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Paper

Magnitude and timescales of Ca isotope variability in human urine: implications for bone mass balance monitoring

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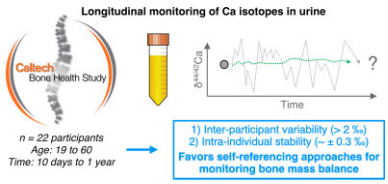
Abstract

Calcium (Ca) isotopes in blood/urine are emerging biomarkers of bone mineral balance (BMB) in the human body. While multiple studies have investigated Ca isotopes in patients suffering from diseases affecting BMB, comparatively little effort has been devoted to understanding the homeostasis of Ca isotopes in healthy individuals. Here, we report on a longitudinal study of the urine Ca isotope composition ($\delta^{44/42}Ca_{Urine}$) from 22 healthy participants (age 19–60) over timescales ranging from days to months. Data from a single participant collected over a 30-day period show that morning urine is an excellent proxy for 24-h pooled urine fractions. Data from all participants reveal large inter-individual variability in $\delta^{44/42}Ca_{Urine}$ (up to 2.2‰), which is partly due to anthropometric differences, as shown by a correlation between the participants' body mass index (BMI) and $\delta^{44/42}Ca_{Urine}$ values. In contrast, intra-individual data reveal encouraging stability (within ~±0.2–0.3‰) over timescales >160 days, indicating that self-referencing approaches for BMB monitoring hold greater promise than cross-sectional ones. Our data confirm that intra-individual $\delta^{44/42}Ca_{Urine}$ variations are mainly a function of Ca reabsorption in the kidney, but also reveal the impact of other (and at times equally important) drivers, such as diet, alcohol consumption, physical exercise, or fasting. We also find that a magnetic resonance imaging contrast agent (gadolinium) can lead to artifacts during Ca isotope analysis. Based on our results, a series of practical considerations for the use of Ca isotopes in urine as tracers of BMB are presented.

Keywords: Ca isotopes; urine; bone mineral balance; mass spectrometry; diagnostic potential; longitudinal study

Graphical abstract



Main scope and findings of the study.

Introduction

Following the initial observation that bones in vertebrates have significantly lighter calcium (Ca) isotope composition than soft tissues (i.e. muscle, blood) [1], Ca isotopes in blood and urine have been proposed as potentially powerful tracers of bone mineral balance (BMB) in the human body. The rationale behind this proposal is that, relative to a healthy control, a subject experiencing net loss of bone mass would exhibit a shift in blood and urine composition toward low, bone-like $\delta^{44/42}Ca$ values (where $\delta^{44/42}Ca$ is the $^{44}Ca/^{42}Ca$ ratio expressed in delta notation, in units of permil). Over the last two decades, pioneering studies have started to lay down the foundation of this novel biomedical application of stable isotopes, exploring the Ca isotopic homeostasis in animal models [2–7], and the human body alike [8], with particular focus on (i) bed rest experiments to simulate and study the effects of spaceflight and microgravity on BMB [9–12], and (ii) the impact of diseases affecting BMB, such as osteoporosis [13–15], multiple myeloma [16], or chronic kidney disease [17,18]. Three studies even documented the impact on Ca isotopes of select treatments meant to stimulate Ca bioavailability and uptake (Vitamin D3) [19], slow down bone resorption (e.g. bisphosphonate drugs) [9], or fight prostate cancer (androgen deprivation therapies) [20].

At its core, the clinical potential of Ca isotopes hinges on our capacity to relate a subject's BMB to the Ca isotopic composition of

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its blood and/or urine ($\delta^{44/42}Ca_{Blood/Urine}$). Because most of the Ca in the body resides in the bones, simple box models accounting for Ca influx (diet) and outflux (mainly through urine), as well as Ca exchanges between soft tissues, bones and kidney, can theoretically be leveraged to gain such insights. In practice, approaches with subtle, yet important, differences have been proposed to relate $\delta^{44/42}Ca_{Blood/Urine}$ and BMB. These can be grouped into three main categories. In the first, it is assumed that the main driver of $\delta^{44/42}Ca_{Blood/Urine}$ fluctuations is a large (~−0.6‰, see review in ref. [21]) fractionation of Ca isotopes during bone formation (and the release of the fractionated bone composition during bone resorption), and therefore, variations in $\delta^{44/42}Ca_{Blood/Urine}$ can be used as direct and faithful proxies of BMB variations [1,9–11,13]. In these models, when kidney function is accounted for [10,11,13], it is assumed to shift the urine composition above that of the blood by a constant value (~1.2‰) regardless of Ca excretion. In this framework, the rapid shift toward light Ca isotope composition seen in the blood/urine of participants in bed rest experiments supposedly reflects the transfer of the low $\delta^{44/42}Ca$ values of the bones to the blood/urine during bone resorption.

Since these early studies, a better understanding of the key role of the kidney in modulating $\delta^{44/42}Ca_{Urine}$ values has emerged, forcing a revision of the framework used to track BMB with Ca isotopes. In particular, because $\delta^{44/42}Ca_{Urine}$ values within a subject have been shown to vary by more than 1‰ as a function of Ca excretion, recent studies no longer interpret $\delta^{44/42}Ca_{Urine}$ variations (including the rapid shifts seen in bed rest experiments) as directly reflecting the transfer of low $\delta^{44/42}Ca$ values from the bones. Instead, most recent work aims to determine if, despite the existence of potentially large variations in $\delta^{44/42}Ca_{Blood/Urine}$ within and between individuals, Ca isotopes retain a diagnostic utility. To do so, the second type of approach uses $\delta^{44/42}Ca_{Blood/Urine}$ as a proxy for assessing the relative strength of Ca fluxes into and out of the bones, and define threshold (or cut-off) values for $\delta^{44/42}Ca_{Blood/Urine}$ marking the transition from a regime of bone formation to one of bone resorption [8,14,15,17]. For instance, the 2019 OsteoGeo study [14] and the 2024 OsteoLabs study [15], support cut-off values of -0.85 ± 0.06‰ for $\delta^{44/42}Ca_{Serum}$, and $+0.23 \pm 0.06$‰ for $\delta^{44/42}Ca_{Urine}$, and interpret patient data below these values as indicative of net bone mass loss. In contrast to the first two approaches, the third one argues that $\delta^{44/42}Ca_{Urine}$ values alone cannot act as a straightforward monitor of BMB, and instead advocates for a "baseline" approach where the impact of renal Ca reabsorption is cancelled out by quantifying both the daily Ca excretion rate and $\delta^{44/42}Ca_{Urine}$ values [12]. Just like in the "cut-off" approach, the goal is still to quantify the relative amounts of bone formation vs resorption, but here an individual's BMB is established by comparison of its Ca excretion vs $\delta^{44/42}Ca_{Urine}$ trend to either that of a healthy control with steady-state bone balance, or preferably, their own trend measured at a time before onset of bone mass loss (i.e. self-referencing).

Common to the two most recent incarnations of Ca isotopes in urine as clinical diagnostic tools is the idea that the $\delta^{44/42}Ca_{Urine}$ values of a given individual are primarily controlled by (i) the ratio of bone formation over bone resorption characteristic of the individual, with (ii) some modulation of this signature that is imparted by the Ca regulatory function of the kidney. The more Ca reabsorbed from the primary urine into the body, the more fractionated (toward heavy values) the excreted urine. As bone resorption increases with age, the $\delta^{44/42}Ca_{Urine}$ value of an individual will decrease in response [8,12]. In the cross-sectional approaches [8,14,15,17], a decline below some universal cut-off value indicates transition to a regime of bone mass loss, while in the self-referencing approach [12], a decline away from a subject's healthy baseline is the diagnostic marker of a change in bone health.

Built into the above frameworks are key assumptions that deserve close examination. The first, which is only relevant to the cut-off value approach, is that all individuals of similar age have a similar blood/serum $\delta^{44/42}Ca$ value, from which the $\delta^{44/42}Ca_{Urine}$ value derives solely as a result of kidney-induced isotopic fractionation. The second is that for a given individual, $\delta^{44/42}Ca_{Blood/Serum}$ is constant over short timescales (hours to months) and changes in $\delta^{44/42}Ca_{Urine}$ values, which are only driven by kidney function, are also generally stable over these timescales. The third is that both the blood and urine Ca isotope composition are only minimally affected by other potential drivers of variability such as diet, demographic, or lifestyle. These assumptions are most certainly wrong to some degree. For instance, recent studies have documented significant inter-subject $\delta^{44/42}Ca_{Urine}$ variability [8,10,12,14,15], as well as elevated values in subjects following a vegan diet [15]. Yet, at this writing, the impact of these assumptions remains to be systematically investigated. In particular, very little remains known about the range of natural variations in the Ca isotope composition of healthy subjects over time, or how demographics and/or lifestyle affect an individual's baseline Ca isotope composition and its temporal variability.

To gain insights into this question, we measured the urine Ca isotope composition from 22 healthy participants over timescales ranging from days to months. The participants were almost evenly split between males ($n = 10$) and females ($n = 12$), and ranged in age from 19 to 60 years old. Below, we briefly describe the methods used for this study and the quality checks performed to validate the data. Our results reveal large inter-individual variability in $\delta^{44/42}Ca_{Urine}$ (up to 2.2‰) but encouraging intra-individual stability (within ~±0.2–0.3‰), indicating that self-referencing approaches for BMB monitoring hold greater promise than cross-sectional ones. Using the answers provided by the participants in their questionnaires, we then evaluate the drivers of inter- and intra-participant $\delta^{44/42}Ca_{Urine}$ variations before discussing the implications for the use of Ca isotopes in urine as tracers of BMB.

