population, we statistically evaluated 2320 study participants comprising 1697 single urine, 143 single serum, and 560 paired urine and serum measurements in a total of 2400 samples as part of a post-market clinical follow-up study of the Osteolabs GmbH, Kiel, Germany, commercially retailing this marker. The purpose of this clinical follow-up study is to refine and confirm the validity of the CIM approach in a larger un-selected cohort from the general population who have a wide range of diseases and receive varying treatments.

2. Materials and methods

2.1. Study participants

Participants of the study were customers of Osteolabs GmbH, Kiel, Germany (https: //www.osteolabs.de) in 2020–2023, who either used the companies' OsteoTest home, OsteoTest med, or OsteoTest med plus to test for Ca loss as the major indicator for osteoporosis. The OsteoTest home is a pure urine test, OsteoTest med is a pure serum test, and OsteoTest med + comprises both urine and serum tests allowing for the calculation of the renal reabsorption value (RN-value defined below) to provide additional information about individual kidney dysfunctionality [13,23]. All participants gave informed consent to participate in our study before testing (https://www.osteolabs.de/de/datenschutz.html). Urine was self-collected by the participants, while blood was collected in a hospital or a doctor's office. Before shipment, urine and blood samples were safely stored in suitable sampling containers following legal regulations. Participants filled out questionnaires of anthropometric measurements, current and previous health status, previous history of fracture for the last 24 and 48 months, current medication, and uptake of supplements, e.g., commercial vitamin D and Ca supplements (see Supplementary Material). All participants resided in Europe during sample acquisition, with 92.9 % residing in Germany and 4.8 % in the UK. Upon arrival in the lab, the samples were assigned new labels following European data protection regulations. Samples were stored frozen to the day of the chemical analysis. The study was conducted in full accordance with the Declaration of Helsinki

2.2. Calcium isotope measurement

Laboratory preparation of serum and urine samples follows already published standardized procedures [12,23]. For CIM measurements, blood serum was used instead of plasma. The primary difference between serum and plasma is that plasma contains clotting factors absent in serum. Ca is enriched in the liquid portion of the blood rather than in the blood cells. To prevent Ca from adhering to surfaces and to minimize Ca sequestration and kinetic isotope fractionation during transport and exposure to higher temperatures, serum (which lacks blood cells) was used. This also avoids kinetic isotope fractionation between blood cells and the liquid portion. Additionally, using serum prevents any chemical reactions between clotting factors and the Ca ion exchange resin during chemical preparation, ensuring more accurate and reliable CIM measurements.

Both serum and urine samples were subject to acid digestion and Ca extraction prior to isotopic measurement. Each batch of sample preparation included 20 samples, standard reference materials (NIST SRM 915a and NIST SRM 1486), in-house standards (urine AK1 or blood SERA-1), and a procedural blank. The samples were shaken to homogenize, and Ca concentration was measured using an Indiko Plus Clinical Chemistry Analyzer (Thermo Fisher Scientific, Bremen, Germany). The digestion was performed in a microwave digestion system MARS 6 (CEM, Matthews, NC, USA) using 50 ml digestion beakers. A mixture of 8 ml of concentrated double-distilled nitric acid and 2 ml of 30 % H_2O_2 (Merck Suprapure®, Merck KGaA, Darmstadt, Germany) were added to 1 ml urine sample and 0.25 ml serum sample. The samples were heated in the microwave digestion system to 180 °C, held at that temperature for 30 min, and subsequently cooled. The solutions were transferred from the digestion beakers to 15 ml perfluoroalkoxy (PFA) beakers (Savillex, Eden Prairie, MN, USA) and evaporated at 120 °C. The dried material was dissolved in 1 ml of 1 mol/l nitric acid and homogenized. The Ca in the digested sample was purified using a prepFAST MC automated column chromatography system (ESI, Omaha, NE, USA). The collected Ca fraction was evaporated at 120 °C, treated with 1 ml of concentrated double-distilled nitric acid and 0.5 ml of 30 % H_2O_2 at 120 °C for 4 h, and evaporated at the same temperature. The Ca fraction was then reconstituted with 0.2 mol/l of nitric acid for the isotope ratio measurements. Ultrapure water (18.2 MΩcm resistivity at 25 °C) produced using a Milli-Q system (Merck KGaA, Darmstadt, Germany) was used throughout the experiment. Procedural blanks were found to be typically around 250 ng. Thus, blanks contribute to <1 % of the measured Ca and do not skew results.

The mass-spectrometer procedure was reported earlier [12]. In brief, Ca isotopic compositions were measured using a Neptune Plus MC-ICP-MS (Thermo Fisher Scientific, Bremen, Germany) equipped with APEX-IR or APEX2-IR (ESI, Omaha, NE, USA) sample introduction system. The isotopes at the mass-to-charge ratio (m/z) of 42, 43, 43.5, and 44 were measured at medium mass resolution (MR, m/Δm = ~4000) on the low mass side of the plateau. To enhance the precision and accuracy of the Ca isotope measurements, we utilized the measurement of mass 43.5, corresponding to doubly positive ionized $(^{87}Sr)^{2+}$. This approach effectively removes the isobaric interference of other relevant doubly ionized Strontium (Sr) isotopes (^{84}Sr, ^{86}Sr and ^{88}Sr) on the singly positively ionized Ca isotopes of ^{42}Ca, ^{43}Ca and ^{44}Ca. This approach ensures a more precise and accurate determination of the Ca isotope ratios.

The sample was measured using the sample-standard-bracketing approach in which the concentration of the sample and standard were matched within ±30 %. NIST SRM 915a was used as the external standard for the sample-standard bracketing purpose. The Ca isotopic composition is expressed as $\delta^{44/42}Ca$ in per mill (‰) as follows:

$$\delta^{44/42}Ca = \left(\frac{\left(^{44}Ca/^{42}Ca\right)_{sample}}{\left(^{44}Ca/^{42}Ca\right)_{reference}} - 1 \right) \quad (1)$$

Each sample was measured at least four times in the same measurement session but not in an adjacent sequence. For data quality reasons, a measurement session is rejected if more than one of the analyzed reference materials deviates >0.2 ‰ from its referenced value or if the data fall outside the mass-dependent fractionation line. A sample measurement was repeated when the 2SE of at least three single measurements was >0.5 ‰. Following Morgan et al. (2011) a single measurement was rejected when the absolute value of $\delta^{44/42}Ca - 2{\cdot}\delta^{43/42}Ca$ was >0.2 ‰. Typically, about 2.5 % of about 200 analytical runs of a measurement session were excluded [24]. Our collection of analyzed reference materials (Table S1) covers all relevant sample matrices, including blood and urine, as well as additional complex matrices such as bone and seawater. Long-term monitoring (>6 years) of our reference material revealed a standard deviation of lower than 0.07 ‰. This value ensures long-term reproducibility and time-independent comparability of the CIM data for precise monitoring of changes in individual health status and long-term therapies. See more details in section 1 of the supplementary material (Table S1).

2.3. Clinical chemical parameters

Clinical parameters were determined in serum samples using a photometric-based Indiko Plus Clinical Chemistry Analyzer (Thermo Fisher Scientific, Bremen, Germany). Vitamin D concentration (nmol/l) and creatinine level (mg/dL) were determined in serum, while Ca concentration (mmol/l) was determined in serum and urine. For participants using the OsteoTest med plus test, the CIM values were measured in both serum and urine, allowing the calculation of the Ca reabsorption

RN-value based on the Ca isotope difference between serum and urine ($\Delta_{urine\text{-}serum}$ = CIM-urine - CIM-serum) as explained in section 2 of the Supplementary Material [13,15]. The calculation of the estimated glomerular filtration rate (eGFR) was performed from the Cockcroft-Gault equation from the creatinine concentration in serum, reported age, and weight, and applied only for men. For women, the calculated value was then multiplied by 0.85 for gender-specific eGFR values [25]. An eGFR value of <90 mL/min indicates renal dysfunction [26], while creatinine value is considered to be abnormal for values higher than 45–84 μmol/l in women and 59–104 μmol/l in men.

2.4. Adjustment of measured isotope values to the intake of calcium supplements

For inter- and intra-individual comparability of CIM values to the equilibrium values, all participants must consume Ca with a fairly constant isotope value. The latter is well justified because, for Europeans, the Ca uptake is dominated by dairy products and vegetables at about 90 % [23]. However, variation of the Ca isotope in blood and urine may occur from the intake of Ca supplements wherein the Ca originates from non-biological inorganic sources (such as carbonate rocks or coral material). They are enriched in the heavy Ca isotopes up to about 1 ‰ higher than the typical Western European diet [27]. As a result, the individual Ca equilibrium value is shifted from the general equilibrium threshold value toward higher values. To accurately assess the influence of calcium supplements, participants were required to disclose specific details about their supplement intake. The self-declaration form requested information on the amount of Ca supplements consumed and the duration of their usage. This data is crucial for evaluating the potential effects of supplemental Ca on the study results and is tailored individually for each participant based on their specific information regarding the duration and amount of Ca supplements taken.

