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Paper

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Investigating apparent drivers of urinary Ca isotope variability in a sample population

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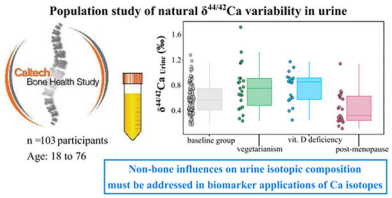
Abstract

Isotopes of calcium (Ca) in blood and urine have been introduced as a potential clinical tool for monitoring bone mineral balance (BMB). While several works support the ability of Ca isotope composition ($\delta^{44/42}$Ca) to capture a shift in BMB in response to external forcings (*e.g.* bed rest) or disease (*e.g.* osteoporosis), the influence of an individual's demographic, health status, diet or lifestyle on $\delta^{44/42}Ca_{Urine/Blood}$ remains largely unconstrained. To gauge the effects of several variables among these four broader categories, we present a population study of $\delta^{44/42}Ca_{Urine}$ from 103 individuals (age 18-76). Age is negatively correlated with $\delta^{44/42}Ca_{Urine}$, and we identify three other attributes (active vitamin D deficiency, vegetarian diet, and being post-menopausal) that lead to systematic differences in an individual's $\delta^{44/42}Ca_{Urine}$. Fluctuations in Ca reabsorption generate significant intra-individual $\delta^{44/42}Ca_{Urine}$ variability. Within a typical range of reabsorption rates, however, the initial isotope composition ($\delta^{44/42}Ca_{Serum}$) exerts the strongest control on inter-individual $\delta^{44/42}Ca_{Urine}$ variability. Using a simple Rayleigh model to express isotopic fractionation associated with Ca reabsorption in the kidneys, we find that a fractionation factor of 0.99972 reproduces the range of isotope ratios and excretion values for several, healthy individuals across three different studies and nearly a 30-year age span. Importantly, $\delta^{44/42}Ca_{Urine}$ variations in three post-menopausal women from three separate studies cannot be explained by the same model, pointing to a different mechanistic control of $\delta^{44/42}Ca_{Urine}$ in these subjects.

Graphical abstract



Introduction

Osteoporosis is a debilitating disease that affects approximately 500 million people worldwide.[1] As no cure exists, the best path for at-risk groups is early detection and treatment to slow the progression of bone loss. The current gold-standard technique for determining bone mineral density (BMD) is dual-energy X-ray absorptiometry (DEXA), which provides a metric ('T-score') of how strongly an individual's BMD deviates from a reference population, with values below -2.5 taken as diagnostic of osteoporosis. While DEXA scans can accurately diagnose true cases of osteoporosis with excellent sensitivity (≥ 90%),

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they struggle to identify true instances of non-diseased patients (specificity ∼ 40%).[2] This is, in part, because (i) DEXA scans are standardized using measurements of the femoral neck, hip, or lumbar spine and therefore may not reflect BMD of the entire skeleton, and (ii) scan resolution only permits detection of bone loss on timescales of 0.5-1 year (at best), over which BMD can deteriorate significantly.[3] Ample opportunity thus exists to enhance the early diagnosis of this life-threatening disease.

In recent years, calcium (Ca) stable isotopes have emerged as a promising potential biomarker of bone health. Indeed, changes in an individual's bone mineral balance (BMB)—the homeostasis between rates of bone formation and resorption—have been shown to lead to acute shifts in blood and urine Ca isotope composition [*e.g.* 4–6]. This is because the skeleton in adults, which contains over 1 kg Ca as hydroxyapatite ($Ca_5[PO4]_3OH$), dominates the body's Ca budget (> 99%).[7] As bones preferentially incorporate light Ca isotopes (*e.g.* ^{42}Ca) over heavy ones (*e.g.* ^{44}Ca), they are isotopically 'light' relative to serum and other soft tissue.[8] Thus, when the skeleton is in an anabolic state and bone formation dominates (*i.e.* net positive BMB), larger amounts of light Ca isotopes are sequestered from the blood into the bones, driving the blood's Ca isotopic composition towards heavier values. This shift is then inherited by the urine. Alternatively, during skeletal catabolism (*i.e.* net negative BMB), the flux of Ca from bones to blood increases. Light Ca isotopes enter the bloodstream and lower both serum and urine isotope ratios. Critically, these shifts are detectable on timescales of days to weeks,[4—6,9] which provides a significant improvement towards early detection of acute bone loss.

Several seminal works support the use of Ca isotopes as tracers of BMB in response to acute bone loss. Skulan et al.[4] were the first to report the resolvable, negative shift in urinary Ca isotopes for 10 people throughout 17 weeks of prolonged bed rest. In another, 30-day bed rest study, Morgan et al.[5] demonstrated that urinary Ca isotopes respond remarkably fast (1-2 weeks), far surpassing other biomarkers. Building on these early studies, Eisenhauer et al.[10] evaluated the effectiveness of Ca isotopes (in both serum and urine) as diagnostic tools for osteoporosis in a cohort of 100 women, 14 of whom had a clinical, DEXA-based diagnosis. They found that urinary Ca isotopes had a sensitivity of 78.6% and a specificity of 71.2% when compared to a cutoff value defined by an age-matched control group. More recent works report stronger metrics, both ∼79%,[11,12] and an updated threshold value of + 0.23 ‰ (reported as the $^{44/42}Ca$ ratio in urine normalized to the SRM 915a standard in units of per mille).

At the same time, the field has begun to recognize and attempt to quantify the impact of mechanisms other than bone dynamics on blood/urine Ca isotopes. Heuser et al.[9] drew attention to the strong isotopic fractionation induced by normal kidney function. They demonstrated that the large, inter-individual variability in pre-bed rest Ca isotope ratios could be accounted for by differences in Ca excretion and showed that urinary Ca isotopes are modulated by more than one process and, thus, cannot be used directly to constrain BMB status. Instead, they suggested the use of both isotopes and excretion to monitor changes in BMB. In a longitudinal study on healthy participants, Tissot et al.[13] documented short-term variability in urinary Ca isotope ratios in response to certain external factors, such as fasting, exercise and alcohol consumption. In combination, these studies show that multiple factors can influence the blood/urine Ca isotope compositions in humans, and that a fundamental understanding of these drivers and their specific impact on $\delta^{44/42}Ca_{Blood/Urine}$ is needed to use Ca isotopes as a diagnostic biomarker.

Here, we aim to identify and investigate drivers of Ca isotope variability among a large and diverse population of primarily healthy participants. To do so, we analyzed 124 urine samples from 81 individuals in basal physiological state, ranging in age from 18 to 76. Using these data, we identify 3 attributes (active vitamin D deficiency, vegetarian diet, and being post-menopausal) that appear to systematically affect Ca isotope ratios in urine. We then model isotopic fractionation induced by Ca reabsorption in the kidneys as a Rayleigh distillation and examine data from each of the 3 groups in the framework of the Rayleigh model.

Materials and methods

Study design

The study aimed at characterizing the natural variability of Ca isotope compositions in urine ($\delta^{44/42}Ca_{Urine}$) among individuals with a putatively homeostatic BMB to assess the influence of several potential drivers of isotopic variability. This study was approved by the Caltech IRB review board (#IR21-1172) after being reviewed in accordance with the requirements of Part 46, "Protection of Human Subjects", of Title 45 of the Code of Federal Regulations and the "U.S. Department of Health and Human Services (DHHS) Federal-Wide Assurance (FWA) for the Protection of Human Subjects for Domestic Institutions," California Institute of Technology Assurance # FWA00003897. Participation was open to all adult volunteers (18 years old or more). Due to biosafety concerns, individuals who were either HIV positive or experiencing chronic urinary tract infections were excluded from this study.

Full details on participant recruitment, urine sampling and self-reporting questionnaires can be found in Tissot et al.[13] In Tissot et al.,[13] we interrogated the short- to medium-term temporal variability of urine Ca isotope ratios in 22 individuals by isolating data from participants who provided > 4 samples over periods of days to months. To complement this longitudinal study, here, we report on samples from a large and diverse pool of individuals ($n = 103$ total, including the 22 from[13]) who did not provide multiple samples, but rather were variably affected by factors that may or may not influence urine Ca isotopes (*e.g.* specific diet or hormonal state).

Sample processing

Our methods for sample processing and mass spectrometric analyses are virtually identical to those reported in Tissot et al.[13] We therefore refer the reader to that work for details not included here and only briefly describe our protocol below. All sample preparation and analysis work was performed at the Isotoparium (Caltech).

Upon initial inspection of urine samples, vials containing visible red blood cells were excluded. For samples passing this screening, urine aliquots of 0.4-1.0 mL were digested in a Teflon beaker in two overnight steps, both using a 50:50 mixture of 15 mol·L^{-1} HNO_3 and 31% H_2O_2. Then, samples were evaporated to dryness and diluted in 2 mol·L^{-1} HNO_3. Chromatography used a fully automated prepFAST MC (ESI) with 1 mL self-packed, resin-filled columns. With every batch of samples (12 to 33 per batch), 2 procedural blanks and 6 reference materials were also processed. Fractions of pure Ca were collected in clean Teflon vials and evaporated to dryness, at which point they were treated with small volumes of 15 mol·L^{-1} HNO_3 to remove any residual organic compounds. Finally, Ca fractions were evaporated to dryness and re-dissolved in 1 mL of 2% (vol.) HNO_3.

Mass spectrometry

Elemental quantification was performed using an iCAP RQ ICP-MS, both before and after chromatography, to determine major element concentrations and assess recovery yields. Urine measurements utilized a 5-point calibration curve from 0 to 1000 μg/mL, with equal proportions of Na, Mg, K and Ca in each standard. Purified Ca fractions were analyzed separately using a mono-elemental calibration curve. Calibration standards and duplicate samples were rerun every ∼30 minutes for quality assurance. Each solution was spiked with indium (In), which was used to correct for instrumental drift. The drift correction was not applied if (i) signal intensities on all analytes remained within 10% for the calibration standards for the entire session and (ii) transmission of the internal standard in each sample was within 5% of the starting intensities.