Materials and methods

Study design

The study aimed at characterizing the natural temporal variability of Ca isotope compositions in urine ($\delta^{44/42}Ca_{Urine}$) in adults. The 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 during the processing of urine samples by the research team, individuals who were either HIV positive or experiencing chronic urinary tract infections were excluded from this study.

Study participants were recruited on the California Institute of Technology campus by members of the research team. All participants provided informed written consent and received a sampling kit containing the following: a sterile urine cup (90 ml), a disposable plastic Pasteur pipette, a sterile 2 ml polypropylene vial labeled with a unique 4-digit identifier code, a hand sanitizing wipe, and a flyer detailing instructions for sample collection. Using this kit, participants sampled a ~2 ml urine aliquot and

placed it into the 2 ml vial, which they then returned to a drop box on campus. At the time of sample collection, the participants filled out an online questionnaire detailing (i) the time of sample collection, (ii) the 4-digit code of their urine sample vial, and (iii) relevant information about their demographic (e.g. age, gender, ethnicity), health and lifestyle, as well as bone health. A copy of the questionnaire template is available in Table S1. Upon submission of the online questionnaire, a unique ID code was automatically created and assigned to each participant. Participants were allowed, but not required, to provide multiple samples over the duration of the study. Several participants provided more than four samples over periods ranging from days to months. The unique ID code was used to group samples from a given participant while maintaining their anonymity.

To assess the natural temporal variability of $\delta^{44/42}Ca_{Urine}$ in adults, we focused on participants that provided four or more samples during the duration of the study. These fall into two categories:

(i) *High-resolution, 30-day sampling.* One participant (male, 36 years old at time of enrollment), hereafter referred to as Participant 1 (Table S2), provided samples for every urination over a 30-day period ($n = 104$). In addition, the participant also provided a 24-h pooled sample for each day ($n = 30$). Finally, the participant submitted another six samples (morning urine), taken 10 months later, over a 2-week period. These samples give us an opportunity to study short- to medium-term variations in $\delta^{44/42}Ca_{Urine}$ in a single individual and quantify the impact of external factors like changes in diet or lifestyle.

(ii) *Low-resolution, week- to month-long sampling.* All other participants (Participants 2–22, Table S3) provided a more limited number of samples (between 4 and 16), over timescales ranging from days to months. In total, 12 female (age 19–60) and 9 male (age 23–35) participants provided 160 samples.

Sample preparation

Reagents and materials

All sample processing and analyses were performed in a Class 1000 clean laboratory at the Isotoparium (California Institute of Technology). Ultrapure water (resistivity >18.2 MΩ cm at 25°C) was obtained from a Milli-Q Advantage A10 water purification system (MilliporeSigma). Reagent grade concentrated nitric acid and hydrochloric acid (Fisher Chemicals) were distilled twice by sub-boiling in Savillex DST-1000 acid purification systems. Optima grade hydrogen peroxide (31%) was purchased from Fisher Chemicals and used as such. All Teflon labware used in this study (i.e. PFA vials and beakers from Savillex) was pre-cleaned using a 4-step protocol with boiling 7 M HNO_3, 6 M HCl, diluted aqua regia (3:1 mixture of HCl:HNO_3), and finally double distilled 1.5 M HNO_3 solution. Metal-free centrifuge tubes were acquired from VWR and Eppendorf. Polypropylene labware was rinsed three times with MQ water and leached with 10% (vol.) HCl by heating overnight.

Sample digestion

Upon receipt, urine samples were visually examined and those containing visible red blood cells were excluded from the study. This additional exclusion criterion was implemented owing to (i) biosafety concerns for the research team during sample processing and (ii) the possibility that the blood's distinct Ca isotope composition could lead to biased results. For samples passing this screening, a 0.4–1 ml aliquot of urine was pipetted into a clean Savillex PFA beaker. The samples were then digested twice via acid attack on a hot plate using 1 ml of concentrated HNO_3 and 1 ml of 31% H_2O_2 at 130°C overnight. After cooling down, the digests were evaporated to dryness and dissolved in 5 ml of 2 M HNO_3, from which 3 ml of the sample solution was used for the Ca purification and 2 ml was used for the elemental quantification.

Table 1. Ca chromatographic extraction protocol on the prepFAST MC (1 ml of DGA resin)

Step	Reagent	Volume (ml)
Column conditioning	2 M HNO_3	5
Sample loading	2 M HNO_3	3
Matrix elution	2 M HNO_3	3
Matrix elution (Sr)	6 M HNO_3	10
Ca elution	0.1 M HCl	6
Column washing	0.1 M HCl	4

Automated Ca purification

Purification of Ca from the sample matrix was done using a fully automated low-pressure chromatography prepFAST MC system from ESI and either (i) pre-packed Sr-Ca columns from ESI (part number CF-1000) or (ii) self-packed DGA resin columns from Eichrom (normal, 50–100 µm). In both cases, a single column was used to purify up to 100 samples. The automated Ca purification protocol is provided in Table 1. Samples were processed in batches of 20–42 samples. To monitor the performance of the prepFAST MC (e.g. recovery yield) and ensure data accuracy, a procedural blank and reference materials were run at the beginning and end of each batch (i.e. at least two blanks and two reference materials per batch). Purified Ca fractions were collected in clean Teflon beakers, dried down on a hot plate and re-dissolved in a 2:1 mixture of concentrated HNO_3 and H_2O_2, heated on a hot plate at 130°C overnight for removal of organic compounds from the resin. Finally, the Ca fractions were evaporated to dryness and re-dissolved in 1 ml of 2% vol. HNO_3 for element quantification and isotope ratio measurements.

Mass spectrometry

Concentration work (iCAP)

Elemental quantification was accomplished using an iCAP RQ ICP-MS (Thermo Fisher Scientific). For this purpose, the sample digests were diluted in 2% vol. HNO_3 50 times for the Ca quantification, and 1000 times for the two most abundant elements in urine: Na and K. The post-chemistry purified Ca fractions were diluted 125 times in 2% vol. HNO_3. Calcium, Na, and K quantifications were accomplished via external calibration using five calibration standards (SPEX CertiPrep), with concentrations ranging from 0 to 500 µg/l in standard mode (no gas) and 0 to 1000 µg/l in He mode with kinetic energy discrimination mode, respectively. Indium (SPEX CertiPrep), at a final concentration of 2 µg/l (no gas mode) or 5 µg/l (KED mode) was used as an internal standard to correct for instrument instability and potential matrix effects.

Isotope analyses

Calcium isotope analyses were performed on a Neptune *Plus* (Thermo Fisher Scientific) in dry plasma conditions using a Jet sample and X skimmer cone (both Ni) for enhanced sensitivity. An *Aridus3* desolvating nebulizer (CETAC) was used throughout this study. To resolve polyatomic interferences, such as $^{40}ArH_2^+$, $^{12}C^{16}O_2^+$, $^{14}N_3^+$, and $^{14}N_2{}^{16}O^+$, measurements were performed

Table 2. Instrument settings and data acquisition parameters for high-precision Ca isotope analysis by MC-ICP-MS

Instrument settings for Ca measurements	
RF power	1200 W
Sample gas	0.7 l/min
Auxiliary gas	0.7 l/min
Cooling gas	16 l/min
Sampler cone	Ni jet-type sampling cone: 1.1 mm aperture Ø
Skimmer cone	Ni skimmer cone: 0.8 mm aperture Ø
Nebulizer	Concentric, 100 μl min^{-1}
Resolution mode	Medium or high
Typical sensitivity	6–13 V of ^{44}Ca for 1 µg/g Ca solution in HR
Mode	Static, multicollection
Idle time	3 s
Integration time	4.194 s
Number of cycles	50
Desolvating system	
Type	*Aridus3*
Ar sweep gas	2.7–4 l/min
N_2 additional gas	6–10 ml/min
Spray chamber T	110°C
Oven T	140°C

Table 3. Cup configuration for Ca isotopic analysis by MC-ICP-MS in MR or HR mode

Cup	L4	L2	L1	C	H1	H2	H3
Resistor (Ω)	10^{11}	10^{11}	10^{11}	10^{11}	10^{11}	10^{11}	10^{11}
Isotope/mass	^{42}Ca	^{43}Ca	43.5	^{44}Ca	^{45}Sc	^{46}Ca	^{47}Ti

in either medium or high mass resolution mode, and a static cup configuration monitoring masses 42 through 47 was used. All Faraday cups were assigned 10^{11} Ω feedback resistors. Cup gains were calibrated before each Ca measurement session (i.e. at least daily). The instrument was tuned for optimal sensitivity and stability, and typical tuning conditions and parameters are summarized in Tables 2 and 3.

Standard and sample solutions diluted to a final Ca concentration of 1 µg/g in 2% vol. HNO_3 were aspired using a PFA nebulizer with a nominal flow rate of 100 μl/min, yielding a typical beam intensity of 6-13 V on ^{44}Ca, corresponding to an average sensitivity of 455 V/ppm. On-peak-zeros were measured before all samples and standards using a 50–60 s uptake and 42 s on-peak measurement (i.e. 10 cycles) of clean acid solution from the same batch used to dilute the samples to monitor memory effects of the sample introduction system and remove background from all measurements. Typical background intensities were 4–9 mV on ^{44}Ca. Sample and bracketing standard measurements consisted of 50–60 s of sample uptake, followed by 50 cycles of 4.192 s integration time each, for a total 209.6 s of static on-peak sample measurement. The introduction system was then rinsed for 60 s between samples using 2% vol. HNO_3 before repeating the cycle for the next standard/unknown. Instrumental mass discrimination was corrected for using the sample-standard bracketing approach. Each unknown measurement was bracketed by measurements of a single element Ca ICP solution from SPEX (CertiPrep, lot #24-71CAY). The SRM 915b was measured in multiple sessions against the SPEX solution and has a $\delta^{44/42}$Ca value of −0.283 ± 0.024‰ (2SE, $n = 7$) relative to SPEX.