Consequently, individuals who routinely take Ca supplements in amounts that are comparable to or even higher than the natural Ca content of their diet will have higher CIM values in serum and urine, eventually increasing above the CIM threshold and indicating an apparent positive BMB. To allow comparability of inter- and intra-individual values and to CIM measurements between individuals, the measured CIM values need to be adjusted for the influence of Ca supplements. Detailed calculations considering the amount of Ca supplement and the duration of uptake are shown in section 3 of the Supplementary Material.

2.5. Statistical analysis

Quantitative data are presented as mean and 1σ-standard deviation, unless otherwise specified. Box plots are presented to show the data distribution, whereby the whiskers indicate the 90th and 10th percentiles. All outliers outside the 90th and 10th percentiles are shown in the box plots. Normality was evaluated with the Shapiro-Wilk test. The difference between groups was calculated using the *t*-test for data with normal distribution or the Mann-Whitney rank-sum test for data that do not have normal distribution. If three or more groups were present, the Kruskal-Wallis test was used, followed by Dwass-Steel-Critchlow-Fligner (DSCF) post-hoc pairwise analysis. The relationship between two continuous variables was assessed using the Pearson correlation test unless otherwise specified. Two-tailed tests with a significance level (p) of 0.05 were used in this study, unless otherwise specified. Statistical analyses were performed using Jamovi version 2.3.28.0 (Jamovi, Sydney, Australia). Graphs were constructed using SigmaPlot 14 (Systat Software Inc., San Jose, CA, USA).

3. Results

3.1. Characteristics of the participants and clinical parameter values

A total of 2320 study participants (1935 women and 385 men) provided 1697 single urine, 143 single serum, 559 paired urine and serum sample, or multiple samples of each kind (Table 1). In total 2960 samples. Among these, 143 multiple urine samples, up to six per individual, were attributed to 63 study participants, providing time series (longitudinal data) for therapy or medication monitoring (see Fig. 5). Descriptive age, weight, and height statistics were calculated based on the number of study participants. For study participants who provided more than one urine and serum sample, the values were not averaged per person but treated like individual measurements. Table 1 summarizes the characteristics of the participants, noting that not all parameters could be fully acquired from the participants. Due to the partially incomplete data, a lower number of participants was considered compared to the total number of participants. Additionally, the clinical parameter values across all groups do not show normally distributed data.

From Table 1 it can be seen that there was no significant difference in age ($p = 0.62$) between women and men. However, weight and height were significantly different ($p < 0.001$ for both). Among the participants 274 (14 %) and 366 (18 %) women experienced at least one or more fractures in the 24 and 48-month intervals before the Ca isotope testing, respectively. Concerning men, 56 (14 %) and 72 (20 %) had at least one fracture in the respective time intervals.

The vitamin D concentration significantly differed between women and men ($p = 0.012$, Table 1). The eGFR values in women (86.1 ± 17.8 mL/min) were significantly lower compared to men (96 ± 22 mL/min) ($p < 0.001$).

Table 1
Characteristics of the participants enrolled in this study†.

Characteristics	Women (*n*, %)	Men (*n*, %)	*p* value
Age (years)	61 ± 12 (1935)	59 ± 17 (385)	0.624
Weight (kg)	68 ± 15 (1784)	84 ± 18 (344)	<0.001*
Height (cm)	167 ± 7 (1790)	180 ± 8 (343)	<0.001*
Number of participants experiencing fracture in the previous 24 months	274 (14 %)	56 (14 %)	n/a
Number of participants experiencing fracture in the previous 24 to 48 months	366 (18 %)	72 (20 %)	n/a
Number of fractures in the previous 24 months	0.20 ± 0.64 (1935)	0.24 ± 0.85 (385)	0.758
Number of fractures in the previous 24 to 48 months	0.17 ± 0.66 (1935)	0.18 ± 1.14 (385)	0.943
Serum Ca (mmol/l)	2.49 ± 0.26 (535)	2.45 ± 0.31 (159)	0.429
Urine Ca (mmol/l)	3.19 ± 2.16 (1892)	3.39 ± 2.60 (358)	0.534
CIM-serum (‰)	−0.85 ± 0.20 (538)	−0.75 ± 0.24 (164)	<0.001*
CIM-urine (‰)	0.24 ± 0.31 (1897)	0.36 ± 0.34 (359)	<0.001*
$\Delta^{44/42}$Ca	1.07 ± 0.23 (433)	1.08 ± 0.24 (127)	0.362
Vitamin D (IU)	93.9 ± 36.2 (237)	81.4 ± 33.1 (58)	0.012*
eGFR (mL/min)	86.1 ± 17.8 (236)	96.0 ± 21.6 (58)	<0.001*
Creatinine (mg/dL)	0.78 ± 0.13 (332)	1.06 ± 0.78 (85)	<0.001*
Ca reabsorption RN-value (%)	96 ± 3 (288)	96 ± 3 (71)	0.482

Note: †Total $N = 2400$ samples with 9 participants who did not identify their gender. The data in all groups do not show a normal distribution. *Mann-Whitney test shows significantly different results on $p < 0.05$.

No statistically significant difference was found in serum Ca concentration between women (2.49 ± 0.26 mmol/l) and men (2.45 ± 0.31 mmol/l) ($p = 0.429$). Similarly, there was no statistical difference in urine Ca concentration between women (3.19 ± 2.16 mmol/l) and men (3.39 ± 2.60 mmol/l) ($p = 0.534$).

The average CIM-serum value was significantly different, with women at −0.85 ± 0.20 ‰ and men at −0.75 ± 0.24 ‰, respectively. The CIM-urine values of men at 0.36 ± 0.34 ‰ and women at 0.24 ± 0.31 ‰ were significantly different ($p < 0.001$ in both cases). The Ca isotope difference between serum and urine in the same individual ($\Delta_{urine\text{-}serum}$ = CIM-urine−CIM-serum) was not significant ($p = 0.362$). Based on that, the average Ca isotope-based calculated renal Ca reabsorption rate (RN-value) calculated from the $\Delta_{urine\text{-}serum}$ values for women and men were both 96 %, which were statistically insignificant ($p = 0.482$). For detailed calculation of the RN-value, see the Supplementary Material.

The comparison of CIM values among individuals and in time series depends on the common Ca isotope value of the diet. Hence, for participants consuming Ca supplements, CIM values must be adjusted to the general Threshold Equilibrium (TE) value for comparability to people not consuming Ca supplements (Table 2, for more details see the Supplementary Material). The average measured CIM-serum value for women taking supplements ($N = 15$) was −0.77 ± 0.15 ‰ and the corresponding supplement adjusted value was −1.11 ± 0.20 ‰ (Table 2), as described in the Supplementary Material. The average measured CIM-urine value for women ($N = 38$) taking up supplements was 0.29 ± 0.29 ‰ and the corresponding adjusted CIM-urine value was −0.01 ± 0.35 ‰ (Table 2). The CIM values for women and men taking supplements were not significantly different neither before nor after correction (see values for men in Table 2). The adjustment for Ca supplements was calculated based on the known amount of Ca supplements consumed by the patient, the duration of supplement intake, the estimated Ca intake from a normal diet (assumed to be 800 mg/day), and the residence time of Ca in the bone (2000 days). The calculated adjustment value was then added to the measured CIM values for serum and urine. This adjustment enables a meaningful comparison of CIM values between participants with and without the intake of dietary Ca supplements. For more detailed calculations, please refer to the Supplement Material.

3.2. Correlations between Ca isotopes and clinical parameters

Correlations between CIM values and clinical data are presented in Table 3 and in the diagrams of Fig. 1 and S4. CIM-urine strongly correlated with CIM-serum ($n = 560$; $R = 0.763$; $p < 0.001$) (Fig. 1). CIM-urine had a significantly moderate to strong correlation with urine Ca concentration and Ca reabsorption (R = -0.499 and $\rho = 0.753$, respectively, $p < 0.001$ in both cases). CIM-serum had significant but weak correlations with urine Ca concentration and Ca reabsorption (R = -0.270 and $\rho = 0.187$, respectively, $p < 0.001$ in both cases). CIM-urine and CIM-serum also only had weak inverse correlations with age and serum Ca concentration ($-0.233 < R < -0.089$, $p < 0.05$) and weak positive correlations with eGFR ($R = 0.253$ for CIM-urine and $R = 0.219$ for CIM serum, $p < 0.001$ in both cases). CIM-serum significantly positively correlated with height ($R = 0.119$, $p = 0.012$), while CIM-urine inversely correlated with serum vitamin D (R = -0.178, $p = 0.002$). No significant correlation was observed between Ca isotopes and both weight and creatinine value.

Table 2
Calcium isotope marker (CIM) values of participants adjusted for the consumption of Ca supplement.

Ca isotope marker	Women (n)	Men (n)	p value
CIM-serum (‰)			
original	−0.77 ± 0.15 (15)	−0.77 ± 0.23 (7)	0.976†
adjusted	−1.11 ± 0.20 (15)	−1.03 ± 0.32 (7)	0.481†
CIM-urine (‰)			
original	0.29 ± 0.29 (38)	0.46 ± 0.53 (11)	0.692*
adjusted	−0.01 ± 0.35 (38)	0.12 ± 0.60 (11)	0.990*

Note: The Ca supplement consumed daily were mostly ranging from 100 to 1000 mg, with a median of 500 mg. †t-test; *Mann-Whitney test; results are significant at $p < 0.05$.