Isotopic analyses were performed using a Neptune *Plus* MC-ICP-MS. Measurements were conducted in medium or high resolution to resolve isobaric interferences (*e.g.* $^{40}ArH_2^+$, $^{14}N_3^+$). Samples and standards were measured at concentrations of 1 μg/mL (MR) or 2 μg/mL (HR). Each analysis consisted of 60 cycles of 4.192 s integration time. Standard-sample bracketing was employed to correct for isotopic fractionation induced by the mass spectrometer. A baseline calibration of all Faraday cups was performed before each sequence and amplifier gains were run at the start of each analytical session.

Isotopic nomenclature and reporting

Isotopic ratios are expressed in delta notation in units of per mille (‰) as:

$$\delta^{44/42}Ca_{SRM915a}(‰) = \left(\frac{^{44/42}Ca_{sample}}{^{44/42}Ca_{SRM915a}} - 1\right) \times 10^3. \quad (1)$$

Calibration of our in-house Ca SPEX solution against SRM 915b was done over 13 analytical sessions and yields a value of + 0.28 ± 0.03 ‰ (2SE, n = 14) relative to SRM 915b. Similarly to Tissot et al,[13] we converted our data to the SRM 915a scale using the well documented offset between SRM 915b and SRM 915a of + 0.35 ± 0.01‰ (2SE, n = 36;[14]), as:

$$\delta^{44/42}Ca_{SRM915a} = \delta^{44/42}Ca_{SPEX} + (0.63 \pm 0.03). \quad (2)$$

All literature data presented in this study have been converted to $\delta^{44/42}$Ca. For example, data originally reported as $\delta^{44/40}$Ca were converted to $\delta^{44/42}$Ca by using the exponential mass law and multiplying by $\ln(M_{44}/M_{42})/\ln(M_{44}/M_{40}) \sim 0.488$, where M denotes the molar mass of the given Ca isotope.[15] Uncertainties for sample data are presented as external reproducibilities, or 2SE of a given sample's replicate analyses. The number of sample replicates herein is 3 or 4. Uncertainties on $\delta^{44/42}$Ca are always better than ± 0.10 ‰, with an average value of ± 0.05 ‰.

Statistical analyses

Unless otherwise specified, data for continuous variables are reported as median values and interquartile ranges. All variables were assessed for normality using the Shapiro-Wilk statistical test. Assessment of covariation between continuous variables used Spearman's rank-order correlation. Comparisons of the same variable between groups were performed using the non-parametric Mann-Whitney test, and *p*-values from these tests below 0.05 are deemed statistically significant.

Augmented dataset

A subset of the samples collected as part of this broader research initiative was used to investigate the variability of $\delta^{44/42}Ca_{Urine}$ values in single individuals over time.[13] Since these samples were analyzed in the same lab under the same conditions as those in the present study, we additionally consider 22 samples: one randomly selected from each of the participants in Tissot et al.[13] Of the 22 total samples, 12 were provided by females (age 19 to 60) and 10 were provided by males (age 23 to 36). Compared to the original dataset, the inclusion of the samples from the longitudinal study does not result in any bias in the distribution of age, elemental concentrations, concentration ratios, delta values, or any demographic parameters like BMI (body mass index) and gender. A full compilation of data from the additional samples is included in Table S1. Our results and discussion utilize this augmented dataset, unless otherwise specified.

Results

Data accuracy and reproducibility

Four metrics are used to assess data accuracy and precision:

Procedural blanks. For each batch of samples, two procedural blanks were also processed. Procedural blank values ranged from 15 to 180 ng of Ca, which is more than two orders of magnitude lower than the sample Ca content, and therefore negligible.

Chromatographic yield. Separation of Ca from other sample matrix components will not impart an isotope effect if > 75% of total Ca is recovered.[16] Samples with worse recoveries were reprocessed or excluded. The typical yield for samples in this study is 81%.

Mass-dependency. On a plot of $\delta^{44/42}$Ca vs $\delta^{44/43}$Ca, mass-dependent isotope fractionation will result in a slope of ∼ 0.488 for natural samples. In Fig. 1a, all samples from this study demonstrate a clear, expected mass-dependent relationship.

Reference materials. Long-term reproducibility of several isotopic reference materials was monitored to ensure data accuracy and reliable inter-laboratory comparisons (Fig. 1b). Both IAPSO seawater and NIST SRM 1486 bone meal were subjected to the same analytical protocol and analyzed alongside samples. We report a value of + 0.92 ± 0.01 ‰ (2SE, $n = 40$) for IAPSO seawater and −0.44 ± 0.02 ‰ ($n = 29$) for SRM 1486 bone meal. These are within error of literature data (*e.g.* [14,16,17]).

Demographic and isotopic variability among the study population

This section describes data collected for this study, and not those included from Tissot et al.[13] In total, samples from 81 volunteers were analyzed: 55 females, 25 males, and one preferring not to disclose their gender (full data summary in Table S1). Sixty-one individuals submitted a single sample, while the remaining 20 each contributed between 2 and 7 samples, for a total of 124 samples. Of these, 81 were contributed by female participants, 41 by males, and 2 by the participant who did not disclose their gender. Participants' age ranged from 18 to 75, with 61 samples contributed by participants younger than 30, and 32 samples by participants over 50. The BMI among the

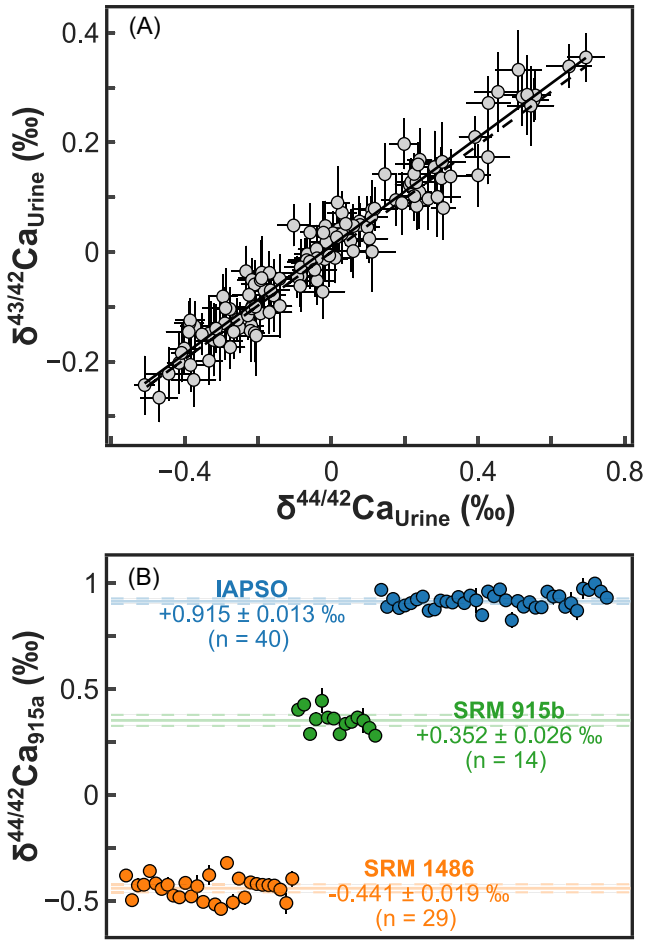

Figure 1 (A) Triple-isotope plot demonstrating mass-dependence of samples analyzed in this study. The dashed line is the theoretical slope of 0.488 and the solid line is a linear regression through the data. (B) Reproducibility of standard reference material $\delta^{44/42}$Ca analyzed during this study.

Table 1 Demographic and Ca data of the samples analyzed in this study.

Variable	Values	Dataset (n = 124)
Age, yr	median (min/max)	29.9 (18.8/75.6)
Female Sex	n (%)	81 (65.3%)
Height, m	median (q1/q3)	1.66 (1.60/1.75)
Weight, kg	median (q1/q3)	65.8 (54.4/72.1)
BMI	median (q1/q3)	22.3 (21.0/25.5)
$\delta^{44/42}Ca_{Urine}$	median (q1/q3)	+0.59 (+0.41/+0.82)
$ln(Ca/K)_{Urine}$	median (q1/q3)	-2.90 (-3.5/-2.28)
$ln(Ca)_{Urine}$	median (q1/q3)	4.23 (3.61/4.92)

∗ Isotopic data reported relative to SRM 915a in units of per mille (‰)

participants extends from 18.6 to 38.0 kg/m^2 with a median value of 22.3 kg/m^2.[18]

A summary of the demographic and Ca data is given in Table 1. The $\delta^{44/42}Ca_{Urine}$ values range from + 0.12 ± 0.05 ‰ to + 1.32 ± 0.04 ‰ with a median value of + 0.61 ‰. Notably, a Shapiro-Wilk test reveals that the distribution of isotopic values is non-normal (W-statistic: 0.967; *P*-value: 0.0043), with a slight skewness toward heavier isotopic values. This hints at the influence of certain factors driving isotopic variability. Table 2 presents a correlation matrix of several continuous variables. As expected, a significant correlation is observed between $\delta^{44/42}Ca_{Urine}$ values and Ca concentrations (see below). Furthermore, $\delta^{44/42}Ca_{Urine}$ values are correlated with age and BMI (*P*-values: 1.8×10^{-5} and 0.0078). Older participants possess systematically lighter $\delta^{44/42}Ca_{Urine}$ values (Fig. 2a), which is expected as bone mass loss increases with age. This observation complicates comparisons between groups whose age distributions differ greatly. Likewise, samples from participants with elevated BMI display light $\delta^{44/42}Ca_{Urine}$ values (Fig. 2b). This observation is not as well-documented in the literature and, in our study, is driven by a small number of individuals with high BMI values (> 28 kg/m^2). The existence of such correlations requires that proper matching of these attributes between groups is a prerequisite of meaningful comparisons (see next section).