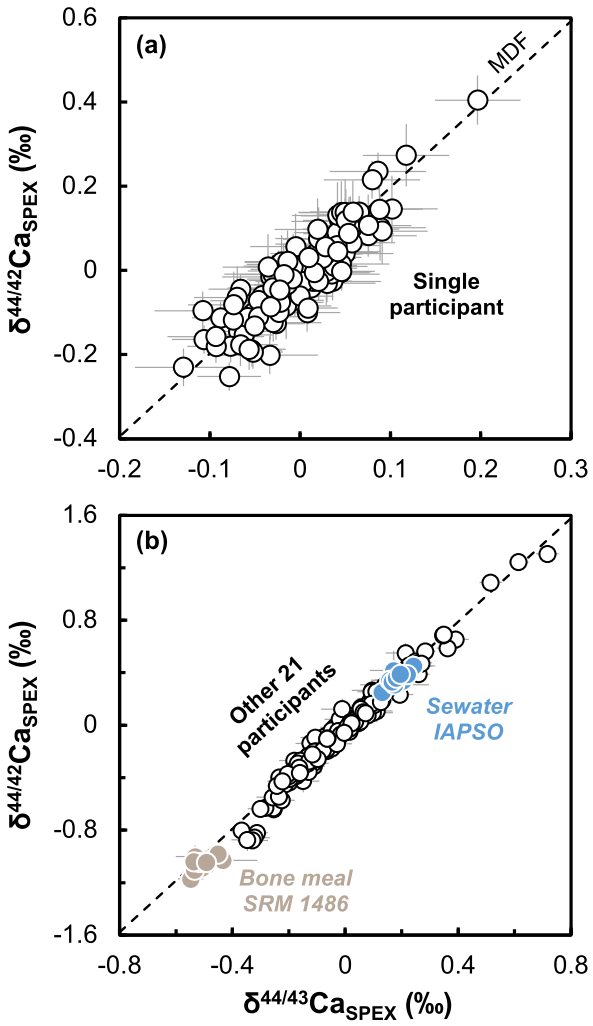

Figure 1. Three isotopes plot showing the $\delta^{44/42}Ca_{SPEX}$ vs $\delta^{44/43}Ca_{SPEX}$ for the data obtained in this study. (a) Samples provided by Participant 1 (36-yr old male) over a 30-day period as well as six additional samples taken 10 months later over a 2-week period. (b) Samples from all other participants and reference materials. The dashed line represents the expected MDF line for measurements performed under ideal conditions: $\delta^{44/42}Ca = 1.973 \times \delta^{44/43}Ca$ (exponential law).

Within each measurement sequence, two secondary reference materials were measured to ensure data accuracy: (i) the NIST SRM 1486 Bone meal reference material and (ii) the IAPSO seawater standard (batch #P165). These materials were subjected to the same chemical processing as the samples and analyzed alongside the samples to monitor the performance of the entire sample processing and analysis. For each batch of sample, a procedural blank was also processed. Full procedural blank values ranged from 40 to 180 ng of Ca, which is more than two orders of magnitude lower than the sample Ca content, and therefore negligible.

Notations

The data in this study were acquired by bracketing against a SPEX single element Ca solution. Values measured relative to the SPEX solution are thus provided in Tables S2 and S3 and shown in Fig. 1 when performing quality control on the measured data. For ease of comparison with literature data, however, Ca isotope compositions shown in all other figures are expressed as $\delta^{44/42}$Ca values relative to the widely used SRM 915a Ca isotopic reference

material:

$$\delta^{44/42}Ca_{SRM915a} = [(^{44}Ca/^{42}Ca)_{sample}/(^{44}Ca/^{42}Ca)_{SRM915a} - 1] \times 10^3. \quad (1)$$

Our data were recast onto the SRM 915a scale using the well-documented isotopic offset between SRM915b and SRM915a (+0.352 ± 0.009‰, 2SE, n = 36) [22], and our calibration of SRM915b against the SPEX solution, as

$$\delta^{44/42}Ca_{SRM915a} = \delta^{44/42}Ca_{SPEX} + (0.634 \pm 0.025). \quad (2)$$

Note that because the SRM915b to SRM915a offset was reported in Ref. [22] as $\delta^{44/40}$Ca, it was converted to $\delta^{44/42}$Ca by multiplying by $\ln(M_{44}/M_{42})/\ln(M_{44}/M_{40}) \sim 0.488$, which assumes that the isotopic fractionation is mass-dependent and follows the exponential (kinetic) law [23]. Uncertainties are reported as '2SE external reproducibility' and calculated as $2 \times \sigma_{Standard}/\sqrt{n}$, where $2 \times \sigma_{Standard}$ is the daily external reproducibility of repeat measurements of a SPEX single element Ca bracketed by itself (measured at the same concentration as the sample), and n is the number of repeat analyses of the same sample solution (typically n = 2–4). Depending on the analytical session and solution concentration, $2 \times \sigma_{Standard}$ varied between ±0.06 and ±0.13‰, resulting in 2SE uncertainties for replicate analysis of samples between ±0.03 and ±0.09‰.

Results

For all samples, a summary of the data is presented in Tables S2 (Participant 1) and S3 (Participants 2–22), including the sample's tube ID, the participants' demographic information, and the concentration and isotopic composition of Ca, as well as the Na and K concentrations.

Data accuracy and reproducibility

Data accuracy on both reference materials and samples was examined by checking the mass-dependency of the isotope effects. Under ideal measurement conditions, delta values obtained on adequately purified Ca fractions should scale with the mass difference of the isotopes considered: i.e. $\delta^{44/42}$Ca ~2×$\delta^{44/43}$Ca. Figure 1 shows that all data reported herein are indeed mass-dependent, indicating that the chromatographic purification protocol efficiently removed the sample matrix and testifying to the data's accuracy. Furthermore, tests conducted on a prepFAST MC system [24] showed no measurable Ca isotope fractionation for yields above 75%, and the measured recovery yields for our samples were consistently high, ranging from 77% to 110%.

To assess data accuracy and reproducibility, 11 replicates of the NIST SRM 1486 Bone meal reference material and the IAPSO seawater standard were processed and measured alongside the urine samples. As shown in Fig. 2, values obtained over 1.5 months and 11 analytical sessions are highly reproducible, giving average values of −0.420 ± 0.032‰ (2SE) for the bone meal and +0.988 ± 0.033‰ (2SE) for the seawater, in reasonable agreement with the average literature data of, respectively, −0.490 ± 0.026‰ (n = 5, 2SE) [25–28] and +0.916 ± 0.009‰ (n = 39, 2SE) [9,22–24,29–56]. Furthermore, the difference between the two materials (−1.408 ± 0.025‰) is indistinguishable from the average value of −1.406 ± 0.027‰ calculated using literature data. In combination, these results confirm that no significant mass-dependent fractionation (MDF) on the column occurred, and that the Ca isotope variations observed are primary to the urine samples.

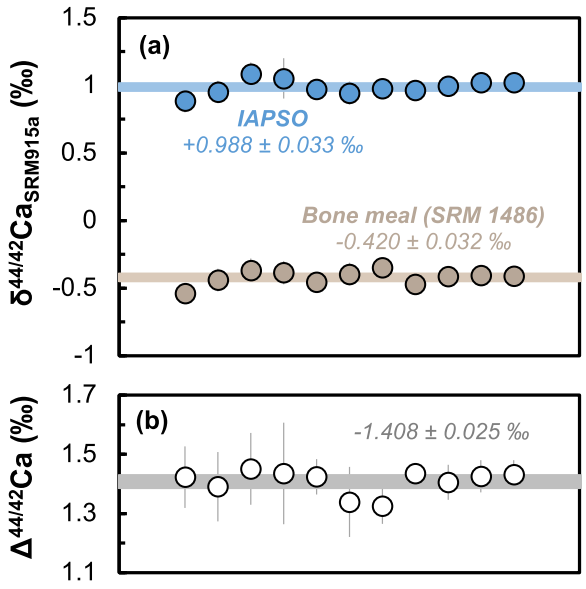

Figure 2. (a) $\delta^{44/42}$Ca values for the IAPSO seawater standard (blue) and the NIST SRM 1486 Bone meal reference material (tan) measured alongside samples in this study. Each data point represents a different analytical session (both materials were measured in each session), while the horizontal bands represent the average values. (b) Difference in $\delta^{44/42}$Ca value between the two reference materials for each analytical session. The average value (−1.408 ± 0.025‰, 2SE) is in excellent agreement with the recommended value of −1.406 ± 0.027‰ (see main text for references).

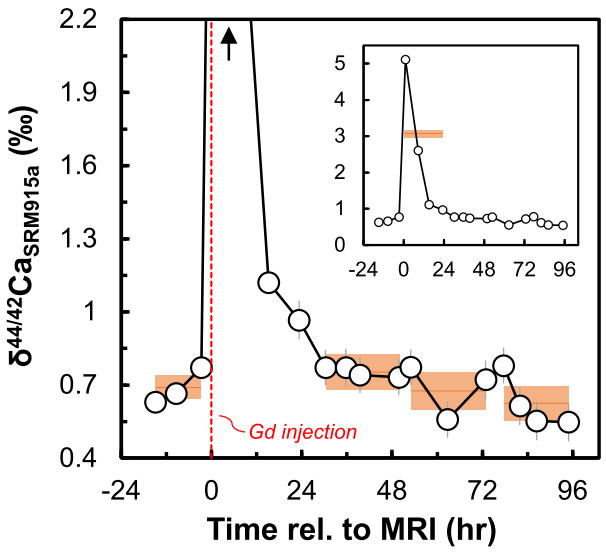

Figure 3. $\delta^{44/42}Ca_{Urine}$ values as a function of time relative to MRI exam, with contrast agent (Gd). Because Gd is not separated from Ca during column chromatography, excretion of Gd into urine post-MRI results in departure of the $\delta^{44/42}Ca_{Urine}$ during isotope analysis (i.e. matrix effects). Individual urine samples (open circles) and 24-h pooled values (orange bands) return to pre-Gd injection levels after 24–48 h.