3.3. Ca isotopes pattern with gender, age and osteoporosis

3.3.1. Women and age

On average, the CIM values for women and men in both serum and urine differ significantly, with women exhibiting about 0.1 ‰ lower CIM value than men in both serum and urine (Table 1, Fig. 2a and c). This aligns with the fact that 85 % of all osteoporosis cases in Germany are women [28]. This difference is observed despite the average age being statistically indistinguishable between genders. This discrepancy likely reflects the impact of menopause, which typically occurs around the age of 51 in women [29], leading to a substantial decline in estrogen production and reduction of bone Ca absorption. The average nominal CIM value of −0.85 ± 0.2 ‰ in serum and of 0.24 ± 0.31 ‰ in urine is compatible with the equilibrium values as determined earlier in the OsteoGeo study. We argue that the decreasing trend of CIM values, coincidence with the equilibrium value of OsteoGeo study, indicates the transition from a balanced to an average imbalanced Ca budget in the women's bodies reflecting the decline of estrogen production in their fifties.

3.3.2. Men and age

Similarly, CIM-urine of men showed a weak inverse relationship to age (Table 3, Fig. 2b and d). The younger age group (<20 years old) did not show any significant difference due to the low number of participants ($n = 1$). However, a significant difference in CIM-urine was observed between the age groups of >20 to 40 years old and > 40 to 60 years old ($p = 0.003$). Concerning CIM-serum in men, the age group of >40 to 60 years old had significantly lower values compared to the age groups of >20 to 40 and > 60 to 80 years old. In comparison to women, the men show higher CIM values in the age intervals of 20 to 40 and 40 to 60 years likely due to higher Ca absorption in young men and the estrogen decline in women. In contrast, in the age interval of 60 to 80 years, there is no significant difference between women and men. This is interpreted to likely indicate that the loss of testosterone in men older than 60 causes a decline of bone Ca absorption and CIM serum values [30–32].

3.3.3. Osteoporosis in women and men

The average Ca isotope values in women and men with self-disclosed osteoporosis showed the lowest value in both urine and serum values (women: CIM-urine = 0.18 ± 0.33 ‰, $N = 71$ and men: CIM-urine = 0.25 ± 0.34 ‰, $N = 10$; women: CIM-serum = −0.91 ± 0.21 ‰, $N = 24$ and men: CIM-serum = −1.11 ± 0.33 ‰, N = 2) compared to the total average (Fig. 2a-d). The CIM-urine and CIM-serum values of women with self-disclosed osteoporosis were significantly below the threshold values (one-sided $p < 0.05$ in both cases). Given the very low number of men with self-disclosed osteoporosis (N = 2), it was not possible to perform any analysis between groups.

3.4. Ca isotope pattern in different medical conditions

All participants, regardless of gender, with self-reported osteoporosis or reported fractures within the last two years showed an average CIM-urine value around the equilibrium threshold for neutral BMB (Fig. 3, Table S3). Although statistically not significant, participants with osteoporosis exhibited the largest negative deviation in their individual CIM-serum values compared to the threshold value. However, the trend of lower CIM-serum in osteoporosis was not reflected in the fracture group. Participants with low Ca reabsorption, based on RN calculation, also

Table 3
Correlation between CIM values and clinical parameters.

Parameters	CIM-urine [‰]			CIM-serum [‰]		
	n	Correlation coefficient (R)	*p value*	*n*	Correlation coefficient (R)	*p* value
CIM-urine				560	0.763	<0.001
Age (years) – all participants	2265	−0.157	<0.001*	704	−0.233	<0.001*
women	1897	−0.176	<0.001*	538	−0.231	<0.001*
men	360	−0.078	0.138	164	−0.153	0.051
Weight (kg)	2008	0.016	0.472	442	0.025	0.595
Height (cm)	2012	0.025	0.269	443	0.119	0.012*
Serum Ca (mmol/l)	557	−0.089	0.036*	696	−0.136	<0.001*
Urine Ca (mmol/l)	2257	−0.499	<0.001*	560	−0.270	<0.001*
Vitamin D (IU)	294	−0.178	0.002*	296	−0.111	0.057
eGFR (mL/min)	294	0.253	<0.001*	295	0.219	<0.001*
Creatinine (mg/dL)	294	0.040	0.498	417	0.089	0.069
Ca reabsorption RN-value (%)†	360	0.753	<0.001*	360	0.187	<0.001*

Note: Pearson correlation was used to evaluate the correlation between Ca isotopes and clinical parameters unless otherwise specified. †The correlation was evaluated using Spearman rank order correlation. *Correlation is significant on $p < 0.05$. The value of $0.1 < |R| < 0.3$ indicates weak correlation, $0.3 < |R| < 0.5$ indicates moderate correlation, and $0.5 < |R| < 1.0$ indicates strong correlation [55].

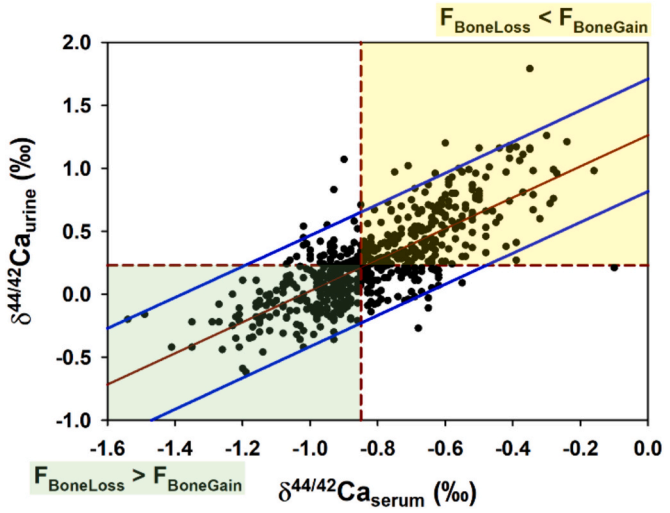

Fig. 1. Correlations between CIM-urine and CIM-serum ($n = 560$; Pearson $R = 0.763$; $p < 0.001$). The solid dark red line represents the regression line. Solid blue lines represent the 95 % prediction interval. Vertical and horizontal dash lines intercept at the respective threshold CIM values for CIM-serum = −0.85 ‰ and CIM-urine = 0.23 ‰, where the fraction of bone loss equals the fraction of bone gain ($F_{BoneLoss} = F_{BoneGain}$) [12]. Therefore, the upper right quadrant indicates $F_{BoneLoss} < F_{BoneGain}$, while lower left quadrant indicates $F_{BoneLoss} > F_{BoneGain}$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

showed significantly lower CIM-urine values than the threshold (Wilcoxon rank test, $p < 0.001$). However, kidney dysfunction and low Ca reabsorption patients tend to correspond to CIM-serum around the equilibrium threshold for neutral BMB. In this study, the relationship between eGFR and RN-value was not significant ($R = 0.087$, $p = 0.137$, $N = 294$, Fig. S5), but all participants with known eGFR and RN-value had relatively good renal condition with RN-value above 70 %.

Cancer patients had different CIM-urine patterns depending on the type of cancer. Breast cancer patients had CIM-urine around the equilibrium threshold for neutral BMB value ($p = 0.272$) but CIM-serum well above the threshold value, although the difference was not significant ($p = 0.126$). On the other hand, prostate cancer patients had lower CIM-urine than the threshold ($p = 0.012$) but the CIM-serum were close to the threshold value ($p = 0.947$). Blood cancer patients have slightly higher levels of both CIM-urine ($p = 0.047$) and CIM-serum ($p = 0.133$) compared to the equilibrium threshold value.

Participants diagnosed with hypo- or hyperthyroidism had CIM-serum and CIM-urine oscillating around the thresholds and the differences were not significant at the 5 % significance level$p > 0.05$ in all cases). However, the spread of the data related to hypothyroid showed a wide range of CIM-urine, tending toward values well below and above the threshold value.

3.5. Effect of medication, diet, height, and smoking on ca isotope pattern

The difference in CIM values between participants with self-reported osteoporosis taking osteoprotective medication (bisphosphonates, denosumab, or Romosozumab) (CIM-urine = 0.44 ± 0.41 ‰ ($N = 39$)) compared to those not taking osteoprotective medication (CIM-urine = 0.21 ± 0.30 ‰ ($N = 38$)) was significant in urine (*t*-test, $p = 0.012$, Fig. 4a). CIM-serum tends to show a similar trend as in urine, but the difference is not significant due to the low number of data ($N = 8$). The positive effect of osteoprotective medication was also seen in the difference in individual Ca isotope values before and after osteoprotective interventions (Fig. 5). Fig. 5b exhibits an individual with osteoprotective intervention of bisphosphonates starting shortly after months 0 and 5 in the context of a breast cancer treatment. This patient started with a CIM-urine value below the threshold (0.11 ‰) at month 0 and received bisphosphonate treatment in month 3. Later Ca isotope measurement in month 7 showed that the CIM-urine value reached a maximum (0.68 ‰), then declined in the following months toward lower values but stayed above the threshold.