Within a given individual, significant isotopic variability in $\delta^{44/42}Ca_{Urine}$ stems from isotopic fractionation during normal kidney function (*e.g.* [9]). It is thus necessary to examine the relationship between $\delta^{44/42}Ca_{Urine}$ values and Ca excretion, or a proxy thereof (such as urine Ca concentration). Table 2 shows a good correlation between isotopes and $[Ca]_{Urine}$, lending support to its use as an excretion proxy. We also report the $(Ca/K)_{Urine}$ ratio as a normalized excretion proxy (*e.g.* [13]). Potassium is useful for normalization given that (i) it is abundant and easily measured in urine, (ii) its main excretion pathway is through urine, and (iii) its concentration in urine is regulated by a different mechanism than Ca, in a distinct region of the nephron.[19] As such, it may mitigate dilution effects and offer complementary information to urinary Ca concentration. Here, $ln(Ca/K)_{Urine}$ values range from -3.50 to -2.28, with a median of -2.90.

Baseline population

Among the studied population we identify a 'baseline' group, whose data serve as the benchmark against which other populations are compared. This group excludes individuals who report any attributes that are argued to influence $\delta^{44/42}Ca_{Urine}$ values in the literature, namely: active vitamin D deficiency, vegetarianism, menopause, osteopenia or active bone therapy treatment (see next section). This approach defines a baseline population of 98 samples from 67 different individuals (Table 3). Since there is a relationship between certain demographic features (*e.g.* age, BMI) and $\delta^{44/42}Ca_{Urine}$ values, the baseline group is further tailored to match the age and BMI distribution of the group to which it is being compared. This yields three baseline subgroups which are matched in age, sex and BMI distributions to three attribute groups (described below), shown in Table 4.

Information regarding specific health conditions was requested from the participants. Several such groups received too few samples to investigate their effects on $\delta^{44/42}Ca_{Urine}$ values, including hyperthyroidism ($n = 2$), cancer ($n = 3$), bariatric surgery ($n = 2$), corticosteroid use ($n = 2$), inflammatory bowel disease ($n = 4$), and pregnancy at the time of urine collection ($n = 4$). While these conditions could theoretically influence an individual's $\delta^{44/42}Ca_{Urine}$ values, those effects are not apparent here, and the isotopic data from these samples do not deviate from others, nor are they aberrant on an isotope-excretion plot (Fig. 3). As such, these 17 samples are included among the baseline population.

Table 2 Correlation matrix of continuous variables.

Index	$\delta^{44/42}Ca_{Urine}$	Ca	Na	K	ln(Ca)	ln(Ca/K)	Age
$\delta^{44/42}Ca_{Urine}$	1	–	–	–	–	–	–
Ca	**-0.502**	1	–	–	–	–	–
Na	0.011	**0.58**	1	–	–	–	–
K	0.04	**0.391**	**0.679**	1	–	–	–
ln(Ca)	**-0.502**	1	**0.58**	**0.391**	1	–	–
ln(Ca/K)	**-0.497**	**0.58**	-0.07	-0.47	**0.58**	1	–
Age	**-0.375**	0.05	-0.062	**0.181**	0.05	-0.077	1
BMI	**-0.238**	0.106	0.169	0.14	0.106	-0.051	0.158

Values are Spearman rank correlation coefficients (r).
Bold values indicate significant correlations.

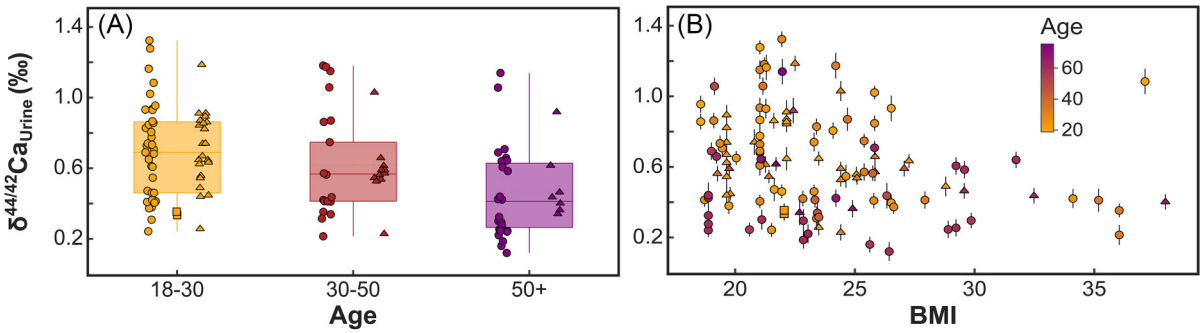

Figure 2 (A) Boxplot of $\delta^{44/42}Ca_{Urine}$ and 3 discrete age bins. (B) Urine Ca isotope ratio plotted against BMI. Color denotes age. Circles denote female, triangles denote male, and squares denote participants who did not disclose their gender.

Table 3 Demographic and Ca data of the augmented dataset (this study + Tissot et al, 2024).

Variable	Values	Full Dataset (n = 146)	Baseline (n = 98)
Age, yr	Median (min/max)	29.7 (18.8/75.6)	29.1 (18.8/70.9)
Female Sex	n (%)	93 (63.7%)	55 (56%)
Height, m	median (q1/q3)	1.68 (1.60/1.75)	1.68 (1.62/1.80)
Weight, kg	median (q1/q3)	65.8 (54.4/72.6)	67.1 (55.3/75.8)
BMI	median (q1/q3)	22.8 (21.0/25.4)	23.2 (21.0/24.9)
$\delta^{44/42}Ca_{Urine}$	median (q1/q3)	+0.59 (+0.41/+0.82)	+0.57 (+0.41/+0.75)
$ln(Ca/K)_{Urine}$	median (q1/q3)	-2.96 (-3.59/-2.35)	-2.96 (-3.52/-2.28)
$ln(Ca)_{Urine}$	median (q1/q3)	4.17 (3.59/4.90)	4.24 (3.70/4.95)

Attributes driving isotopic variability

Nine individuals (8 females, 1 male) reported active vitamin D deficiency. Of the 17 samples they contributed, 3 are from post-menopausal women and excluded, as menopause appears to induce a contrasting isotope effect (see below). The remaining 14 samples have significantly (*p*-value: 0.004) higher $\delta^{44/42}Ca_{Urine}$ values (+0.87 ± 0.46 ‰, 2SD) compared to their baseline group (+0.57 ± 0.50 ‰; Fig. 4).

Eleven individuals (8 females, 3 males) reported consuming no meat. Of their 23 samples, 7 were provided by post-menopausal women and excluded here (as above). The remaining 16 samples show markedly heavier isotopic values (+0.86 ± 0.60 ‰, 2SD) compared to their age-matched baseline group (+0.61 ± 0.47 ‰, *P*-value: 1.9e-4; Fig. 5).

Sixteen participants who reported having experienced menopause provided 23 samples. While 10 samples come from individuals who report vitamin D deficiency or vegetarianism, the above effects of these attributes are not apparent in these women's isotopic data and menopause appears to exert the strongest control (Fig. 6). Two individuals, however, report osteopenia and bone therapy treatment, and are thus omitted. The 21 remaining samples are isotopically lighter than the full baseline group, in line with observations from previous studies (*e.g.* [10,12]). However, when compared to an age-matched baseline group (Table 4), neither $\delta^{44/42}Ca_{Urine}$ values nor excretion proxies of the post-menopause group differ significantly. This is true whether we also sex-match the baseline group (*i.e.* only include samples from female participants) or not.

The 2 females who reported osteopenia (dark circles in Fig. 6), and 2 males who reported both osteopenia and bone therapy treatment (dark triangles in Fig. 6), possess seemingly unaffected $\delta^{44/42}Ca_{Urine}$ values. This is perhaps due to successful implementation of bone therapies, as 3 of the 4 participants (2 males, 1 female) report active Ca and vitamin D supplementation, and both males report past bisphosphonate treatment. Ipriflavone plus Mg and Sr supplementation are additionally reported by the female participant. The remaining

Table 4 Group comparisons.

		Vitamin D Deficiency		
Variable	**Values**	**vit. D baseline (n = 33)**	**vit. D (n = 14)**	***p-value***
Age, yr	median (min/max)	30.6 (18.8/49.0)	20.5 (19.6/49.0)	0.300
Female Sex	n (%)	19 (57.6%)	8 (57.1%)	1.000
Height, m	median (q1/q3)	1.65 (1.59/1.75)	1.69 (1.62/1.75)	0.549
Weight, kg	median (q1/q3)	64.9 (59.0/79.4)	68.0 (65.8/68.0)	0.243
BMI	median (q1/q3)	23.5 (21.6/25.8)	23.2 (22.1/26.3)	0.507
$\delta^{44/42}Ca_{Urine}$	median (q1/q3)	+0.57 (+0.40/+0.76)	+0.87 (+0.65/+0.93)	*0.004*
$\ln(Ca/K)_{Urine}$	median (q1/q3)	-2.97 (-3.53/-2.31)	-2.67 (-4.06/-2.46)	0.954
		Vegetarian diet		
		veg. baseline (n = 70)	**veg. (n = 16)**	***p-value***
Age, yr	median (min/max)	25.6 (18.8/42.5)	23.1 (19.5/41)	0.233
Female Sex	n (%)	40 (57.1%)	8 (50%)	0.718
Height, m	median (q1/q3)	1.68 (1.62/1.80)	1.71 (1.60/1.75)	0.789
Weight, kg	median (q1/q3)	66.4 (55.3/70.3)	68.0 (56.2/68.0)	0.612
BMI	median (q1/q3)	22.0 (21.0/24.4)	22.1 (21.1/22.4)	1.000
$\delta^{44/42}Ca_{Urine}$	median (q1/q3)	+0.61 (+0.43/+0.81)	+0.86 (+0.73/+1.16)	*2e-04*
$\ln(Ca/K)_{Urine}$	median (q1/q3)	-2.86 (-3.46/-2.16)	-2.95 (-4.25/-2.56)	0.136
		Post—menopausal		
		meno. baseline (n = 12)	**menopause (n = 21)**	***p-value***
Age, yr	median (min/max)	60.0 (45.8/70.9)	60.3 (53.7/64.0)	0.807
Female Sex	n (%)	5 (41.7%) ∗	21 (100%)	*0.005*
Height, m	median (q1/q3)	1.70 (1.55/1.84)	1.62 (1.57/1.68)	0.189
Weight, kg	median (q1/q3)	72.6 (53.1/92.1)	56.7 (50.6/73.1)	0.183
BMI	median (q1/q3)	23.8 (22.6/28.3)	22.8 (19.0/29.0)	0.268
$\delta^{44/42}Ca_{Urine}$	median (q1/q3)	+0.43 (+0.35/+0.59)	+0.30 (+0.24/+0.59)	0.303
$\ln(Ca/K)_{Urine}$	median (q1/q3)	-3.14 (-3.51/-2.80)	-2.85 (-3.14/-1.80)	0.120

∗ Males are considered among post-menopausal baseline (see text for details).