Impact of MRI contrast agent (gadolinium)

During the 30-day sampling period, Participant 1 underwent a magnetic resonance imaging (MRI) procedure with a contrast agent (gadolinium, Gd). Following injection, Gd is excreted from

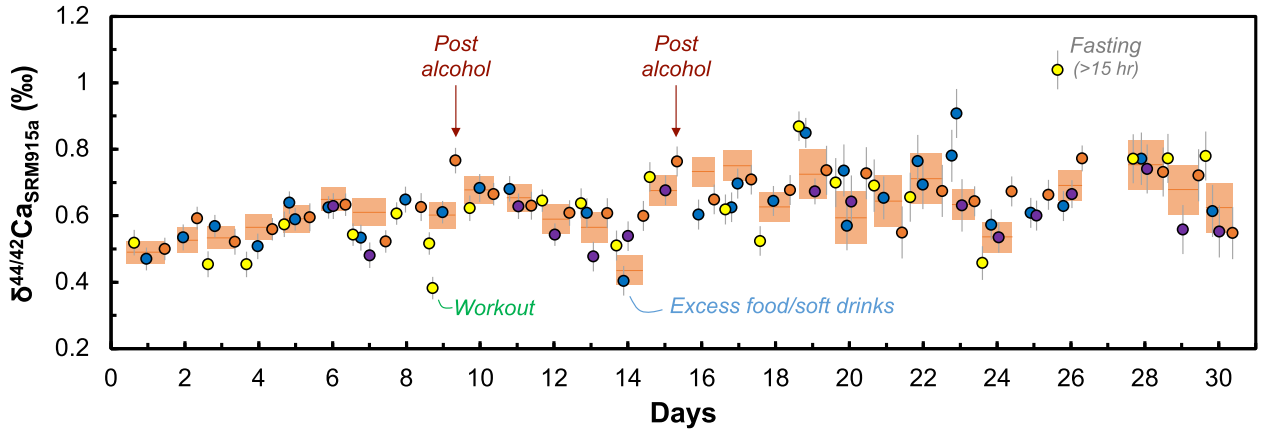

Figure 4. $\delta^{44/42}Ca_{Urine}$ values from Participant 1 (36-yr old male) over a 30-day period. Symbols are color coded according to the time of collection (morning = orange, afternoon = yellow, evening = blue, and night = purple). Morning is defined as first urination after waking up (or until 12.30 p.m.), afternoon is 12.31 p.m. to 6 p.m., evening is 6 p.m. to 11.59 p.m., and night is 12 a.m. till first urination. Orange bands denote the 24-h pooled values for each day (the missing band for day 8 and 25 are due to lost samples). Data affected by matrix effects due to the presence of Gd in urine following the MRI exam (Fig. 3) are not plotted here (day 27).

the body through urine over the next few days. Because Gd is not separated from Ca during column chromatography, the purified Ca fraction of urine samples following the MRI procedure contained both Ca and Gd in high abundances, and the latter could theoretically affect the isotopic analyses (i.e. matrix effects). As shown in Fig. 3, the presence of Gd in urine indeed induces systematic biases in the measured Ca isotope composition. After injection, $\delta^{44/42}Ca_{Urine}$ values increased by up to ~4.5‰ relative to the pre-injection value, and the data departed significantly from the mass-dependent line ($\delta^{44/42}Ca_{SPEX}$ = +4.5‰, $\delta^{44/43}Ca_{SPEX}$ = +17.98‰). Individual and 24-h pooled $\delta^{44/42}Ca_{Urine}$ values returned to pre-Gd injection levels and mass-dependent behavior after 24–48 h.

Short-term variability: 30-day experiment

Figure 4 shows the $\delta^{44/42}Ca_{Urine}$ values of all samples from Participant 1 (36-year-old male) over a 30-day period. During this period, the participant's diet was not fixed, nor was his daily routine or the frequency of activities that might affect Ca uptake and regulation (e.g. physical exercise, alcohol consumption, food intake frequency).

Over the 30-day period, the data from all individual urine samples reveal limited variability of the $\delta^{44/42}Ca$ values about a mean value of +0.63 ± 0.22‰ (2SD). Similar average values, but smaller variances, are observed in both the 24-h pooled samples (+0.62 ± 0.16‰, 2SD) and in the morning urine samples (+0.64 ± 0.16‰, 2SD), indicating that some of the variations seen in individual samples are high-frequency noise due to secondary controls (e.g. diet) that is averaged out in the morning urine/24-h pooled fractions. This averaging effect is clearly visible when the Ca isotope composition is plotted against the time since last urination: the scatter in the data decreases as the time since urination increases (Fig. 5). While individual urine samples were collected at intervals ranging from 2 to 13 h, the morning urine samples were all collected at least 6.5 h after the previous sample.

Figure 4 also allows examination of the data at the daily scale. For each day, individual urine samples are plotted as circles (colors denote time of day, see figure caption) and the corresponding 24-h pooled samples are shown as orange bands. In many cases, individual urines from a given day have Ca isotope compositions

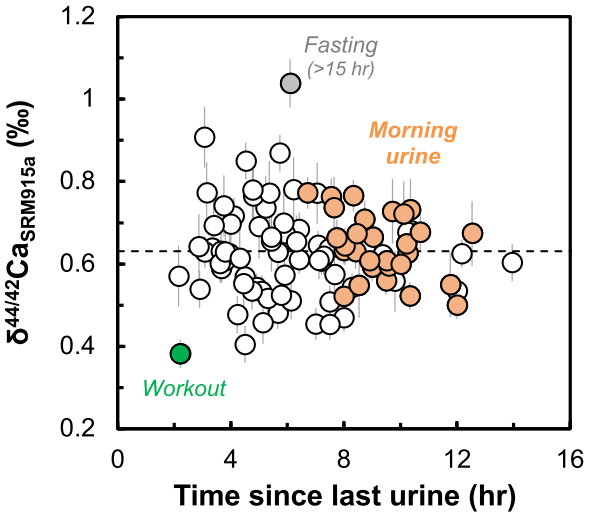

Figure 5. $\delta^{44/42}Ca_{Urine}$ plotted against time since last urination for all samples from Participant 1 (36-yr old male) over a 30-day period. While the average value calculated using all samples (dashed line) and only the morning urine samples is the same (+0.63 vs +0.64‰, respectively), the range of variation is smaller in the morning urine (±0.16‰, 2SD) compared to all urine samples (±0.22‰, 2SD).

indistinguishable from each other as well as from the 24-h pooled fraction. Samples collected throughout the day/night can, however, show significant variability around the 24-h pooled value (see for instance afternoon samples on day 9, including the sample labeled *workout*). In contrast, $\delta^{44/42}Ca$ values in morning urines seldom deviate from the 24-h pooled value (Fig. 6). Ignoring the samples affected by the MRI contrasting agent (which are not plotted in any figure except Fig. 3), only a few notable departures exist:

— The most striking is visible on day 26, where a urine sample collected in the afternoon has a $\delta^{44/42}Ca_{Urine}$ of +1.04 ± 0.06‰, far above the monthly average value of +0.63‰. This sample was taken more than 15 h after the last food consumption by Participant 1 and is thus labeled as *fasting*.

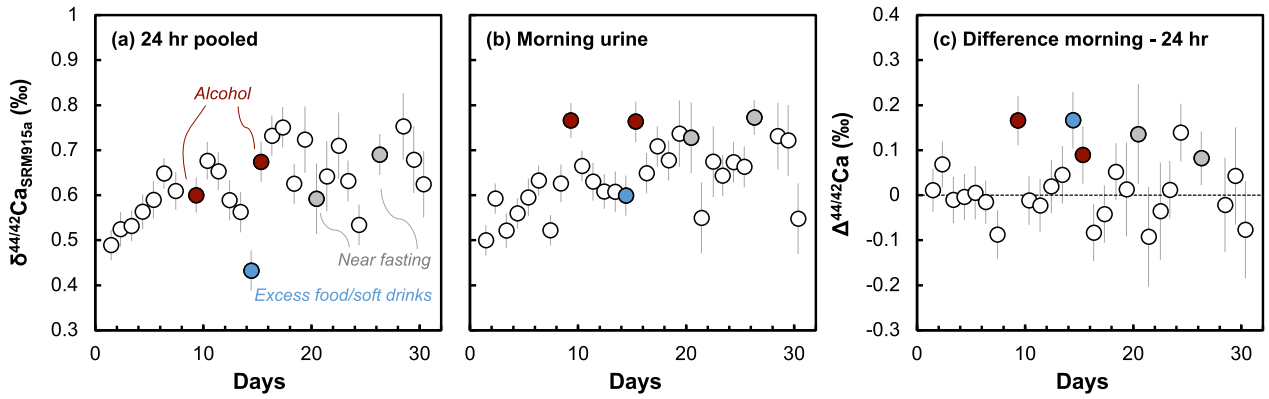

Figure 6. $\delta^{44/42}Ca_{Urine}$ values of (a) 24-h pooled fractions and (b) morning urine, from Participant 1 (36-yr old male) over a 30-day period. (c) Difference ($\Delta^{44/42}Ca$) between the morning urine and the 24-h pooled fraction. In most cases, $\delta^{44/42}Ca$ values in morning urines seldom deviate from the 24-h pooled value. Notable exceptions are shown as filled symbols. Dark red symbols denote days in which the participant consumed alcohol. Blue symbols denote days in which the participant consumed excess food/soft drinks. Gray symbols denote days in which the participant was nearly fasting by the time the morning urine was collected (i.e. almost no food for dinner).