There is no difference in the CIM values of the participant groups consuming Ca supplements or vitamin D compared to those not consuming the supplements (Table S4). This result is independent of the adjustment of those study participants who took Ca supplements. Study participants with a vegetarian and in particular vegan diet showed significantly higher CIM-urine relative to those with a normal diet. Two vegan patients were taking Ca supplements which have been adjusted to normal dietary Ca values. Unfortunately, there is only one vegan CIM-serum value available so that no inferences can be made.

A weak positive correlation exists between body height and CIM-serum (Table 3). Body height and Ca absorption efficiency are linked, with taller individuals potentially requiring more Ca to support their larger bone structure. There is no statistical linkage between CIM-urine and height.

There is no significant difference between smokers and non-smokers (Table S3) in their Ca isotope composition although it is reported that smoking has a direct impact on bone health, contributing to an increased risk of osteoporosis and fractures.

4. Discussion

To further test the clinical applicability of the CIM method, we tested

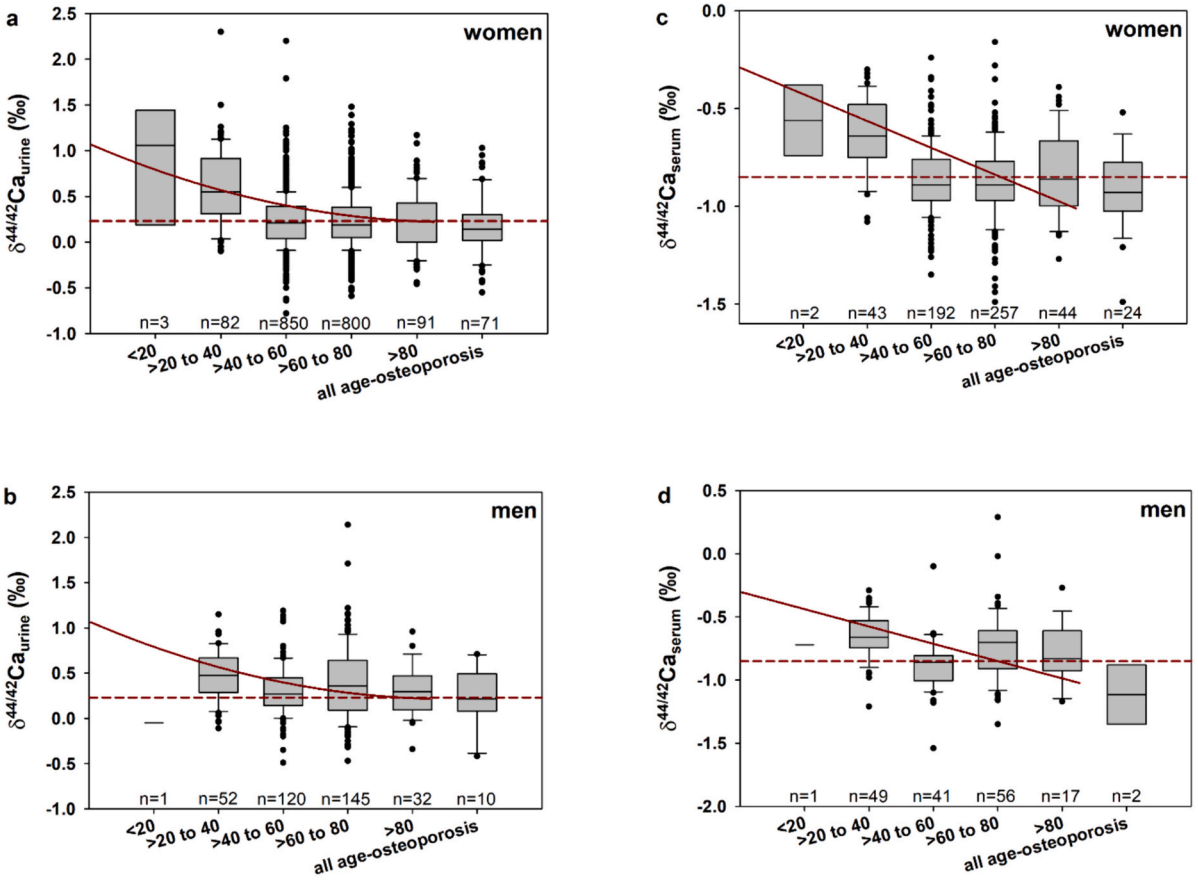

Fig. 2. Box plots of CIM-urine and CIM-serum in women (a and c, respectively) and men (b and d, respectively) at different age groups and in participants with reported osteoporosis. The red horizontal dashed lines represent the threshold CIM-urine and CIM-serum values in the respective figures, where the fraction of bone loss equals the fraction of bone gain ($F_{BoneLoss} = F_{BoneGain}$) [12]. The red lines represent the approximated drop of the Ca isotope values in urine and serum as a function of age ($\delta^{44/42}Ca_{urine} = 0.0000933 \times Age^2 - 0.018 \times \text{Age} + 1.047$ and $\delta^{44/42}Ca_{serum} = -0.008 \times \text{Age} - 0.2675$ for urine and serum, respectively). There was a significant difference in the CIM-urine and CIM-serum values between groups in women and men (Kruskal-Wallis tests, $p < 0.001$ and $p = 0.010$, respectively). Pairwise comparisons of the CIM-urine and CIM-serum in women showed a similar pattern, in which the Ca isotope values in the age group of >20 to 40 years old were significantly different to the age groups of >40 to 60 years old, >60 to 80 years old, >80 years old, and those with osteoporosis ($p < 0.05$ in all case). In men, the CIM-urine values in the age group of >20 to 40 years old were significantly different compared to the age group of >40 to 60 years old ($p = 0.003$), while the other pairwise comparisons of the CIM-urine values did not provide significant results ($p > 0.05$). The CIM-serum values in men showed a significant difference between >40 to 60 years old and > 60 to 80 years old ($p = 0.026$), and between >20 to 40 years old and > 40 to 60 years old ($p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

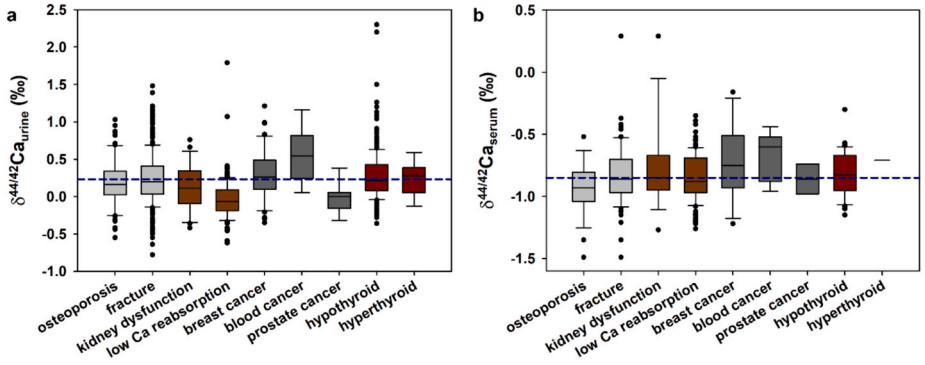

Fig. 3. Box plots of CIM-urine (a) and CIM-serum (b) in the participants with various pre-existing medical conditions as indicated in their questionnaires. The horizontal dashed lines represent the threshold CIM-urine and CIM-serum values in the respective figures, where the fraction of bone loss equals the fraction of bone gain ($F_{BoneLoss} = F_{BoneGain}$) [12]. The health status was also assessed based on the reported medication and clinical parameters. Participants who had low eGFR (<60 ml/min) were considered to suffer from kidney dysfunction. Participants who had Ca reabsorption (RN-value) below 95 % and were not diagnosed with kidney dysfunction (based on the questionnaire and clinical parameters) were grouped in low Ca reabsorption.