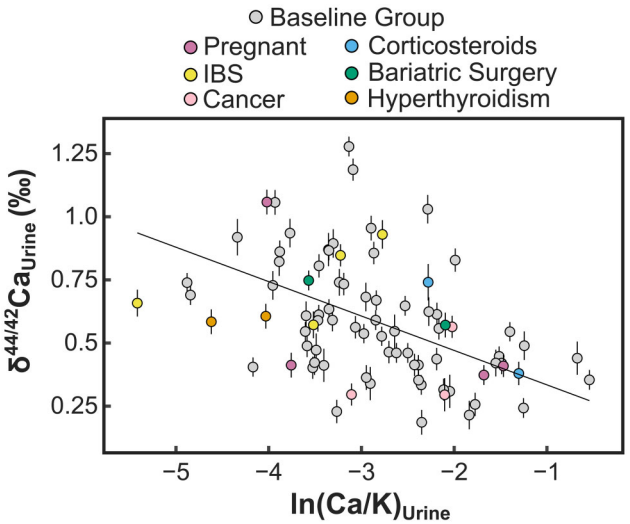

Figure 3 Scatterplot of $\delta^{44/42}Ca_{Urine}$ vs $\ln(Ca/K)_{Urine}$ for the baseline group (in grey) and individuals with various health conditions (colorful points).

female participant does not report active treatment yet, despite her osteopenia diagnosis, possesses a relatively heavy $\delta^{44/42}Ca_{Urine}$ value of $+ 0.64 \pm 0.04$ ‰. This is unexpected and additional drivers may be at play for this individual; however, none are apparent from the questionnaire responses.

Several other drivers were considered among questionnaire responses, though these attributes do not appear to influence isotopic variability. These include groupings of dairy, alcohol, and timely (past 48 hr) multivitamin consumption. Data for these groups are included in Table S2.

Discussion

Drivers of $\delta^{44/42}Ca_{Urine}$ variability among healthy individuals

Current models of Ca reabsorption from the primary urine into the bloodstream describe this kidney function as a Rayleigh distillation, with the Ca isotope composition of the excreted urine ($\delta^{44/42}Ca_{U,f}$) expressed as a function of the isotopic composition of the primary urine ($\delta^{44/42}Ca_0$) and the fraction of Ca remaining in the excreted urine (f) as:

$$\delta^{44/42}Ca_{U,f} \approx \delta^{44/42}Ca_0 + 1000 * \left(f^{\alpha_{kidney}-1} - 1 \right). \quad (3)$$

In this equation, α_{kidney} is the fractionation factor associated with Ca reabsorption in the kidney. As the primary urine travels through the nephron, f decreases as more Ca^{2+} is reabsorbed into the bloodstream leaving the secondary urine fractionated with respect to its starting composition.

Three terms in Eq. 3 control $\delta^{44/42}Ca_{Urine}$ variability—α_{kidney}, f and $\delta^{44/42}Ca_0$—and their relative importance is a subject of debate. Heuser

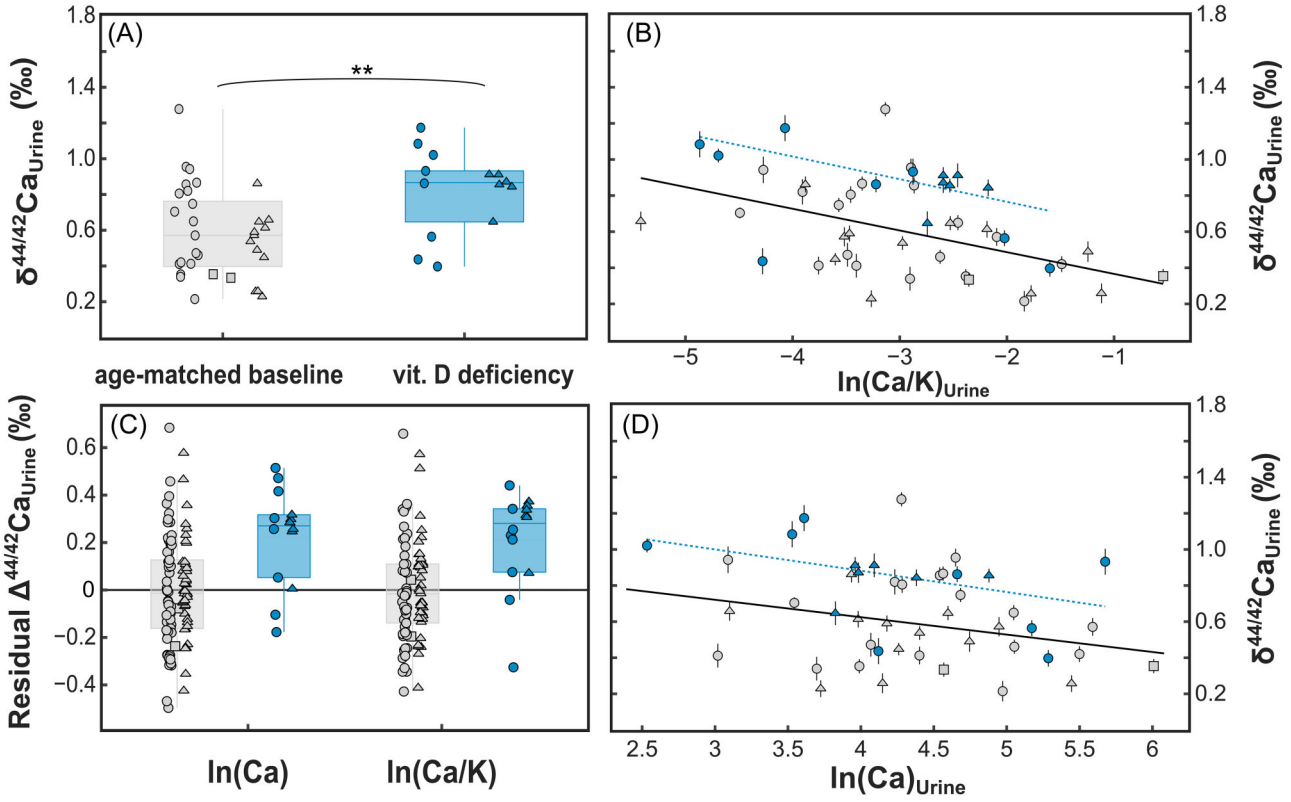

Figure 4 (A) Boxplot of $\delta^{44/42}Ca_{Urine}$ values from the vitamin D group (blue) and their age-matched baseline (grey). (B) Urine Ca isotope ratio plotted against $\ln(Ca/K)_{Urine}$. (C) Y-residuals (*i.e.* distance to regression line, in ‰) of the $\delta^{44/42}Ca_{Urine}$—$\ln(Ca)_{Urine}$ and $\delta^{44/42}Ca_{Urine}$—$\ln(Ca/K)_{Urine}$ trends. (D) Urine Ca isotope ratio plotted against $\ln(Ca)_{Urine}$. The solid and dashed lines are linear regressions through the baseline and vitamin D deficient group's data, respectively. Symbols reflect gender, as in Fig. 1.

and Eisenhauer[20] analyzed a time series of urine samples from a boy (4 yr-old) and a woman (63 yr-old) and observed a difference in their $\delta^{44/42}Ca_{Urine}$ vs $[Ca]_{Urine}$ relationships. Assuming a typical 98% of Ca reabsorption ($f = 0.02$) and that $\delta^{44/42}Ca_0$ is equal to the participants dietary compositions, the authors calculated distinct α_{kidney} values for the boy ($\alpha_{boy} = 0.99969$) and the woman ($\alpha_{woman} = 0.99983$). They further suggested that α_{kidney} changes over time, and is a function of age, gender and/or health status. In a later study, Heuser et al.[9] analyzed urine from several healthy male patients before, during and after prolonged bed rest (3 weeks). They found that (i) variable Ca excretion (f) readily explained the large (0.8 ‰) range in $\delta^{44/42}Ca_{Urine}$ values but also that (ii) urine samples prior to and post bed rest plot on separate, yet nearly parallel, lines in a log-log Ca isotope vs concentration plot. Given the similar age and health status of the participants, a change in α_{kidney} values was not invoked. Instead, the authors attributed this shift to an increased flux of light Ca isotopes from bone dissolution, thereby decreasing the serum composition (*i.e.* $\delta^{44/42}Ca_0$). Lastly, an age dependence of $\delta^{44/42}Ca_0$ was documented by Shroff et al.[11] in healthy adults. Whereas serum $\delta^{44/42}Ca$ values remain relatively constant in children aged 0-18 ($n = 66$), 51 adults show a systematic decrease in serum values from age 18-40. While this age-dependence likely results from increasing bone resorption rates, there is considerable scatter imparted from dietary differences and perhaps other factors.