— The next one is seen on day 9, where the morning urine (orange circle) is isotopically heavier than the individual urines of the day and the 24-h pooled fraction by 0.17 ± 0.05‰. This sample was collected the morning following consumption of alcohol by Participant 1. A smaller increase is seen in the morning urine of day 15 after consumption of alcohol. Except for these two days, Participant 1 entirely abstained from alcohol, and these two samples are labeled as *post-alcohol*.
— Similarly, on day 14, the morning urine was isotopically heavier than the 24-h pooled fraction by 0.17 ± 0.06‰. Unlike on day 9, however, it is not the morning urine value that is offset toward higher value relative to other samples, but the 24-h pooled fraction value that is unusually low, as it displays the lowest value of any 24-h pooled fraction, at +0.43 ± 0.04‰. On that day (Christmas day), Participant 1 consumed excessive food and non-alcoholic drinks, and the 24-h value appears to be dominated by the evening urination (first post-dinner urination).
— Finally, on day 24, the morning urine was isotopically heavier than the 24-h pooled fraction by 0.14 ± 0.06‰. No obvious external driver was identified for this anomalously elevated morning urine value. Like for day 14, the 24-h value seems controlled by the day and night urines: indeed, the 24-h value overlaps with all other samples taken that day, which have a uniform composition, different from the morning urine.

In Fig. 7, Ca isotope compositions are plotted as a function of Ca excretion (or a proxy thereof, such as the Ca/K ratio). For the 24-h pooled samples, the total weight of urine collected was measured, and Ca excretion can be calculated (Fig. 7a). For other samples, no weighing was done, and the Ca concentration is used as a rough proxy for Ca excretion (Fig. 7b). For all samples (pooled and individual urines), $\delta^{44/42}Ca_{Urine}$ values are plotted against Ca/K ratios as well (Fig. 7c and d). Potassium was chosen as the normalizing element as it is (i) primarily excreted in urine where it is therefore very abundant, and (ii) shows the least amount of correlation with Ca: i.e. the normalization to potassium mitigates dilution effects without canceling out potentially significant variability in the data. The concentration data obtained on the samples reveal a negative correlation between $\delta^{44/42}Ca_{Urine}$ values and (proxies of) Ca excretion. As posited by earlier works [9–13,17], this trend is likely to reflect isotopic fractionation in the kidney during Ca reabsorption from the primary urine into the body. The linear trend seen in the log-linear space of Fig. 7 is consistent with a Rayleigh distillation process whereby the more intensely Ca is being reabsorbed into the body, the more the Ca excreted in the urine is isotopically heavy.

While the 24-h pooled data show a single population in $\delta^{44/42}Ca_{Urine}$ vs Ca excretion space, two outliers are seen in the individual urine samples. The first is the *fasting* sample, identified based on its elevated $\delta^{44/42}Ca_{Urine}$ value, and which plots here slightly above the rest of the samples. The second is the *workout* sample (green circle in Fig. 7b and d), identified in Fig. 4 owing to its low $\delta^{44/42}Ca_{Urine}$ value, which stands out in Fig. 7 due to its unusually low Ca concentration (and Ca/K ratio). This sample was collected less than 2.5 h after the previous urine sample (Fig. 5), but in the interval, Participant 1 underwent an intense workout of 1.5 h, hydrating only with water and consuming no food. Interestingly, the other outliers identified solely based on $\delta^{44/42}Ca_{Urine}$ values (i.e. post-alcohol and excessive food/soft drinks) all plot within the general trend defined by the rest of the individual samples, indicating that their isotopically high and low values are most likely to be controlled by normal kidney regulation. This is in contrast to the *workout* outlier, and to a lesser extent the *fasting* outlier as well, which seem to be affected by other biological functions.

Long-term and inter-participant variability

Ten months after the 30-day high resolution sampling experiment, Participant 1 provided six additional morning urine samples collected over a 2-week period. The $\delta^{44/42}Ca$ values for these new samples range from +0.44 ± 0.04‰ to +0.59 ± 0.04‰ (Fig. 8). While within error of the average morning urine values measured during the 30-day experiment (+0.64 ± 0.16‰, 2SD), half of these new samples have the lowest Ca isotope composition measured for any morning urine samples from Participant 1. At face value, this small shift toward lower $\delta^{44/42}Ca$ values could represent (i) a slight underestimation of the long-term Ca isotope variability in the participant's urine based on the 30-day data or (ii) the impact of prolonged changes in diet (specifically increase in dairy consumption), as the additional six samples were collected 1.5 month after Participant 1 had spent 2.5 months abroad.

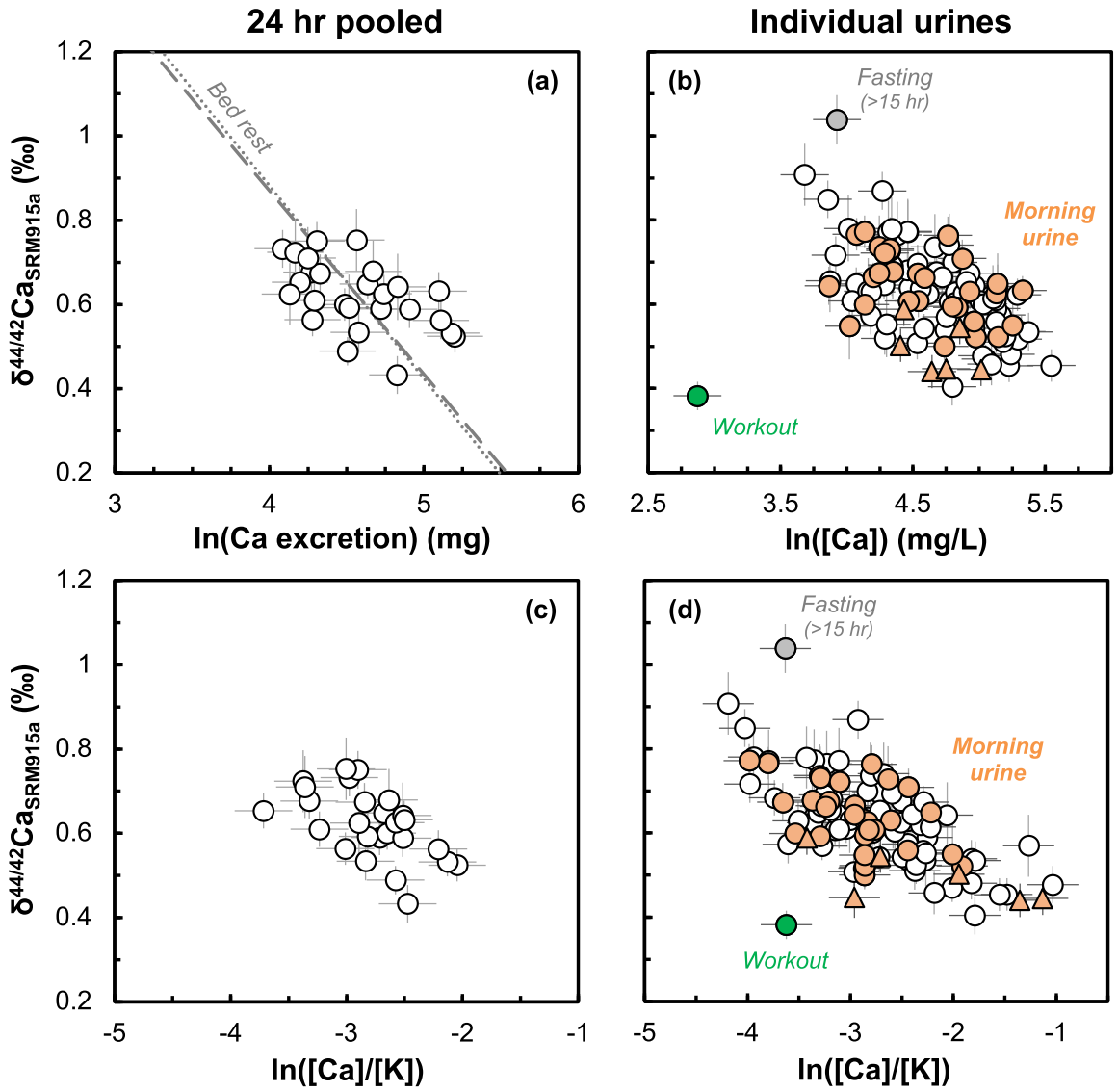

Figure 7. $\delta^{44/42}$Ca values in urine samples from Participant 1 (36-yr old male) plotted against Ca excretion (or a proxy thereof). Left and right panels show values for, respectively 24-h pooled fractions and individual urines. The data define a negative trend, which previous works have interpreted as reflective of renal functions. Dashed and dotted lines in panel (a) show the slopes obtained in bed rest experiments by, respectively, Heuser *et al.* (2019) and Morgan *et al.* (2012). Amongst individual urines, two outliers are seen: one sample collected after > 15 h of fasting (gray), and another collected less than 2.5 h after the previous sample during which the participant underwent an intense 1.5-h workout (green). Orange symbols denote morning urines, with circles showing samples taken during the 30-day high-resolution experiment, and triangles showing samples collected 10 months later over a 2-week period.