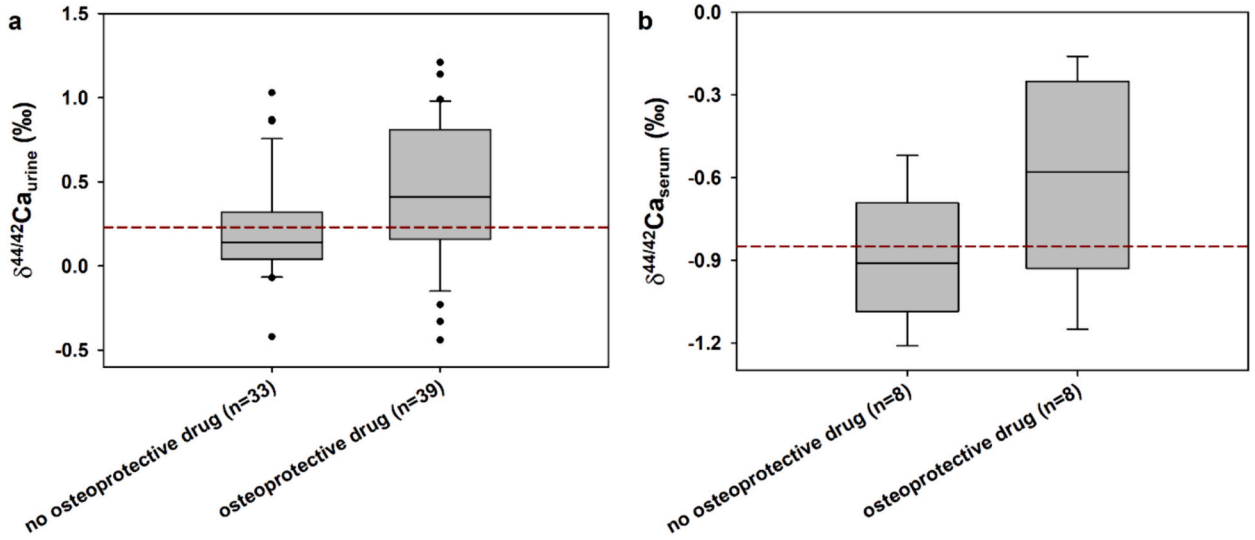

Fig. 4. Box plots of CIM-urine (a) and CIM-serum (b) in participants who had osteoporosis and those who were treated with osteoprotective medication to prevent bone loss (bisphosphonate, denosumab, and Romosozumab). The horizontal dashed lines represent the threshold CIM-urine and CIM-serum values in the respective figures, where the fraction of bone loss equals the fraction of bone gain ($F_{BoneLoss} = F_{BoneGain}$) [12]. There was a significant difference in the CIM-urine values of participants with osteoporosis but not taking osteoprotective medication compared to those who were treated with osteoprotective medication (t-test, $p = 0.012$), indicating that those not taking osteoprotective drugs showed low CIM values and a negative BMB. In contrast, those with CIM-urine above the threshold indicate a positive BMB. However, the CIM-serum values were not significantly different between the two groups (*t*-test, $p = 0.082$) because of the low number of participants ($n = 8$).

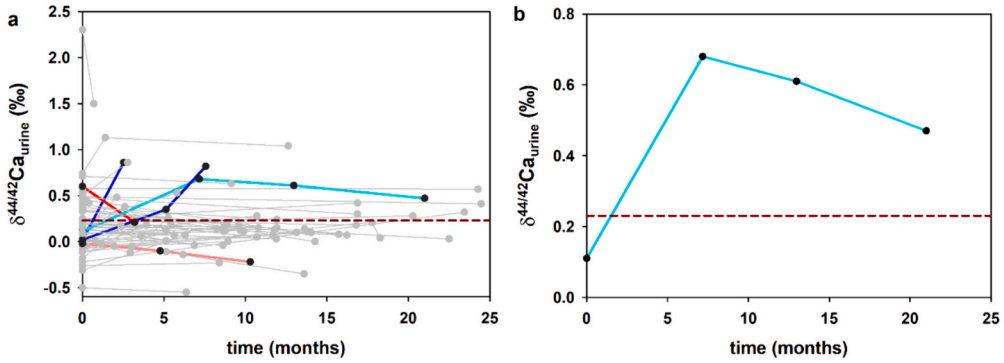

Fig. 5. Measured CIM-urine values of the same individuals who were repeatedly tested (a) and selected time series of a participant monitoring the intervention of an osteoprotective measure applying a bisphosphonate in the context of an aromatase inhibitor cancer treatment (b). Each line indicates $\delta^{44/42}$Ca time series of individual participants. Time at zero refers to the first sampling, and the subsequent sampling is counted from the first sampling time. Dark blue lines represent participants consuming Romosozumab, and the light blue line represents a participant consuming bisphosphonate after the first sampling. The dark red line represents a participant diagnosed with osteoporosis but did not take osteoprotective drugs. Grey lines indicate participants without special remarks. The light red line represents a participant diagnosed with osteoporosis at the third (last) sampling. The horizontal dashed lines represent the threshold CIM-urine and CIM-serum values in the respective figures, where the fraction of bone loss equals the fraction of bone gain ($F_{BoneLoss} = F_{BoneGain}$) [12]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the hypotheses that the threshold values for Ca isotopes in serum and urine that were determined in the OsteoGeo study can distinguish between positive and negative BMB even in a larger group of people independent of age, gender and diet and with a wide variety of medical conditions, drugs and therapies.

4.1. Comparison of the Osteolabs to the OsteoGeo study

In particular, the Osteolab's CIM diagnostic test is based on a single clinical pilot study (OsteoGeo, NCT02967978) with 100 study participants pre-selected by applying distinct inclusion and exclusion criteria. Specifically, the OsteoGeo study did not include men and solely focused on women [12].

The average CIM values for women with DXA confirmed osteoporosis in the OsteoGeo study (CIM-serum = −0.99 ± 0.10 ‰; CIM-urine = 0.10 ± 0.21 ‰; $N = 14$) were in accordance with those of the self-reported osteoporotic women of the Osteolabs study of CIM-serum = −0.91 ± 0.21 ‰ ($N = 24$) and of CIM-urine = 0.18 ± 0.33 ‰ ($N = 71$). These results indicate that the findings of the OsteoGeo study also apply to the much larger Osteolabs data population. The Osteolabs study average CIM-serum and CIM-urine tend to be about 0.08 ‰ higher than the OsteoGeo study's values. It probably reflects that the Osteolabs study participants were about ten years younger, corresponding to commonly better bone health conditions. Furthermore, the OsteoGeo study applied relatively strict inclusion and exclusion criteria to improve the selection of women with osteoporosis, i.e., including female patients with at least one (above 60 years old) or two (above 50 years old) osteoporosis risk factors. The pre-selection of study participants based on their age shifts their average value toward lower average CIM values in the OsteoGeo study compared to the Osteolabs data where the participants are on

average about ten years younger.

4.2. CIM value changes as a function of age

It is a common knowledge that both men and women experience continued bone loss with aging after having passed the peak-bone stage at an age between 25 and 35. Women are at a higher risk due to lower peak bone mass and accelerated bone loss post-menopause. Women experience a rapid decline in bone density following menopause due to a significant drop in estrogen levels, which is critical for maintaining bone density. This period sees the highest rate of bone loss, with a substantial risk of developing osteoporosis. By the age of 65–70, the rate of bone loss in men and women begins to equalize. This is exactly what is seen and reflected in the CIM data.

4.3. Time series data and medication monitoring

Besides diagnosing BMB and the pathology of osteoporosis, CIM is potentially very useful to monitor the effect of osteoporosis therapy. Generally, the Osteolabs study data showed that osteo-protective medication increased the CIM values in the participants (Fig. 4), corresponding to an improved BMB. Time series data of a selected number of participants proved that the CIM-urine steeply rose shortly after the administration of the osteo-protective drug. In particular, Romosozumab increased the CIM-urine up to at least 0.8 ‰ in less than eight months. Similarly, bisphosphonate consumption increased CIM-urine by 0.57 ‰ ($N = 1$) at month 7 (Fig. 5). This result agrees well with an earlier study of oral bisphosphonate consumption for bed-rest participants, which had a maximum increase of ~0.5 ‰ for the $\delta^{44/40}Ca_{urine}$ in a time interval of 17 weeks, comparable to an increase of ~0.3 ‰ for CIM-urine, at month 2 [18]. A higher increase in the CIM-urine of the patients with lower BMB after bisphosphonate consumption is expected as the whole body has sensed Ca inadequacy for a longer time-period, in comparison to a non-resting control group. This is in agreement with current studies which tend to show that Romosozumab is more effective than oral bisphosphonate in reducing fracture risk [33].

4.3.1. The uptake of vitamin D, calcium supplementation, and CIM values

The role of vitamin D and the uptake of Ca is controversial. Yao et al. (2019) conducted a systematic review and meta-analysis to assess the effectiveness of vitamin D and Ca supplementation in preventing fractures [34]. Key findings of this study were that the combined supplementation of Ca (800 to 1200 mg/day) and vitamin D (400 to 800 IU/day) was associated with a 6 % reduction in the risk of any fracture and a 16 % reduction in the risk of hip fractures. However, the study also confirmed that vitamin D alone did not provide significant benefits in fracture prevention. Yao et al. (2019) also noted that while the combined supplementation showed some effectiveness, five of the six trials they referred to had a high risk of bias, suggesting that the evidence should be interpreted with caution. The study emphasizes that while there is some evidence supporting the combined use of vitamin D and Ca to reduce fracture risk, the potential benefits should be weighed against the risks, such as an increased likelihood of kidney stones and possible cardiovascular issues in elderly patients [34,35].

In general, the evidence on the effectiveness of Ca and vitamin D supplementation for preventing osteoporosis-related fractures is mixed. Large-scale studies, such as the Women's Health Initiative (WHI) found no statistically significant reduction in fractures with daily supplementation of 400 IU of vitamin D and 1000 mg of Ca [36]. Moreover, the U.S. Preventive Services Task Force (USPSTF) even concluded that there is inadequate evidence to support higher doses of these supplements for fracture prevention [37].