Which parameter, then, drives $\delta^{44/42}Ca_{Urine}$ and $[Ca]_{Urine}$ variability in our dataset? To investigate, we fit a Rayleigh distillation model to our data and literature data. First, we target single participants who are nominally healthy. We estimate f using $[Ca]_{Urine}$ (or daily Ca excretion, if available) and typical Ca filtration rates (ϕ_{Ca}) in the kidney (~10 ± 2 g·d^{-1};[21]). Assuming a representative Ca concentration and daily miction volume near 1 L, f is estimated as: $[Ca]_{urine}/\phi_{Ca}$. This leaves two unknowns in Eq. 3: α_{kidney} and $\delta^{44/42}Ca_0$. There are few constraints on whether α_{kidney} remains constant between individuals or through time. Therefore, we first assume a nominal α_{kidney} value, derived from two independent methods. In the first, we leverage data from 12 healthy adults subjected to 30 days of bed rest[5,6] to calculate α_{kidney} empirically. Using measured $\Delta^{44/42}Ca_{Serum\text{-}Urine}$ of -1.2 ‰ and $f \sim 1.5\%$ from pre-intervention serum and urine samples yields α_{kidney} of 0.99972. The second method leverages the predictability of isotopic fractionation during diffusion processes.[22] Nearly 90% of Ca^{2+} reabsorption occurs *via* paracellular transport in the proximal tubule and thick ascending limb of the loop of Henle in the nephron,[23—25] with much of the remaining flux accounted for by transcellular transport in the distal convoluted tubule. Thus, the majority of Ca loss from the nephron occurs by diffusive loss through highly Ca^{2+}-permeable tight junctions between epithelial cells.[26] The difference in diffusivity between two isotopes is directly proportional to their relative mass difference as: $D_2/D_1 = (M_1/M_2)^{\beta}$, where β is a unitless term derived experimentally. Bourg et al.[27] report an experimental β-value (0.0045 ± 0.0005) for Ca^{2+} diffusion in an aqueous solution at 75°C. Using their Eq. 2, and accounting for the lower temperature of the human body (36.6°C), we calcu-

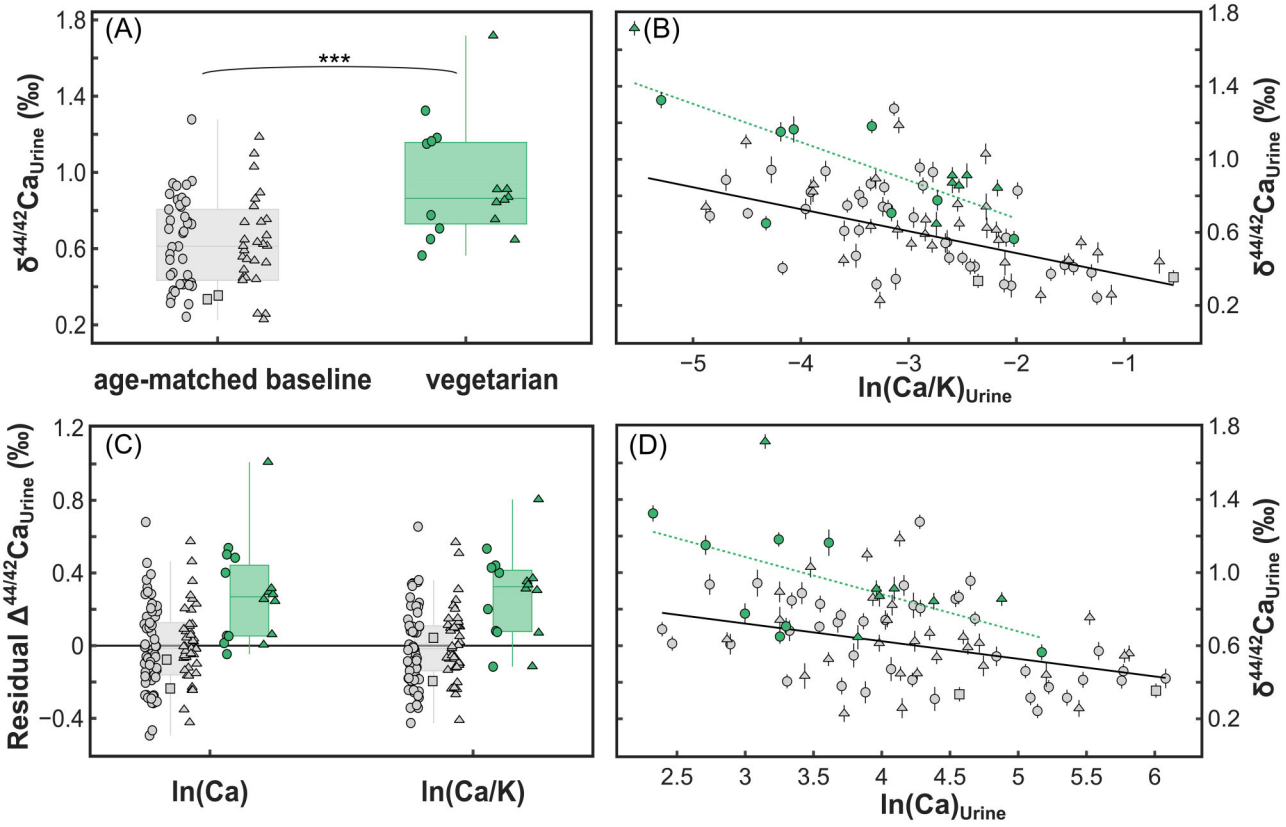

Figure 5 (A) Boxplot of $\delta^{44/42}Ca_{Urine}$ values for the vegetarian group (green) and their age-matched baseline (grey). (B) Urine Ca isotope ratio plotted against $ln(Ca/K)_{Urine}$. (C) Y-residuals (*i.e.* distance to regression line, in ‰) of the $\delta^{44/42}Ca_{Urine}$—$ln(Ca)_{Urine}$ and $\delta^{44/42}Ca_{Urine}$—$ln(Ca/K)_{Urine}$ trends. (D) Urine Ca isotope ratio plotted against $ln(Ca)_{Urine}$. The solid and dashed lines are linear regressions through the baseline and vegetarian group's data, respectively. Symbols reflect gender, as in Fig. 1.

late an $\alpha_{diffusion} = 0.999736 \pm 0.00004$. This value is consistent with the empirical value above and provides an independent constraint on the isotopic fractionation that occurs as Ca^{2+} is reabsorbed in the nephron. Therefore, we adopt $\alpha_{kidney} = 0.99972$ in our Rayleigh models. Keeping $\delta^{44/42}Ca_0$ as a free parameter, we first assess how well this value explains the $\delta^{44/42}Ca_{Urine}$ variability across several individuals.

Figure 7 shows Rayleigh models for 4 individuals who provided between 7 and 30 samples. These individuals span over 30 years in age (from 4 to 36), fall within a narrow BMI range (19.3-23.4; BMI is not available for the 4 y/o boy, though he is reported to be in good health) and none of them possess attributes observed to affect $\delta^{44/42}Ca_{Urine}$ values. If α_{kidney} was variable from person to person, or over time, we would expect our estimate of α_{kidney} to fail to explain the $\delta^{44/42}Ca_{Urine}$ variability of all individuals. However, an α_{kidney} of 0.99972 can reproduce the $\delta^{44/42}Ca_{Urine}$ variability of all 4 individuals. This supports the idea that α_{kidney} remains relatively constant with age, at least from childhood to early adulthood, and is comparable between nominally healthy individuals. Furthermore, the range of estimated f values is similar between the 4 individuals and falls within literature estimates (*e.g.* [24]). This suggests that, for healthy individuals, both kidney function and $\delta^{44/42}Ca_0$ will impact $\delta^{44/42}Ca_{Urine}$ values. For these 4 individuals, $\delta^{44/42}Ca_0$ values vary by ~ 0.2 ‰ and are aligned with $\delta^{44/42}Ca_0$ values reported by Shroff et al.[11] for 100 healthy individuals. We note that the assumptions needed for Rayleigh modelling here invite uncertainty, and future studies will benefit from investigating this behavior when certain assumptions (*e.g.* GFR_{Ca}) can be directly constrained.

Two observations support a regime with near constant α_{kidney} and variable $\delta^{44/42}Ca_0$, rather than a variable α_{kidney}, to explain the large inter-individual $\delta^{44/42}Ca_{Urine}$ variability. First, modelling significantly different (*e.g.* $\pm$ 0.0001) α_{kidney} values requires improbable $\delta^{44/42}Ca_0$ values. For instance, using an α_{kidney} of 0.99969 for the 4 y/o boy yields a $\delta^{44/42}Ca_0$ value of -0.75 ‰, which is isotopically light for a healthy child in a state of skeletal anabolism. Furthermore, it is outside the range reported for children of similar ages.[11] Second, there is no a priori reason why large variations in α_{kidney} would be expected between healthy individuals. Whereas a range in $\delta^{44/42}Ca_0$ values is documented and a natural outcome of age, diet and/or lifestyle differences, invoking significant changes to α_{kidney} requires a mechanism. Assuming α_{kidney} is governed by the diffusion of Ca^{2+} in the proximal tubule and loop of Henle, invoking large variations in α_{kidney} requires variations in how Ca^{2+} is transported at the cellular level. One cannot invoke selective differences in the behaviors of cells without quickly entering a disease state. Furthermore, nephron epithelial cells are highly specialized, and it is not expected that metal cation reabsorption is achieved in unique ways from person to person. We therefore cannot identify a plausible mechanism for variations in α_{kidney} among healthy individuals. Instead, we posit that (i) α_{kidney} is nearly constant at ~0.99972, and (ii) differences in Ca excretion (f) and initial Ca isotope composition ($\delta^{44/42}Ca_0$)—which depend on age, diet, and