Support for the second hypothesis comes from considering the Ca concentration (Ca/K ratio) data. Indeed, in $\delta^{44/42}$Ca vs Ca (or Ca/K) space, the six additional sample samples (triangles in Fig. 7b and d) plot on the lower edge of the domain defined by the rest of the morning urine (i.e. small systematic offset).

Twenty-one other participants provided multiple samples (4–16) over timescales ranging from days to months. The data, shown in Fig. 9a, reveal significant inter-participant variability in $\delta^{44/42}Ca_{Urine}$ values with the entire population covering a range from −0.24 ± 0.06‰ to +1.94 ± 0.04‰. In contrast, intra-participant variability (i.e. the spread in $\delta^{44/42}Ca_{Urine}$ values between samples from a given participant) is often limited. Using the variance of the data from Participant 1 (±0.22‰) as a reference, most participants (13 out of 21) have similarly stable $\delta^{44/42}Ca_{Urine}$ values over timescales up to 160 days (Fig. 9b). The increased variability observed in the data from the remaining eight participants (Fig. 9c) is discussed in the 'Discussion' section. A general decrease in $\delta^{44/42}Ca_{Urine}$ values is observed with increasing age (Fig. 9a), although clear departures from this general trend are seen (e.g. Participants 4, 16, 17, or 9).

In addition to providing samples of individual urine, one participant (Participant 10) also provided a 24-h pooled urine sample. Compared to the 24-h sample (+0.58 ± 0.03‰, 2SE, $n = 11$), individual urine samples have higher $\delta^{44/42}$Ca values (up to +0.90‰). One final sample from Participant 10, collected after 5 days of vegan diet, displays a $\delta^{44/42}Ca_{Urine}$ value of +1.04 ± 0.04‰ and a characteristically low Ca/K ratio (0.008).

In Fig. 10, the $\delta^{44/42}$Ca values in urine samples from all participants are plotted against Ca concentration (Fig. 10a) and Ca/K

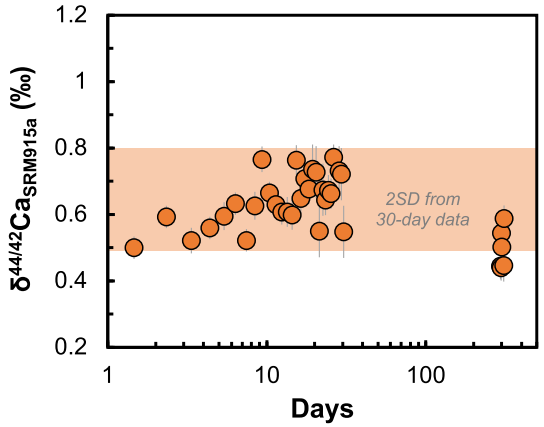

Figure 8. $\delta^{44/42}Ca_{Urine}$ values for morning urines from Participant 1 (36-yr old male) over a 10-month period. The orange horizontal band denotes the average and 2SD defined by the data during the first 30-day period. Samples collected nearly 10 months later have similar Ca isotope compositions, indicating a stability of the participant's $\delta^{44/42}Ca_{Urine}$ values over nearly a year.

ratio (Fig. 10b). The data from most participants (both male and female; dark blue symbols in Fig. 10) define the same negative trend seen in the data from Participant 1, but expand it 2-fold, from ~0.50‰ to more than 1‰. At the same time, the data from some participants (shown as bright colored symbols in Fig. 10) deviate from the general trend defined by most participants. For some of these, useful context (and potential origins of these departures) was provided by the participants in the questionnaires associated with each sample. For instance, moving from the highest to the lowest Ca isotope ratios:

- Participants 9 (violet circles) and 17 (green triangles with white outline), whose samples have $\delta^{44/42}$Ca values mostly around 1.1‰, are the only participants who reported consuming more than 10 units of alcohol per week.
- Participant 21 (blue triangles), whose samples have nearly identical $\delta^{44/42}$Ca values despite their Ca concentrations spanning one order of magnitude (and Ca/K ratios spanning two orders of magnitude), is one of only two participants who reported consuming dairy and milk every day.
- Participant 12 (pink circles) is a 60-year-old woman who has experienced menopause. The strong departure of data from her samples toward negative $\delta^{44/42}$Ca values nearly perpendicularly to the trend defined by the other participants could be related to bone mass imbalance.

Discussion

Magnitude and timescales of $\delta^{44/42}Ca_{Urine}$ variability

Our systematic study of Ca isotopes in urine from 22 participants reveals large inter-subject variability with $\delta^{44/42}Ca_{Urine}$ values covering a range of nearly 2.2‰. While a general decrease in $\delta^{44/42}Ca_{Urine}$ is observed as participants' age increases, a virtually identical range of variations is seen between participants of similar age: e.g. Participants 4 and 16 are 25 and 26 years old, respectively, and their $\delta^{44/42}Ca_{Urine}$ values differ by 2.1‰ (Fig. 9a). In contrast to the large inter-subject variations, our data reveal remarkable intra-subject stability, with 14 out of 22 participants having $\delta^{44/42}Ca_{Urine}$ values constant within ±0.30‰ over timescales of 100 days or more (Fig. 9b). A similar result was very recently reported in the OsteoLabs study [15] for 63 individuals over periods of up to 2 years. Such stability over monthly to yearly timescales supports one of the key assumptions behind the use of $\delta^{44/42}Ca_{Urine}$ as tracers of BMB: i.e. the mid-term average $\delta^{44/42}Ca_{Urine}$ value of a participant (with stable dietary, behavioral and physiological states) can be established by measuring the $\delta^{44/42}$Ca value of a single urine sample. At the same time, our data show that small to large variations in $\delta^{44/42}Ca_{Urine}$ do exist within some individuals, with seven participants showing intra-variability between 0.5 and 0.6‰, and two participants pushing this number up to ~0.8‰. Below we systematically consider the primary and secondary controls affecting urine Ca isotope compositions.

Factor controlling $\delta^{44/42}Ca_{Urine}$

Age

The relative importance of bone mineralization and bone resorption in humans varies drastically over an individual's lifetime. Bone mineralization dominates during childhood and teenage years as the skeleton is rapidly being built and continues to do so until the mid-twenties, at which point the peak bone mass is reached and a near steady-state between mineralization and resorption is established. Past the peak bone mass and as humans age, bone resorption tends to slightly exceed mineralization, which in the more extreme cases will lead to osteopenia and osteoporosis [57,58]. As bones are isotopically light relative to soft tissues for Ca (by ~−0.3‰) [1,10,13,14], the expectation is that a general decrease in $\delta^{44/42}$Ca values will be observed in the serum/urine of individuals of increasing age. Our data conform to this general expectation, with $\delta^{44/42}Ca_{Urine}$ values decreasing by ~0.6‰ going from participants in their early 20s to early 60s (Figs 9 and 11), in good agreement with results from previous work [8,13,15]. This broad evolution across individuals indicates that $\delta^{44/42}Ca_{Urine}$ values do reflect, to some extent, the BMB in the human body. At the same time, the large differences seen amongst individuals of similar ages clearly indicate that an individual's urine Ca isotope composition is also controlled by other, equally important, factors.

Taken at face value, our results on 22 participants seem to suggest that monitoring of BMB with Ca isotopes cannot simply be done by comparing an individual's $\delta^{44/42}Ca_{Urine}$ value to that of a hypothetical healthy control of similar age or to a threshold value (i.e. cut-off approaches). An obvious limitation of our dataset, however, is that it is small and biased toward younger participants, with 19 out of 22 of them being less than 40 years old. To know if the same extent of variability seen in our data extends to all ages, we turn to previous studies [8,14], and particularly the recent OsteoLab study [15], which reports on a large dataset of 2320 participants. Significant inter-participant $\delta^{44/42}Ca_{Urine}$ variations are seen in children and young adults (~1–1.5‰) as well as in adults above 40-year-old (~2–3‰). Focusing on data from women, who constitute the vast majority of the participants in the OsteoLab study ($n = 1935$) [15] and thus carry the strongest statistical significance, nearly identical ranges, medians and distributions of $\delta^{44/42}Ca_{Urine}$ values are observed for all age brackets above 40 (i.e. the ">40–60", the ">60–80", and the "> 80" bracket). All three age sub-groups show the same range of $\delta^{44/42}Ca_{Urine}$ variability seen in our data (~2 to 3‰), indicating that, while limited, our dataset is reasonably representative of larger adult populations. Importantly, while the OsteoLab study proposed a $\delta^{44/42}Ca_{Urine}$ value of +0.23 ± 0.06‰ as a statistically sound threshold for the diagnosis of osteoporosis, we note that the median $\delta^{44/42}Ca_{Urine}$ values of all age sub-groups above 40 are either equal to, or below, this cut-off value. In isolation, the cut-off $\delta^{44/42}Ca_{Urine}$