Our finding that the uptake of Ca supplements and vitamin D was not reflected by an increase of the CIM values ($p > 0.05$ in both cases) aligns with previous literature indicating no clear evidence that Ca and vitamin D supplementation significantly impacts osteoporosis or fracture prevention [35–37]. This suggests no significant improvement in BMB and overall bone health with Ca and vitamin D treatment. These results are in contrast with the current guidelines for osteoporosis treatment, which consider vitamin D uptake essential for osteoporosis prevention [38].

4.3.2. The influence of specific diseases on the BMB and Ca isotope values in serum and urine

The data analysis shows that certain diseases are reflected in serum and urine CIM values (Fig. 3, Table S3). Osteoporosis cases are characterized by average CIM values below equilibrium levels in both urine and serum. However, there is a significant number of outliers, especially with urine CIM values above the equilibrium level which cause the difference to the threshold value not be significant. We speculate that these outliers likely reflect the influence of secondary effects related to other diseases superimposing osteoporosis as the primary disease (Fig. 3). Various conditions, such as vitamin D intoxication and sarcoidosis, may cause renal upregulation of Ca reabsorption. The most prominent condition is probably hyperparathyroidism, which is associated with elevated levels of parathyroid hormone (PTH or PTHrP), reduced calcitonin concentrations, and increased renal Ca reabsorption. This typically results in increased CIM urine values due to renal upregulation but decreased CIM serum values due to the release of bone Ca. Additionally, the degree of osteoporosis at the time of sampling also influences the wide CIM values of the participants with osteoporosis.

The average CIM values for fractures of the last 24 months, kidney dysfunction, and prostate cancer with low PSA levels showed values that were below the equilibrium threshold value, indicating an osteoporotic situation with negative BMB, Ca loss, and bone demineralization. In contrast, breast and blood cancers displayed CIM values above the equilibrium threshold and, therefore, indicated positive BMB associated with Ca gain and bone mineralization.

The negative amplitude in prostate cancer below the equilibrium threshold value was probably related to the intervention with ADT therapies which have a negative impact on the Ca balance since ADT can cause bone demineralization [14,16]. Additionally, kidney dysfunction reduces renal Ca reabsorption, leading to systemic Ca deficiency and a negative bone mineral balance (BMB). This imbalance is counteracted by increased Ca resorption from the bones, which further negatively affects the BMB. This process is reflected in Ca and inorganic mineral (CIM) values falling below the equilibrium threshold [13,15,20].

The positive CIM amplitudes in breast and blood cancer (leukemia and lymphoma) above the equilibrium threshold may be due to standard osteoprotective measures with anti-resorptive agents substances such as denosumab, Romosozumab, and/or bisphosphonates [39], which have a positive effect on Ca absorption and bone mineralization [40]. The latter is reflected by CIM values in serum and urine that were above the equilibrium threshold, indicating an enhanced mineralization and increased bone mass. Moreover, other factors potentially increase CIM values in cancer patients, such as osteoblastic bone lesions, which could be found in 20 % of breast cancer bone metastasis and > 50 % of prostate cancer bone metastasis [41,42]. This result is in contrast to those observed in multiple myeloma, a different type of blood cancer, which showed lower CIM-serum as a manifestation of bone destruction commonly regarded as the hallmark of the disease [43].

Participants with thyroid diseases showed a wide range of CIM-urine values, from −0.4 to 2.3 ‰. Untreated hyperthyroidism causes enhanced bone turnover, accompanied by higher osteoclast activity and a higher risk for osteoporosis [44]. In contrast, hypothyroidism results in low bone turnover but higher bone Ca mineralization which resulted in a wide spread of the data toward higher CIM-urine above the threshold values (CIM-urine = 0.28 ± 0.33, $N = 268$, Fig. 3a), indicating enhanced mineralization and bone mass accumulation for a large number of participants. As most of the participants with pathologies received thyroid hormones, the CIM values may reflect the difference in disease progression or medication effectiveness. Unlike the application of the CIM

values that showed the effect of thyroid disease, applying conventional diagnostic methods did not show a direct relationship between thyroid hormone levels and bone mineral density (BMD) [45,46].

4.4. The influence of diet on the calcium isotope values in serum and urine

Study participants with a vegetarian (mean value: 0.31 ± 0.22 ‰, $N = 72$) and in particular vegan diet (mean value: 0.53 ± 0.35 ‰, $N = 17$) showed average significantly higher CIM-urine values relative to those with a conventional diet (mean value: 0.26 ± 0.29 ‰, p-value: 0.049 and < 0.001 for vegetarian and vegan, respectively), for details see Table S3 in the appendix. Unfortunately, there is only one vegan for whom we have a serum sample. There is no paired CIM-urine and CIM-serum value available.

The result that vegan CIM-urine show higher values is surprising and contradictory because, according to a large cohort study [47], the calculated average daily Ca intake for a vegan diet was in the order of less than ~525 mg Ca and the vegans simultaneously showed the lowest plasma levels of vitamin D on average of only about 55.8 nmol/l. Based on the same cohort, it was also reported that vegans had a 30 % higher risk of bone fractures [48]. Therefore, a low Ca intake of less than ~525 mg for vegan [48] is expected to result in a low bone mineralization rate and low CIM-urine values which is in contrast to our observation of high CIM-urine in vegans.

Unfortunately, except for information about the uptake of supplements, we do not have specific data on the vegan diet of the study participants. Therefore, it cannot be excluded that vegans consume a special vegan-related diet like Ca-set tofu, Ca-fortified milk imitation, or a similar enriched diet to account for their Ca deficiency. To reconcile relatively high CIM-urine values and low Ca consumption, we have to take into account that some, if not all vegans eat a Ca-enriched Ca-set tofu or Ca-fortified milk imitation to ensure adequate Ca intake to account for their diet-related Ca deficiency. Ca-set tofu is a type of tofu that has been coagulated using a Ca salt, typically calcium sulfate (gypsum, $CaSO_4$) or calcium chloride ($CaCl_2$), during the tofu-making process which increases its Ca content from the usual Ca level of about 100 to 150 mg/100 g [49] to about 350 to 450 mg/100 g and up to 680 mg/100 g [50,51], significantly. Ca-fortified milk imitation is plant-based "milk" derived from low-contained almond drinks, soy drink, coconut drink, etc., that is added with Ca to match the Ca content of cow milk [52]. Calcium from biological sources would not change the Ca isotope composition of serum and urine. In contrast, Gypsum and calcium chloride are of non-biological origin and can also be considered to be just like normal Ca supplements (see Table S2) also about 1 ‰ higher than the supplement-free and normal diet. This kind of diet would shift from the general equilibrium threshold value toward higher values as we see in vegan urine. In the future, the impact of a Ca-enriched diet on the serum and urine CIM values has to be examined in detail.

Without a Ca-enriched diet, a vegan diet may interfere with PTH and PTHrP levels, potentially leading to secondary hyperparathyroidism associated with increased bone Ca resorption and reduced bone mineralization rates, resulting in net bone Ca loss [53,54]. To confirm secondary hyperparathyroidism, we would expect to see low CIM-serum values (indicating bone Ca loss) but high CIM-urine values (indicating increased renal Ca reabsorption). However, we currently lack paired CIM-serum and CIM-urine values for vegans, and thus, this hypothesis remains to be validated by future research.

5. Weakness of the study

The Ca isotope value of a diet depends primarily on the type of diet, mainly reflecting the daily intake of dairy products and, to a lesser extent, vegan or vegetarian diets.

Measured CIM-serum and CIM-urine values are adjusted to a normal diet if Ca supplements are consumed. Future studies have to evaluate the role of Ca-fortified non-dairy kinds of milk and tofu products on the CIM values.

It has not yet been tested whether socioeconomic status and different dietary behaviors might also influence the Ca isotope composition of the diet.

This study relied on self-declaration of diseases, therapies and medications. Inaccuracies in reporting may distort data.

Participants of this study may not represent the general population, hence results may not be generalized.

6. Conclusions

The threshold values as reported earlier in the OsteoGeo study were confirmed by the Osteolabs study presented here. The Osteolabs study also showed that all factors positively or negatively affecting the musculoskeletal metabolism were reflected by a change of the CIM value in urine and serum. Diseases, such as osteoporosis, prostate cancer, or thyroid diseases, medications, and hormonal therapies (e.g., ADT and thyroxine) related to a negative BMB were reflected by CIM values significantly below the threshold value. Osteoprotective medications like bisphosphonates, denosumab, and Romosozumab were related to a positive BMB, reflected by CIM values significantly above the threshold values. Other individual factors like age and sex were also reflected by the CIM values. The Osteolabs study also revealed interesting results for vegans showing that their CIM-urine is the highest compared to non-vegans.

This study supports earlier findings that CIM values are a strong predictor of BMB [13], reflect renal function, and indicate side effects of diseases and medical therapies influencing the musculoskeletal system. The significant correlations between individual attributes like gender, age, and health status offer the possibility of performing a predictive, minimally-invasive, easily repeatable and early detection of Ca loss and metabolic diseases affecting the musculoskeletal metabolism. It also offers the possibility of performing minimally invasive 'liquid biopsies' to assess bone health. Although Osteolabs is commercially distributing Ca isotope diagnostics, the clinical application is still in its infancy and is currently treated more as a sophisticated research tool. However, as part of a comprehensive bone biomarker panel, it proves to be an innovative and excellent adjunct to current diagnostics, enabling the early identification of patients who may benefit from osteoporosis treatment, allowing real-time monitoring of the therapeutic response, and predicting fracture risk. Isotope diagnostics to monitor the effect of bone anabolic medications may allow personalized treatments to improve bone health, early identification of bone metastasis, monitoring the response to certain chemotherapeutic agents and reducing fracture risk in patients with kidney impairment.