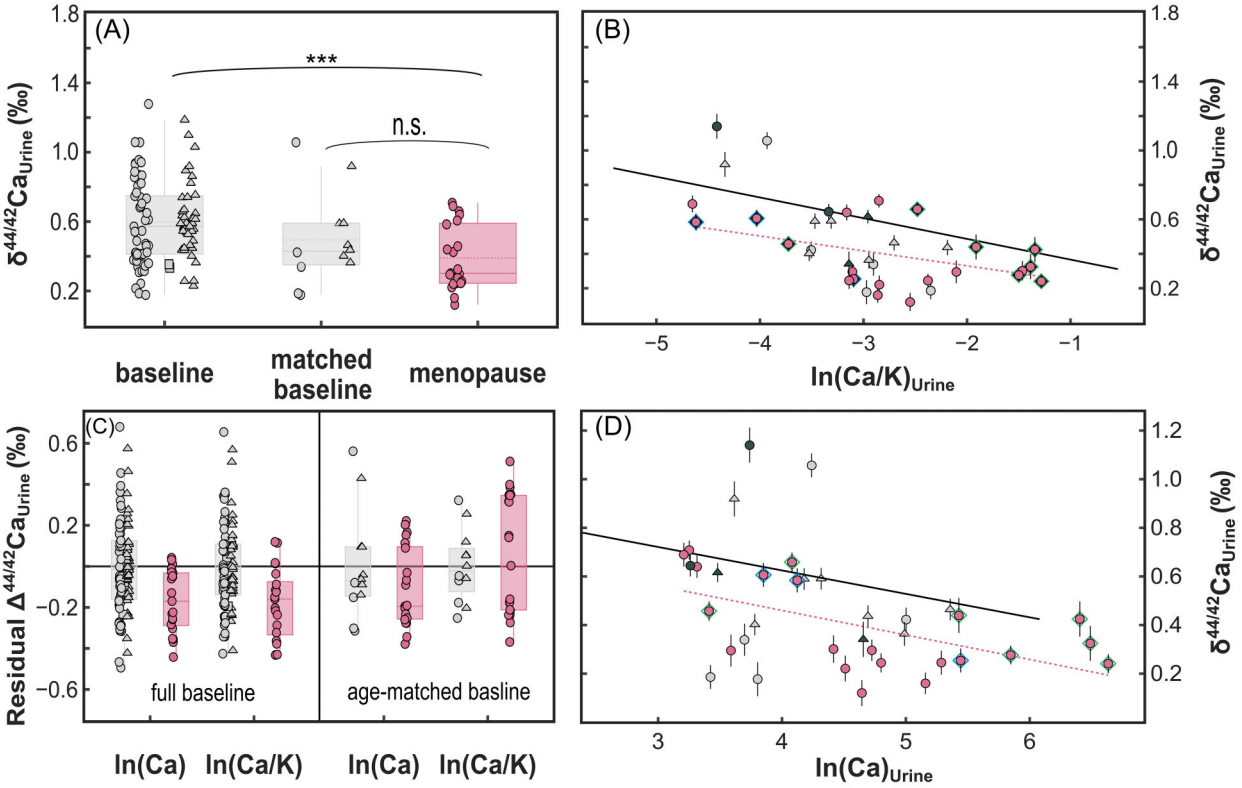

Figure 6 (A) Boxplot of $\delta^{44/42}Ca_{Urine}$ values for the post-menopause group (pink), their age-matched baseline, and the full baseline group. (B) Urine Ca isotope ratio plotted against $ln(Ca/K)_{Urine}$ for the post-menopause group and their age-matched baseline. (C) Y-residuals (*i.e.* distance to regression line, in ‰) of the $\delta^{44/42}Ca_{Urine}$—$ln(Ca)_{Urine}$ and $\delta^{44/42}Ca_{Urine}$—$ln(Ca/K)_{Urine}$ trends; left panel is the full baseline and right panel is the age-matched baseline group. (D) Urine Ca isotope ratio plotted against $ln(Ca)_{Urine}$. Vegetarianism (green diamonds), vitamin D deficiency (blue diamonds), and osteopenia (dark grey symbols) were also reported. The solid and dashed lines are linear regressions through the age-matched baseline and post-menopause group's data, respectively. Symbols reflect gender, as in Fig. 1. n.s.= not significant.

other factors—exert the strongest controls on $\delta^{44/42}Ca_{Urine}$ variability between individuals.

Drivers of $\delta^{44/42}Ca_{Urine}$ variability among the attribute groups

Body mass index

The relationship between BMI and BMD is complex and has received much attention due to the heightened risk of fractures associated with both high and low BMI values.[28] Both mechanical and biochemical mechanisms should lead to greater bone density in high-BMI individuals. However, a greater number of fractures are consistently reported among obese individuals. Among other factors, researchers attribute this to bone fragility associated with metabolic parameters and the type of adiposity. Certain adipose tissues in the abdomen are more commonly associated with low BMD (*e.g.* visceral adipose tissue; VAT), whereas others are less so.[29] Similarly, the type of mass (lean vs fat mass) can affect the strengthening of bone under increased weight loads. Thus, it is difficult to predict BMD when considering BMI alone.

The relationship between BMI and Ca isotopes has received little attention, though as with BMD, the relationship appears complex. One bed rest study reports BMI values of 21.4-27.4 kg/m^2 for 7 males age 23 to 33 and documents no correlation with $\delta^{44/42}Ca_{Urine}$ values.[10] On the other hand, Tissot et al.[13] reported a positive correlation between BMI and the (average) $\delta^{44/42}Ca_{Urine}$ of 19 individuals, with a change of $\sim$ 0.1 ‰ per kg/m^2. This correlation does not include data from the participant with the highest BMI (30.9 kg/m^2), who displays a light $\delta^{44/42}Ca_{Urine}$ value. In Fig. 2, $\delta^{44/42}Ca_{Urine}$ exhibits a weak, negative correlation with BMI for 81 individuals (124 samples) over a large range (18-38 kg/m^2). However, this trend is driven by a small number of individuals with high-BMI (> $\sim$28 kg/m^2), and low $\delta^{44/42}Ca_{Urine}$. The sample density below 28 kg/m^2 is considerably higher and shows no covariation. It is possible that individuals above a certain BMI threshold possess a negative BMB, causing lighter $\delta^{44/42}Ca_{Urine}$ values. This would be consistent with the heightened bone fragility and fracture rates reported among these individuals.[30] Our sampling resolution is, however, too heavily biased towards lower BMI values, so further data are needed to confirm this relationship.

The opposite trends between BMI and $\delta^{44/42}Ca_{Urine}$ are not necessarily contradictory. Enhanced weight-bearing within a 'normal' BMI range (18-25 kg/m^2;[18]) may feasibly contribute to greater BMD,[31] heightened Ca fluxes to bone, and heavier $\delta^{44/42}Ca_{Urine}$ values. Above this range, it is possible that other factors (*e.g.* VAT) outweigh this phenomenon, resulting in enhanced bone fragility and lighter $\delta^{44/42}Ca_{Urine}$ values. Furthermore, obese individuals show marked hormonal differences that cannot be ignored.[32] These may combat or compound osteological effects or additionally influence the way in which Ca is metabolized in the gut and/or reabsorbed in the kidney. Lastly, given that a change in BMI is a coincidental risk factor of many other diseases (*e.g.* non-alcoholic fatty liver disease),[33] putative correlations between BMI and $\delta^{44/42}Ca_{Urine}$ must control for

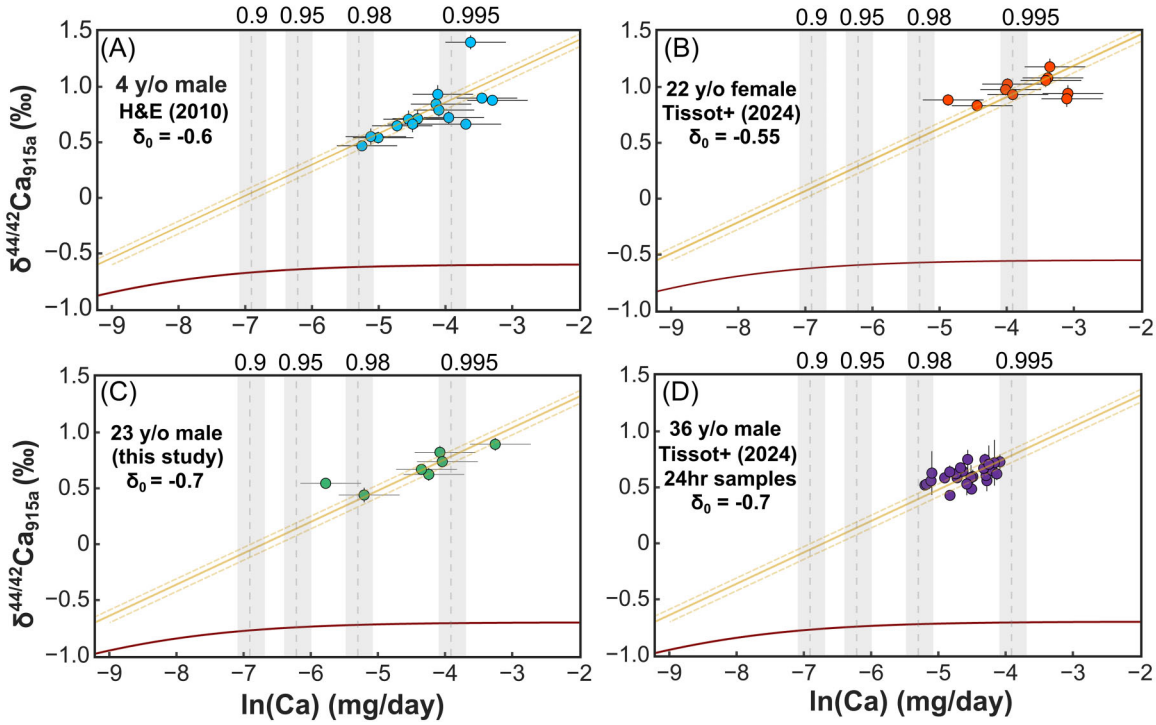

Figure 7 $\delta^{44/42}Ca_{Urine}$ values plotted against $\ln(Ca)_{Urine}$ for a) a 4 y/o boy (Heuser and Eisenhauer, 2010), b) a 22 y/o female (P2 from Tissot et al, 2024), c) a 23 y/o male (this study), and d) a 36 y/o male (P1 from Tissot et al, 2024). A Rayleigh model (Eq. 3) is fit to their data (yellow lines) and used to estimate $\delta^{44/42}Ca_{Serum}$, listed in each figure panel (δ_0). The cumulative reabsorbed Ca is shown in red. An α_{kidney} of 0.99972 is used for all models. Vertical dashed grey lines are estimates of f. Uncertainties of GFR_{Ca} (2 g Ca·d^{-1}) are shown as dashed yellow lines (for the model) and grey shaded regions (for f estimates).

additional health variables and a causal relationship cannot be guaranteed.