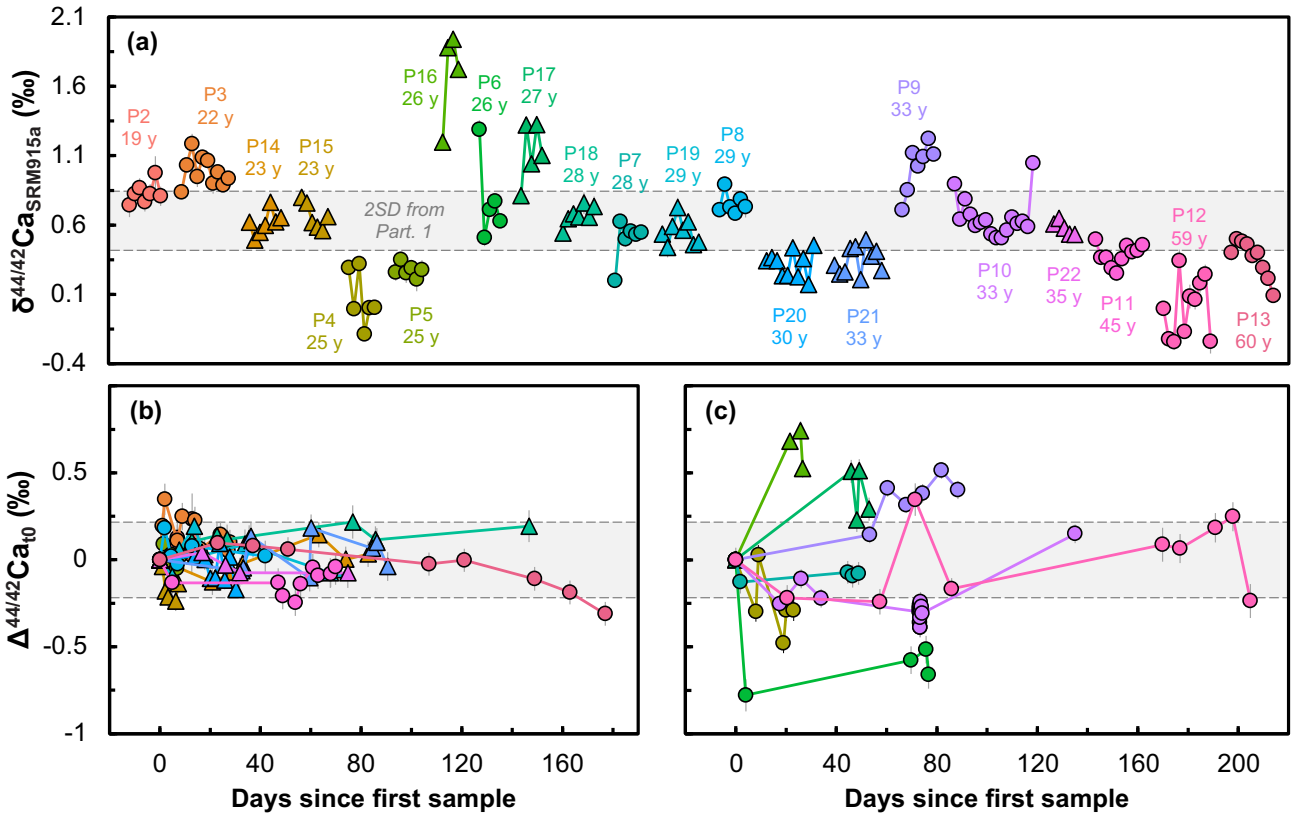

Figure 9. (a) $\delta^{44/42}Ca_{Urine}$ values from Participants 2 to 22. Participants' age increases from left to right. Circles and triangles denote, respectively, females and males. (b and c) Same data but shown as the difference from the first sample ($\Delta^{44/42}Ca_{t0}$) and plotted as a function of time. Panel (b) shows participants whose data are similarly stable to those of Participant 1 (± 0.22‰, 30-day high-resolution experiment), while panel (c) shows participants whose data are significantly more variable. In all panels, the horizontal gray band denotes the range of variations in the data from Participant 1.

approach would seemingly lead to a nearly 50% rate of false positive for women over 40 years old, raising questions about the efficiency of such an approach, and highlighting the need to further understand what other controls affect $\delta^{44/42}Ca_{Urine}$ values.

Body mass index

To investigate the origin of inter-participant variability, $\delta^{44/42}Ca_{Urine}$ values were plotted against key anthropometric measurements of the participants: height, weight, and body mass index (BMI = weight/height^2 in kg/m^2) (Fig. 12). The high correlation of the BMI with total body fat makes it a useful proxy for epidemiological purposes, and standard ranges (e.g. underweight, normal weight, overweight, obesity) have been defined based on correlations between BMI and mortality rates. In height and weight spaces, $\delta^{44/42}Ca_{Urine}$ data from men and women define mostly distinct populations, consistent with males being generally taller and heavier than females. These differences disappear in BMI space, where the data from both males and females largely overlap as most participants display BMI values considered healthy (i.e. between 18.5 and 25) [59].

Figure 12 reveals interesting structure in the data, with $\delta^{44/42}Ca_{Urine}$ values in female participants being broadly correlated with height, weight, and BMI values, while more scatter is observed in samples from male participants. In BMI space in particular (Fig. 12c), the data from all but two female participants (Participants 5 and 12) define a strong positive relationship. Participant 12 is one of only two participants who reported having experienced menopause and the offset toward lower $\delta^{44/42}Ca_{Urine}$ values seen in her data could possibly reflect the impact of a postmenopausal increase in rate of bone mass loss. Participant 5 is a 25-year-old female with a BMI of 30.9, a score that is in the Obesity Class I category according to the WHO classification [59]. While it is tempting to propose that the low $\delta^{44/42}Ca_{Urine}$ values of Participant 5 might reflect an increased rate of bone mass resorption due to the participant's elevated BMI/weight, the connections between BMI and bone are complex, with high BMI (i.e. obesity) having both positive and negative feedback on BMB [60]. It is therefore possible that the low $\delta^{44/42}Ca_{Urine}$ values of Participant 5 reflect some aspect of her BMB, but we cannot rule out other potential drivers (e.g. kidney function).

In Fig. 12c, the gray dashed line shows a regression fit [and 95% confidence interval (CI) envelope] through the average data from both male and female participants. This fit excludes Participants 5 and 12 (for the reasons discussed above), as well Participant 20, a 30-year-old male participant whose $\delta^{44/42}Ca_{Urine}$ values plot below the trend defined by other participants. This participant reported consuming significant amounts of dairy products, which could have affected his data [61–63]. The positive relationship shown in Fig. 12c indicates that, over a BMI range of 18 to 28 kg/m^2 (i.e. from normal weight to overweight, but excluding obesity), an individual's $\delta^{44/42}Ca_{Urine}$ value will increase (decrease) by ~0.10‰ for each unit of BMI gained (lost). This positive trend, although preliminary, is in line with the documented positive effect of BMI on bone mineral density, resulting from higher physical constraints and estrogen production by adipose tissues [60].

The recognition of a broad correlation between $\delta^{44/42}Ca_{Urine}$ values and BMI provides a potential path toward canceling some of the inter-participant Ca isotope composition variations. Indeed,

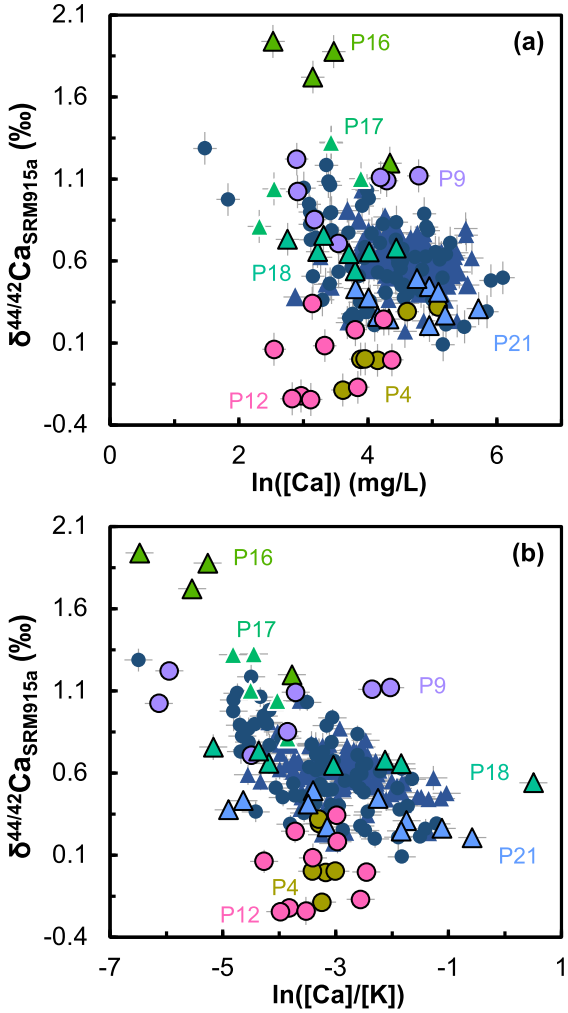

Figure 10. $\delta^{44/42}$Ca values in urine samples from all participants plotted against (a) Ca concentrations and (b) Ca/K ratios. Circles and triangles denote, respectively, females and males. Brightly colored symbols denote participants whose data deviate from the general trend defined by most participants. See text for details.

while the average $\delta^{44/42}Ca_{Urine}$ values of all participants vary by more than 1.75‰, the spread at a given BMI value is typically less than 0.5‰. This statement excludes Participants 5 and 12, whose respective obesity and menopause are likely confounding factors driving their Ca isotope composition toward light values. While more data from a larger population will be necessary to confirm these findings, our data suggest that an individual's BMB could potentially be determined by comparing their $\delta^{44/42}Ca_{Urine}$ value to that of a healthy control of similar BMI (or after normalization to a reference BMI using the slope of 0.10‰/unit of BMI shown in Fig. 12c).