Data sharing statement

The data collected for the study, including individual patient data and a data dictionary that defines each field in the data set, will be made available as deidentified participant data to researchers who propose to use the data for individual patient data meta-analysis. Data will be shared following approval of the proposal by the corresponding author and a signed data access agreement.

CRediT authorship contribution statement

A. Eisenhauer: Writing – review & editing, Writing – original draft, Project administration, Investigation, Conceptualization. **A. Hastuti:** Writing – original draft, Investigation, Formal analysis, Conceptualization. **A. Heuser:** Writing – review & editing, Validation, Software, Methodology. **A. Kolevica:** Methodology. **B. Brandt:** Validation. **R. Shroff:** Writing – review & editing, Validation. **J. Oehme:** Writing – review & editing. **M. Müller:** Writing – review & editing, Validation, Conceptualization.

Declaration of competing interest

AE, AK, and MM are co-founders of Osteolabs GmbH. AH and JO are part-time employees of the Osteolabs GmbH. The remaining authors have declared that no conflict of interest exists.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2024.117210.

References

[1] K. Diem, C. Lenter, Scientific Tables 5th ed., vol. 565, Ciba-Geigy Limited, Basel, 1970.
[2] M. Peacock, Calcium metabolism in health and disease, Clin. J. Am. Soc. Nephrol. 5 (2010) 23–30, https://doi.org/10.2215/CJN.05910809.
[3] M.B. Moor, O. Bonny, Ways of calcium reabsorption in the kidney, Am. J. Physiol. Ren. Physiol. 310 (2016) F1337–F1350, https://doi.org/10.1152/ajprenal.00273.2015.
[4] A. Vakiti, C. Anastasopoulou, P. Mewawalla, Malignancy-Related Hypercalcemia. http://www.ncbi.nlm.nih.gov/pubmed/31086686, 2024.
[5] M. Librizzi, F. Naselli, G. Abruscato, C. Luparello, F. Caradonna, Parathyroid hormone related protein (PTHrP)-associated molecular signatures in tissue differentiation and non-Tumoral diseases, Biology (Basel). 12 (2023) 950, https://doi.org/10.3390/biology12070950.
[6] L.J. Suva, P.A. Friedman, PTH and PTHrP actions on bone, in: P.H. Stern (Ed.), Bone Regul. Osteoporos. Ther. Handb. Exp. Pharmacol vol. 262, Springer, Cham, 2020, pp. 27–45, https://doi.org/10.1007/164_2020_362.
[7] E.F. Kranioti, A. Bonicelli, J.G. García-Donas, Bone-mineral density: clinical significance, methods of quantification and forensic applications, Res. Reports Forensic Med. Sci. 9 (2019) 9–21, https://doi.org/10.2147/rrfms.s164933.
[8] T.T. Hlaing, J.E. Compston, Biochemical markers of bone turnover - uses and limitations, Ann. Clin. Biochem. 51 (2014) 189–202, https://doi.org/10.1177/0004563213515190.
[9] O. Al Musaimi, L. Lombardi, D.R. Williams, F. Albericio, Strategies for improving peptide stability and delivery, Pharmaceuticals 15 (2022) 1283, https://doi.org/10.3390/ph15101283.
[10] J.E. Compston, SP0201 usefulness and limitations of DXA for diagnosing osteoporosis, Ann. Rheum. Dis. 73 (2014) 52, https://doi.org/10.1136/annrheumdis-2014-eular.6150.
[11] P. Choksi, K.J. Jepsen, G.A. Clines, The challenges of diagnosing osteoporosis and the limitations of currently available tools, Clin. Diabetes Endocrinol. 4 (2018) 12, https://doi.org/10.1186/s40842-018-0062-7.
[12] A. Eisenhauer, M. Müller, A. Heuser, A. Kolevica, C.C. Glüer, M. Both, C. Laue, U. V. Hehn, S. Kloth, R. Shroff, J. Schrezenmeir, Calcium isotope ratios in blood and urine: a new biomarker for the diagnosis of osteoporosis, Bone Reports. 10 (2019) 100200, https://doi.org/10.1016/j.bonr.2019.100200.
[13] R. Shroff, M. Fewtrell, A. Heuser, A. Kolevica, A. Lalayiannis, L. McAlister, S. Silva, N. Goodman, C.P. Schmitt, L. Biassoni, A. Rahn, D.C. Fischer, A. Eisenhauer, Naturally occurring stable calcium isotope ratios in Body compartments provide a novel biomarker of bone mineral balance in children and young adults, J. Bone Miner. Res. 36 (2021) 133–142, https://doi.org/10.1002/jbmr.4158.
[14] K. David, G. Devos, N. Narinx, L. Antonio, W. Devlies, L. Deboel, D. Schollaert, A. Eisenhauer, E. Cavalier, D. Vanderschueren, F. Claessens, S. Joniau, B. Decallonne, Changes in bone and mineral homeostasis after short-term androgen deprivation therapy with or without androgen receptor signalling inhibitor – substudy of a single-Centre, double blind, randomised, placebo-controlled phase 2 trial, EBioMedicine 97 (2023) 1–11, https://doi.org/10.1016/j.ebiom.2023.104817.
[15] R. Shroff, A.D. Lalayiannis, M. Fewtrell, C.P. Schmitt, A. Bayazit, V. Askiti, A. Jankauskiene, J. Bacchetta, S. Silva, N. Goodman, L. McAlister, L. Biassoni, N. Crabtree, A. Rahn, D.C. Fischer, A. Heuser, A. Kolevica, A. Eisenhauer, Naturally occurring stable calcium isotope ratios are a novel biomarker of bone calcium balance in chronic kidney disease, Kidney Int. 102 (2022) 613–623, https://doi.org/10.1016/j.kint.2022.04.024.
[16] R. Khalil, L. Antonio, M.R. Laurent, K. David, N.R. Kim, P. Evenepoel, A. Eisenhauer, A. Heuser, E. Cavalier, S. Khosla, F. Claessens, D. Vanderschueren, B. Decallonne, Early effects of androgen deprivation on bone and mineral homeostasis in adult men: a prospective cohort study, Eur. J. Endocrinol. 183 (2020) 181–189, https://doi.org/10.1530/EJE-20-0348.
[17] J.L.L. Morgan, J.L. Skulan, G.W. Gordon, S.J. Romaniello, S.M. Smith, A.D. Anbar, Rapidly assessing changes in bone mineral balance using natural stable calcium isotopes, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 9989–9994, https://doi.org/10.1073/pnas.1119587109.
[18] J. Skulan, T. Bullen, A.D. Anbar, J.E. Puzas, L. Shackelford, A. LeBlanc, S.M. Smith, Natural calcium isotopic composition of urine as a marker of bone mineral balance, Clin. Chem. 53 (2007) 1155–1158, https://doi.org/10.1373/clinchem.2006.080143.
[19] A. Cranney, S.A. Jamal, J.F. Tsang, R.G. Josse, W.D. Leslie, Low bone mineral density and fracture burden in postmenopausal women, Can. Med. Assoc. J. 177 (2007) 575–580, https://doi.org/10.1503/cmaj.070234.
[20] K.M. Hill Gallant, X.-Y. Zheng, Natural stable calcium isotope ratios: a new gold standard for bone balance? Kidney Int. 102 (2022) 473–476, https://doi.org/10.1016/j.kint.2022.06.017.
[21] M.J. Joyner, N. Paneth, Promises, promises, and precision medicine, J. Clin. Invest. 129 (2019) 946–948, https://doi.org/10.1172/JCI126119.
[22] B. Röhrig, J.B. Du Prel, D. Wachtlin, R. Kwiecien, M. Blettner, Fallzahlplanung in klinischen studien: Teil 13 der serie zur bewertung wissenschaftlicher publikationen, Dtsch. Arztebl. 107 (2010) 552–556, https://doi.org/10.3238/arztebl.2010.0552.
[23] A. Heuser, A. Eisenhauer, A pilot study on the use of natural calcium isotope (44Ca/40Ca) fractionation in urine as a proxy for the human body calcium balance, Bone 46 (2010) 889–896, https://doi.org/10.1016/j.bone.2009.11.037.
[24] J.L.L. Morgan, G.W. Gordon, R.C. Arrua, J.L. Skulan, A.D. Anbar, T.D. Bullen, High-precision measurement of variations in calcium isotope ratios in urine by multiple collector inductively coupled plasma mass spectrometry, Anal. Chem. 83 (2011) 6956–6962, https://doi.org/10.1021/ac200361t.
[25] W.M. Michels, D.C. Grootendorst, M. Verduijn, E.G. Elliott, F.W. Dekker, R. T. Krediet, Performance of the Cockcroft-gault, MDRD, and new CKD-EPI formulas in relation to GFR, age, and Body size, Clin. J. Am. Soc. Nephrol. 5 (2010) 1003–1009, https://doi.org/10.2215/CJN.06870909.
[26] International Society of Nephrology, KDIGO 2012 clinical practice guideline for the evaluation and Management of Chronic Kidney Disease, Kidney Int. Suppl. 3 (2013).
[27] N. Chu, G.M. Henderson, R.E.M. Hedges, Ca isotope variations in modern dietary systems and their potential to assess the importance of dairying in past cultures, Geophys. Res. Abstr. 7 (2005) 07426.
[28] J.A. Kanis, N. Norton, N.C. Harvey, T. Jacobson, H. Johansson, M. Lorentzon, E. V. McCloskey, C. Willers, F. Borgström, SCOPE 2021: a new scorecard for osteoporosis in Europe, Arch. Osteoporos. 16 (2021) 82, https://doi.org/10.1007/s11657-020-00871-9.
[29] G.A. Greendale, N.P. Lee, E.R. Arriola, The menopause, Lancet 353 (1999) 571–580, https://doi.org/10.1016/S0140-6736(98)05352-5.
[30] H.A. Fink, S.K. Ewing, K.E. Ensrud, E. Barrett-Connor, B.C. Taylor, J.A. Cauley, E. S. Orwoll, Association of Testosterone and Estradiol Deficiency with osteoporosis and rapid bone loss in older men, J. Clin. Endocrinol. Metab. 91 (2006) 3908–3915, https://doi.org/10.1210/jc.2006-0173.
[31] S. Khosla, L.J. Melton, B.L. Riggs, Estrogens and bone health in men, Calcif. Tissue Int. 69 (2001) 189–192, https://doi.org/10.1007/s00223-001-1044-8.
[32] S.M. Harman, E.J. Metter, J.D. Tobin, J. Pearson, M.R. Blackman, Longitudinal effects of aging on serum Total and free testosterone levels in healthy men, J. Clin. Endocrinol. Metab. 86 (2001) 724–731, https://doi.org/10.1210/jcem.86.2.7219.
[33] M.N. Händel, I. Cardoso, C. von Bülow, J.F. Rohde, A. Ussing, S.M. Nielsen, R. Christensen, J.-J. Body, M.L. Brandi, A. Diez-Perez, P. Hadji, M.K. Javaid, W. F. Lems, X. Nogues, C. Roux, S. Minisola, A. Kurth, T. Thomas, D. Prieto-Alhambra, S.L. Ferrari, B. Langdahl, B. Abrahamsen, Fracture risk reduction and safety by osteoporosis treatment compared with placebo or active comparator in postmenopausal women: systematic review, network meta-analysis, and meta-regression analysis of randomised clinical trials, BMJ (2023) e068033, https://doi.org/10.1136/bmj-2021-068033.
[34] P. Yao, D. Bennett, M. Mafham, X. Lin, Z. Chen, J. Armitage, R. Clarke, Vitamin D and calcium for the prevention of fracture, JAMA Netw. Open 2 (2019) e1917789, https://doi.org/10.1001/jamanetworkopen.2019.17789.
[35] D.C. Grossman, S.J. Curry, D.K. Owens, M.J. Barry, A.B. Caughey, K.W. Davidson, C.A. Doubeni, J.W. Epling, A.R. Kemper, A.H. Krist, M. Kubik, S. Landefeld, C. M. Mangione, M. Silverstein, M.A. Simon, C.-W. Tseng, Vitamin D, calcium, or