Vegetarianism

We employ the above Rayleigh model to investigate why vegetarian participants show systematically higher $\delta^{44/42}Ca_{Urine}$ relative to non-vegetarians. Figure 8 shows a good model fit for this group using $\alpha_{kidney} = 0.99972$, despite additional scatter from considering multiple individuals at once (given the large, natural variability of $\delta^{44/42}Ca_{Serum}$;[11]). Notably, vegetarians appear to display higher Ca reabsorption rates (up to 99.8%) as inferred from their lower urine Ca concentrations, which is consistent with lower urinary Ca excretion observed in some vegetarian populations.[34,35] The exponential increase in $\delta^{44/42}Ca_{Urine}$ values at low f values would then account for the heavier isotopic ratios of this group. However, the results from our simple Rayleigh model are at odds with Fig. 5 and the observation that vegetarians do not deviate in their Ca/K_{Urine} ratios from non-vegetarians (Table 4). The Ca/K_{Urine} proxy thus suggests no significant difference in Ca excretion for this group. Without a true measure of Ca excretion for these spot-urine samples, the isotopic offset of vegetarians cannot be unambiguously attributed to systematically lower f values. In addition to a lack of direct constraints on Ca excretion (*i.e.* creatinine, pooled sampling), this ambiguity may also reflect the limitations of our relatively simple modelling approach.

Recently, Eisenhauer et al.[12] reported heavier $\delta^{44/42}Ca_{Urine}$ values for 72 vegetarians and 17 vegans relative to those with conventional diets. The authors suggest the influence of Ca-fortified foods (*e.g.* tofu), which source Ca from primarily inorganic reservoirs and are thus enriched in heavy Ca isotopes (*e.g.* [11,36]). Vegan $\delta^{44/42}Ca_{Urine}$ values are thus potentially impacted in two ways: by a decreased intake of light Ca from dairy and an increased intake of heavy Ca from fortified foods. The same rationale does not apply to vegetarians, as those in our study report the consumption of dairy 4.7 days per week (from 3 to 7 days/week). Therefore, the majority of their dietary Ca still comes from an isotopically light source, as in a typical Western diet,[37] and there is no reason to expect that their $\delta^{44/42}Ca_{Diet}$ should be systematically heavier than that of omnivores. Given that only 4-6% of Ca is sourced from meat in a typical Western diet,[20,37] isotopic mass balance yields a theoretical difference in $\delta^{44/42}Ca_{Diet}$ of only 0.02 ‰, an order of magnitude smaller than the 0.25 ‰ difference observed in our dataset. Enhanced Ca reabsorption in the kidneys is an alternative, given that Rayleigh models accurately predict the ~ 0.2 ‰ offset between the vegetarian and baseline groups when f is lowered by 1%.

The data presented here confirm the previously documented observation that individuals with vegetarian diets possess systematically heavier $\delta^{44/42}Ca_{Urine}$ values. At this writing, however, we lack a conclusive explanation to account for their isotopic offset. Importantly, and as a result, the use of Ca isotopes as a diagnostic tool for BMB and/or osteoporosis may be complicated by such dietary habits. A more complex assessment of the vegetarian Ca cycle is a need of future studies. In addition to collecting direct measurements of Ca excretion, which would better constrain the uncertainties of Fig. 8, such studies should assess the duration of an individual's current dietary habits and the cumulative extent of their (lack of) dairy and fortified-Ca consumption.

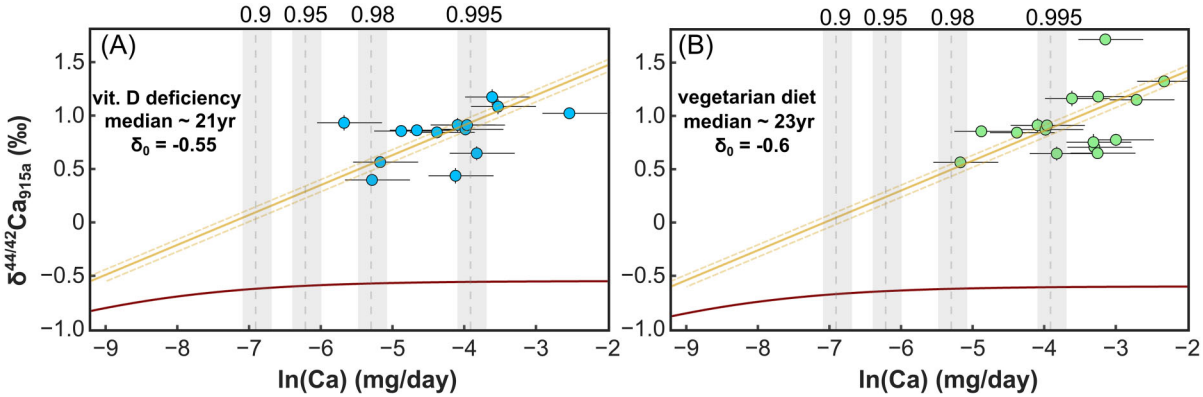

Figure 8 $\delta^{44/42}Ca_{Urine}$ values from individuals with active vitamin D deficiency (A) and vegetarian diets (B) plotted against $\ln(Ca)_{Urine}$. A Rayleigh model (Eq. 3) is fit to both groups (yellow lines). The cumulative reabsorbed Ca is shown in red. An α_{kidney} of 0.99972 is used for both groups. Vertical dashed grey lines are estimates of f. Uncertainties of GFR_{Ca} (2 g Ca·d^{-1}) are shown as dashed yellow lines (for the model) and grey shaded regions (for f estimates).

Vitamin D deficiency

In agreement with previous work by Rangarajan et al.[38] individuals in this study with active vitamin D deficiency possess systematically heavier $\delta^{44/42}Ca_{Urine}$ values relative to the baseline group. A Rayleigh model (Fig. 8) confirms that $\alpha_{kidney} = 0.99972$ can also reproduce the values of this group, despite the additional scatter that results from natural variability in $\delta^{44/42}Ca_{Serum}$ between individuals. Unlike vegetarians, their estimated Ca reabsorption rates are largely within the typical range (98-99.5%). This suggests that the heavier $\delta^{44/42}Ca_{Urine}$ values in vitamin D deficient individuals are caused by systematically elevated $\delta^{44/42}Ca_{Serum}$ values, as opposed to other parameters.

Vitamin D levels, and particularly the concentration of calcitriol ($1,25[OH]_2D$) following hydroxylation in the kidney, critically regulate $[Ca]_{Serum}$ values. Calcitriol suppresses the gene transcription leading to parathyroid hormone (PTH) synthesis and promotes the absorption of Ca from the gut.[39] Thus, a deficiency in vitamin D would result in lower Ca absorption from the gut, insufficient Ca uptake to maintain a constant bone mass, and therefore lighter $\delta^{44/42}Ca_{Serum}$ values from enhanced bone resorption overtime. This is in conflict with the urine data presented both here and in Rangarajan et al.,[38] the only other study to investigate Ca isotopic data in vitamin D deficient individuals. Alternatively, PTH also regulates Ca reabsorption in the kidneys and an overactive PTH response may result in greater Ca retention rates. In Fig. 8, our Rayleigh model instead indicates normal reabsorption rates of 98-99.5% for this group, and there is no deviation in $[Ca]_{Urine}$ or $(Ca/K)_{Urine}$ values from baseline. Thus, none of the data we present suggests that Ca reabsorption is systematically different for this group. However, the lack of direct constraints on Ca excretion in this study means that this mechanism cannot be definitively ruled out.

As a final alternative, we explore diminished fractionation upon Ca absorption from the GI tract as a potential culprit for the elevated $\delta^{44/42}Ca_{Serum}$ values in vitamin D deficient individuals. The fractionation of Ca upon metabolization from the gut is difficult to measure in humans; however, data from animal models (*e.g.* [8,40]) suggest a preferential absorption of light Ca isotopes. This fractionation occurs as Ca travels across gut epithelia in the small intestine *via* transcellular and paracellular transport,[39] and it is consistent with the ~ -0.3 ‰ fractionation that occurs as Ca diffuses across epithelia in the nephron by similar means. Lower vitamin D levels may induce systematic differences in how or where Ca is absorbed in the small intestine. The transient receptor potential cation channel V6 (TRPV6) facilitates Ca entry into intestinal epithelial cells and is regulated by calcitriol levels. Importantly, TRPV6 is significantly more active in the duodenum over the jejunum or ileum.[41] Diminished Ca absorption in the duodenum *via* transcellular transport owing to lower calcitriol levels may feasibly result in a different net fractionation factor attending Ca metabolization. At present, it is uncertain whether the predicted change would result in a greater or lesser extent of fractionation; however, to explain the observations, an α_{GI} closer to unity is needed.

Vitamin D deficiency appears to influence the Ca isotope composition in human body compartments, though at this writing, a robust explanation for these heavy isotope ratios is still lacking. Importantly, and similar to the vegetarian group, this observation will complicate the use of Ca isotopes as a biomarker of BMB in deficient individuals. Moreover, this complication would affect the use of both $\delta^{44/42}Ca_{Urine}$ and $\delta^{44/42}Ca_{Serum}$ values, if higher serum values are indeed responsible as our data suggest. In futures studies, a more complex modelling approach is needed to fully disentangle the ways in which vitamin D levels may impact Ca cycling, and thus Ca isotope fractionation, in the human body. In particular, isotopic fractionation associated with Ca metabolization in the gut is poorly constrained.

Menopause

Following age-matching, no isotopic offset is observed for the post-menopausal group (Fig. 6). This would suggest that, in our dataset, the light $\delta^{44/42}Ca_{Urine}$ values of post-menopausal women result from the natural increase in bone loss with age. In Fig. 9, Ca isotope and concentration data are shown for the post-menopausal women of this study and fit with our Rayleigh model. The model predicts a low $\delta^{44/42}Ca_0$ value ~ -1.1 ‰, consistent with increased bone loss. One participant from the present study, however, shows a different behavior, whereby $\delta^{44/42}Ca_{Urine}$ plateaus despite increasing $[Ca]_{Urine}$ (Fig. 9b). Literature data from two post-menopausal women (one with osteoporosis) also show this behavior. In all studies, $\delta^{44/42}Ca_{Urine}$ data plateau at approximately the same value ($+0.3 \pm 0.2$ ‰), for estimated reabsorption rates between 90 and 99%. This is enigmatic, as enhanced excretion with-

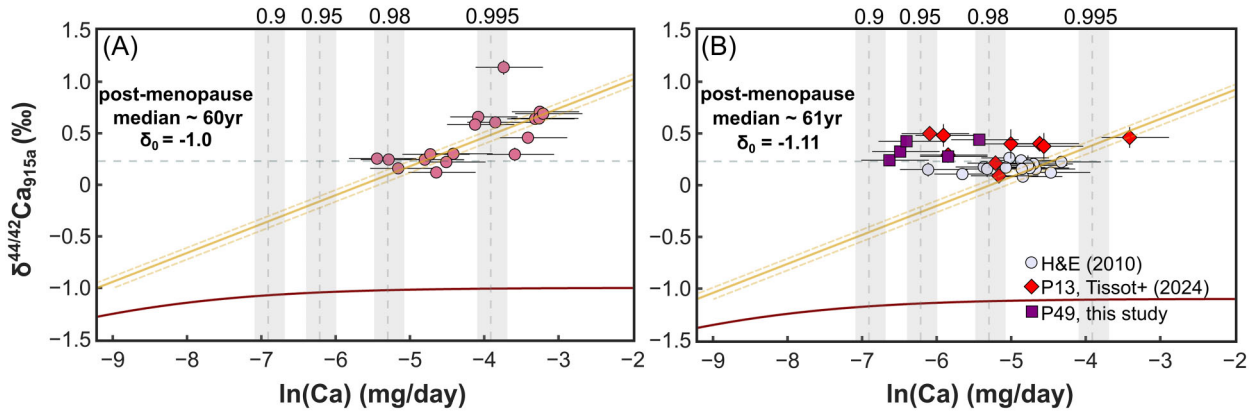

Figure 9 (A) $\delta^{44/42}Ca_{Urine}$ values from 20 post-menopausal women in this study plotted against $ln(Ca)_{Urine}$. Our Rayleigh model (in yellow) predicts a $\delta^{44/42}Ca_{Serum}$ of -1.0 ‰. An α_{kidney} of 0.99972 is used. (B) Data from 3 post-menopausal women, which are unable to be reproduced by our Rayleigh model. Vertical dashed grey lines are estimates of f. Uncertainties of GFR_{Ca} (2 g Ca·d^{-1}) are shown as dashed yellow lines (for the model) and grey shaded regions (for f estimates). Horizontal dashed lines are the threshold value of Eisenhauer et al, 2024 (+0.23 ‰).

out a commensurate isotope effect is not allowed by current models of Ca reabsorption in the kidney. Thus, matching the measured isotopic data of these three women is impossible without invoking unrealistic α_{kidney} ($\geq$ 0.9999) or (very heavy) $\delta^{44/42}Ca_0$ values, given that these samples come from the same people over the span of days to weeks.

For the 3 post-menopausal women whose data are shown in Fig. 9b, only $[Ca]_{Urine}$ data are presented and there are no direct constraints on Ca reabsorption rates. Therefore, the large spread in their $[Ca]_{Urine}$ values may result from (i) a constant flux of Ca to the kidneys and variable reabsorption rates (*i.e.* large spread in f values), (ii) typical reabsorption rates of 98-99.5% and variable fluxes of Ca to the kidney (*i.e.* variable GFR_{Ca}), or a combination thereof. Regardless of the likelihood of either case for these women, both should present a noticeable shift in $\delta^{44/42}Ca_{Urine}$ values—through Rayleigh distillation in the first case and through the large flux in light Ca isotopes from bone in the second case. Given that constant $\delta^{44/42}Ca_{Urine}$ and variable $[Ca]_{Urine}$ values are observed for post-menopausal women in three separate studies, the current paradigm for Ca isotope fractionation in the kidneys and its response to menopause is somewhat upended. Importantly, the use of $\delta^{44/42}Ca_{Urine}$ values as a biomarker for BMB or as a diagnostic tool for osteoporosis will suffer as a result of this observation. This is of particular concern for post-menopausal women, given their greater chances of developing osteoporosis and thus greater need for early detection.

An alternative explanation for this behavior may be osmotic concentration of urine solutes as water is reabsorbed in the collecting duct.[42] This process occurs downstream of Ca reabsorption in the nephron. Therefore, Ca would follow routine isotopic fractionation (as in Fig. 9a), yet the final concentration could increase as fluid is recycled. A common way to correct for the influence of such effects is by adjusting the measured urine specific gravity (USG) to expected levels (~1.024;[43]) and correcting solute concentrations accordingly. Unfortunately, USG is not a parameter so far reported in biomedical Ca isotope studies. Yet, given the good Rayleigh model fits, this does not appear to influence the use of $[Ca]_{Urine}$ as an excretion proxy for other individuals (*e.g.* Fig. 7), or even most post-menopausal women considered here (Fig. 9a). Regardless, osmotic concentration may impact the use of $[Ca]_{Urine}$ as an excretion proxy in any instance where spot-urine samples are analyzed. To properly contextualize effects such as this, future studies will benefit from such a correction, and from quantifying true measures of Ca excretion (*i.e.* GFR, creatinine, 24 hr urine collection).

Implications for the Ca isotope biomarker

Currently, two approaches are discussed in the literature for the use of Ca isotopes as a biomarker. The first employs a cross-sectional approach, in which an individual's Ca isotope ratio is compared to a reference value (*e.g.* [10,12]). Values below the reference threshold (*e.g.* +0.23 ‰ in urine) are taken as diagnostic of osteoporosis. The second instead suggests a self-referencing approach, in which Ca isotope ratios are compared to a reference value established for that same individual at an earlier time, when BMB was presumably homeostatic (*e.g.* [9,13]). Currently, both approaches have limitations. For the former, the large inter-individual differences in serum and urine isotope ratios call into question whether a cross-referencing approach is feasible, and whether a universal threshold value can be defined. In this work, we additionally document several attributes which show a demonstrable effect on urinary Ca isotopes, irrespective of bone dynamics. Kidney function is a first order control on $\delta^{44/42}Ca_{Urine}$ values and must be addressed prior to BMB estimates using urine isotope ratios (*e.g.* [9]). Further, we document the influence of several demographic, dietary and health status factors, which are highly variable among the general population. Such variables must be constrained, and their effect on $\delta^{44/42}Ca_{Urine}$ understood, to implement a cross-referencing approach.

The self-referencing approach mitigates some issues outlined above as many variables will remain stable between analyses. As a result, $\delta^{44/42}Ca_{Urine}$ values for a given healthy individual are expected to be relatively constant over time, an idea supported by the limited (± 0.3 ‰) variations seen in data from healthy individuals over monthly timescales.[12,13] However, this approach is also susceptible to, for example, changes in diet or vitamin D levels over time (*i.e.* drivers of isotopic variability other than BMB). Therefore, lacking in both approaches is a robust understanding of which variables or attributes must be monitored and their relative importance

regarding Ca isotope variability. Future investigations of Ca isotope fractionation at homeostasis, and in response to common perturbations, will undoubtedly improve the predictability of Ca isotopes under disease states.

Concluding remarks

Here, we consider variations in Ca concentration and isotopic composition of nearly 150 urine samples in tandem with self-reported data on the participant's demographic, health status, diet and lifestyle. Several variables—namely age, vitamin D, vegetarianism, menopause and perhaps BMI—appear to systematically influence $\delta^{44/42}Ca_{Urine}$ values among the studied population. The $\delta^{44/42}Ca_{Urine}$ values herein decrease with age, as expected from enhanced bone resorption through time. Participants with high BMI values also possess lighter $\delta^{44/42}Ca_{Urine}$ values among the study population, yet this observation does not persist at lower BMI values (< 28 kg/m^2), suggesting a nuanced and perhaps non-monotonic relationship. Vegetarianism and vitamin D deficiency both result in systematically heavy $\delta^{44/42}Ca_{Urine}$ values among the study population, in agreement with data presented in previous studies. Lastly, post-menopausal women herein are observed to have significantly lighter $\delta^{44/42}Ca_{Urine}$ values. This matches prevailing observations in the literature, yet here appears to be largely driven by enhanced bone resorption rates with age. Surprisingly, there is no difference between the $\delta^{44/42}Ca_{Urine}$ values of non-osteoporotic post-menopausal women in this study ($+0.30 \pm 0.37$ ‰, 2SD, $n = 21$) and those of osteoporotic post-menopausal women reported in the literature ($+0.18 \pm 0.33$ ‰, 2SD, $n = 71$[12]).

We further employed a model of Rayleigh distillation, expressing the isotopic fractionation associated with Ca reabsorption in the kidneys, to investigate the mechanistic drivers associated with the observed isotopic variability. We find that a near-constant α_{kidney} of 0.99972 accurately reproduces the data for many individuals across a wide age range and thus suggest that α_{kidney} does not vary from person to person or over time, at least through young adulthood. In employing this model for the vegetarian and vitamin D deficient groups, we find the underlying cause of their isotopic offsets may be associated with altered kidney function, heavier $\delta^{44/42}Ca_{Serum}$ values, or mechanisms that have yet to be considered in Ca isotope metallomics studies. Importantly, this exercise reveals the need for (i) caution when applying the Ca isotope biomarker to these individuals, (ii) more complex modelling to assess how these variables influence Ca cycling in the human body, and (iii) larger datasets to allow for more statistically robust assessments of these groups.

Lastly, data from 3 post-menopausal women across 3 different studies are shown to not adhere to the current understanding of Ca isotope variability in urine samples. Given the high risk of osteoporosis for this group, future studies must consider how to overcome such behavior when implementing this new biomarker. While these observations favor the use of a self-referencing approach over a cross-referencing one, a detailed understanding of Ca isotope responses to common perturbations remains essential to implementing any approach using Ca isotopes as a biomarker. While the present work reaffirms the utility of Ca isotopes in identifying shifts in bone homeostasis and other effects of osteoporosis, we also document areas of uncertainty to be addressed in future studies and provide important considerations for this rising proxy of BMB.

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Supplementary material

Supplementary material is available at *Metallomics* online.

Conflicts of interest

None declared.

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Data availability

The data underlying this article are available in the article and in its online Supplementary material. Additional data related to this paper may be requested from the authors.

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