combined supplementation for the primary prevention of fractures in community-dwelling adults, JAMA 319 (2018) 1592, https://doi.org/10.1001/jama.2018.3185.
[36] R.L. Prentice, G.L. Anderson, The Women's Health Initiative: lessons learned, Annu. Rev. Public Health 29 (2008) 131–150, https://doi.org/10.1146/annurev.publhealth.29.020907.090947.
[37] V.A. Moyer, Vitamin D and Calcium Supplementation to Prevent Fractures in Adults: U.S. Preventive Services Task Force Recommendation Statement, Ann. Intern. Med (2013), https://doi.org/10.7326/0003-4819-158-9-201305070-00603.
[38] D.A. Hanley, A. Cranney, G. Jones, S.J. Whiting, W.D. Leslie, D.E.C. Cole, S. A. Atkinson, R.G. Josse, S. Feldman, G.A. Kline, C. Rosen, Vitamin D in adult health and disease: a review and guideline statement from osteoporosis Canada, Can. Med. Assoc. J. 182 (2010) E610–E618, https://doi.org/10.1503/cmaj.080663.
[39] R. Bartl, B. Frisch, E. von Tresckow, C. Bartl, Bisphosphonates in Medical Practice, Springer Berlin Heidelberg, Berlin, Heidelberg, 2007. doi:https://doi.org/10.1007/978-3-540-69870-8.
[40] G.D. Roodman, Mechanisms of bone metastasis, N. Engl. J. Med. 350 (2004) 1655–1664, https://doi.org/10.1056/NEJMra030831.
[41] K. Venetis, R. Piciotti, E. Sajjadi, M. Invernizzi, S. Morganti, C. Criscitiello, N. Fusco, Breast cancer with bone metastasis: molecular insights and clinical management, Cells 10 (2021) 1–11, https://doi.org/10.3390/cells10061377.
[42] J.-C. Janssen, N. Woythal, S. Meißner, V. Prasad, W. Brenner, G. Diederichs, B. Hamm, M.R. Makowski, [68Ga]PSMA-HBED-CC uptake in osteolytic, osteoblastic, and bone marrow metastases of prostate Cancer patients, Mol. Imaging Biol. 19 (2017) 933–943, https://doi.org/10.1007/s11307-017-1101-y.
[43] G.W. Gordon, J. Monge, M.B. Channon, Q. Wu, J.L. Skulan, A.D. Anbar, R. Fonseca, Predicting multiple myeloma disease activity by analyzing natural calcium isotopic composition, Leukemia 28 (2014) 2112–2115, https://doi.org/10.1038/leu.2014.193.
[44] G.R. Williams, J.H.D. Bassett, Thyroid diseases and bone health, J. Endocrinol. Invest. 41 (2018) 99–109, https://doi.org/10.1007/s40618-017-0753-4.
[45] A.P. Delitala, A. Scuteri, C. Doria, Thyroid hormone diseases and osteoporosis, J. Clin. Med. 9 (2020) 1034, https://doi.org/10.3390/jcm9041034.
[46] D. Apostu, O. Lucaciu, D. Oltean-Dan, A.-D. Mureşan, C. Moisescu-Pop, A. Maxim, H. Benea, The influence of thyroid pathology on osteoporosis and fracture risk: a review, Diagnostics 10 (2020) 149, https://doi.org/10.3390/diagnostics10030149.
[47] F.L. Crowe, M. Steur, N.E. Allen, P.N. Appleby, R.C. Travis, T.J. Key, Plasma concentrations of 25-hydroxyvitamin D in meat eaters, fish eaters, vegetarians and vegans: results from the EPIC–Oxford study, Public Health Nutr. 14 (2011) 340–346, https://doi.org/10.1017/S1368980010002454.
[48] P. Appleby, A. Roddam, N. Allen, T. Key, Comparative fracture risk in vegetarians and nonvegetarians in EPIC-Oxford, Eur. J. Clin. Nutr. 61 (2007) 1400–1406, https://doi.org/10.1038/sj.ejcn.1602659.
[49] AlgaeCal Inc, Calcium in Tofu – Did you Know Tofu Is High in Calcium?. https://www.algaecal.com/calcium/foods/tofu/, 2024. (Accessed 13 July 2024).
[50] C. Milmine, Calcium in Tofu: Detailed Nutrition from a Dietitian. https://plantpoweredyou.com/calcium-in-tofu/, 2024. (Accessed 13 July 2024).
[51] U.S. Department of Agriculture, Tofu, raw, firm, prepared with calcium sulfate, (2018). https://fdc.nal.usda.gov/fdc-app.html#/food-details/172475/nutrients (accessed July 13, 2024).
[52] K.E. Scholz-Ahrens, F. Ahrens, C.A. Barth, Nutritional and health attributes of milk and milk imitations, Eur. J. Nutr. 59 (2020) 19–34, https://doi.org/10.1007/s00394-019-01936-3.
[53] J. Menzel, K. Abraham, G.I. Stangl, P.M. Ueland, R. Obeid, M.B. Schulze, I. Herter-Aeberli, T. Schwerdtle, C. Weikert, Vegan diet and bone health—results from the cross-sectional rbvd study, Nutrients 13 (2021) 1–16, https://doi.org/10.3390/nu13020685.
[54] R.M. Merrill, S.G. Aldana, Consequences of a plant-based diet with low dairy consumption on intake of bone-relevant nutrients, J. Women's Heal. 18 (2009) 691–698, https://doi.org/10.1089/jwh.2008.1020.
[55] J. Cohen, Statistical power analysis for the behavioral sciences, Routledge (1988), https://doi.org/10.4324/9780203771587.