Renal function

The kidney plays a critical role in regulating Ca homeostasis in the body [64]. In a healthy individual, most of the Ca present in the primary urine is reabsorbed into the body before urine excretion. This process involves a combination of passive and active transporters, and while the exact drivers of isotopic fractionation are still unclear [65], the overall effect is that the light isotopes of Ca are preferentially reabsorbed in the kidney. Most often, the kidney is described as the seat of a Rayleigh distillation process

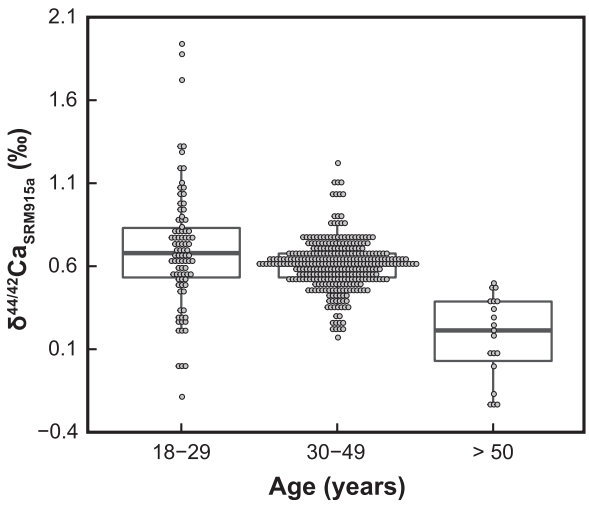

Figure 11. Boxplot showing the $\delta^{44/42}Ca_{Urine}$ values from all 22 participants as a function of age. A decreasing trend is observed in agreement with the expected higher rates of bone resorption in older adults.

[12,13,17], and the Ca isotope composition of the urine can thus be 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

$$\delta^{44/42}Ca_{Urine}(f) = \delta^{44/42}Ca_0 + (f^{(\alpha-1)} - 1) \times 1000. \quad (3)$$

For a typical Ca reabsorption of 98% (f = 0.02), the urine excreted from the body is typically ~1.2‰ heavier than blood [11,14]. Assuming that there is no fractionation between the blood and the primary urine [65], and using Equation 3, yields a value of α~0.9997, equivalent to a fractionation factor $\Delta^{44/42}$Ca of ~−0.30‰: i.e. at each point in time, the instantaneously reabsorbed Ca is 0.3‰ lighter than the Ca in the urine it is reabsorbed from. In such a framework, even limited variations in the extent of Ca reabsorption can significantly impact the excreted urine $\delta^{44/42}$Ca values. For instance, varying the Ca excretion by 1 natural log unit around 2% (half a log unit in both directions) would yield anticorrelated $\delta^{44/42}Ca_{Urine}$ variations covering a range of ~0.30‰ (i.e. the fractionation factor describing the Rayleigh distillation).

In excellent agreement with these first-order predictions, the 24-h data from Participant 1 for which Ca excretion could be calculated, cover a range of $\delta^{44/42}Ca_{Urine}$ of ~0.3‰, while Ca excretions vary by a factor 3 (~1 natural log unit) (Fig. 7a and c). Using the Ca/K ratio as a proxy for Ca excretion, a similar picture is painted by the individual urine samples of Participant 1, albeit with a shallower slope, whereby the $\delta^{44/42}Ca_{Urine}$ values vary by 0.45‰ over a Ca/K ratio spread of 3 natural log units (Fig. 7b and d). This observation extends to most of the other 21 participants of the study as well, whose combined $\delta^{44/42}Ca_{Urine}$ values mainly range from +0.1 to +1.1‰, while the corresponding Ca/K ratios vary by ~5 natural log units (or a factor ~150) (Fig. 10b). A handful of participants have $\delta^{44/42}Ca_{Urine}$ vs Ca/K relationship that deviate from this kidney-controlled slope, with some showing much steeper (Participants 16 and 17) or shallower slopes (Participants 9, 18, and 21) and even two participants showing $\delta^{44/42}Ca_{Urine}$ variations unrelated, or positively correlated, to Ca excretions (Participants 4 and 12). For these participants, controls other

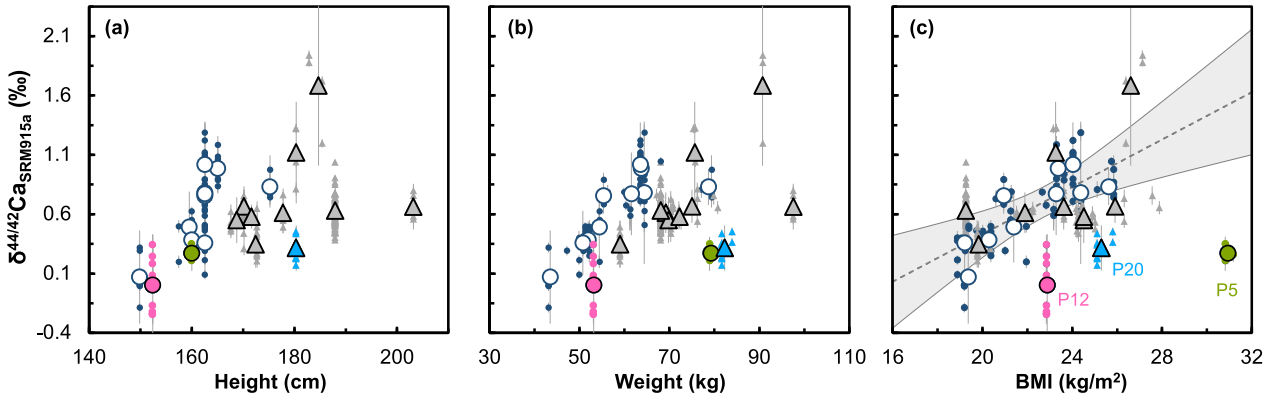

Figure 12. $\delta^{44/42}$Ca values in urine samples from all participants plotted against (a) height, (b) weight, and (c) BMI. Circles and triangles denote, respectively, females and males. Small symbols denote individual urine samples, while larger symbols show the average value for each participant. Brightly colored symbols with black outline denote participants whose data deviate from the general trend defined by most participants (gray dashed lines and 95% CI envelope, slope of ~0.10‰/unit of BMI). See text for details.

than healthy kidney function (like the impact of menopause for Participant 12) must be at play (see below).

Given the significant impact of BMI on an individual's $\delta^{44/42}Ca_{Urine}$ values ('Body mass index' section), removing this effect from the data could be beneficial to clarify the extent and possible origin of inter-participant Ca isotope variations. In Fig. 13, we compare the data before (top panel) and after (bottom panel) normalization of the $\delta^{44/42}Ca_{Urine}$ values to an arbitrary BMI value of 22 (i.e. the median of the healthy BMI range). Significant differences can be seen in the data distribution before and after BMI-normalization. (i) Participants 16, 17, and 9, who stood out because of their elevated 'measured' $\delta^{44/42}Ca_{Urine}$ values, plot together within the range defined by most participants after BMI-normalization. (ii) The data from Participant 5 (BMI~31), which used to plot at the low end of the region defined by most participants, clearly stand out after BMI-normalization, plotting ~1‰ below most other participants. (iii) Participants 4 (25 years old) and 12 (60 years old), which despite being 35 years apart had data that directly overlapped at the lowest $\delta^{44/42}Ca_{Urine}$ values among all participants, now display average $\delta^{44/42}Ca_{Urine}$ values nearly 0.5‰ apart. The shift is entirely due to the low BMI of Participant 4, whose data were shifted up into the region defined by most participants as a result of the BMI-normalization. (iv) Finally, Participant 20, whose data did not stand out before BMI-normalization, is now showing very low $\delta^{44/42}Ca_{Urine}$ values, even overlapping with Participant 12. At present, we cannot say whether the low $\delta^{44/42}Ca_{Urine}$ values for Participant 20 testify to a net bone resorption or the impact of high-dairy consumption by this participant.

Time since last urination

As shown by the data from Participant 1 (Fig. 5), the Ca isotope composition of an individual urine sample depends on the time since last urination. Urine samples collected soon (<6 h) after the previous urination cover a range of $\delta^{44/42}$Ca values ~50% greater than samples collected a long time (>8 h) after the last urination. This increased variability in $\delta^{44/42}Ca_{Urine}$ is accompanied by an increased variability in Ca concentration and Ca/K ratio, and for the most part reflects the impact of Ca reabsorption in the kidney (Fig. 7d). Indeed, samples collected shortly after a previous sample are more likely to capture the short-term fluctuations in Ca homeostasis in the body (higher and lower levels of Ca excretion), and by extension, fluctuations in Ca isotope composition. Practically, these results imply that a urine sample will be most representative of the BMB if taken a long time after the last urination (e.g. morning urine), and not directly after an activity likely to influence Ca excretion (eating, physical exercise).

Fasting

One sample from Participant 1 was collected more than 15 h after last food consumption and is characterized by a low Ca concentration (and Ca/K ratio) as well as an elevated $\delta^{44/42}Ca_{Urine}$ value (+1.04 ± 0.06‰) (Fig. 4). To better understand this result, in Fig. 14, the isotopic composition and Ca content of all individual urines from Participant 1 are plotted as a function of the time since last food. Samples collected more than 12 h after eating tend to have low [Ca] and Ca/K ratios, testifying to a high level of Ca reabsorption into the body. Isotopically, however, only the sample taken 15 h after the last food shows a clear departure from its corresponding 24-h pooled fraction (i.e. the daily averaged Ca isotope value). In a Ca isotope vs Ca excretion plot, this sample plots above the general trend corresponding to the normal kidney regulation (Fig. 7b and d), suggesting that the elevated $\delta^{44/42}Ca_{Urine}$ value reflects a specific regime of Ca regulation triggered by prolonged fasting.

Alcohol consumption

Data from Participant 1 show an increase in $\delta^{44/42}Ca_{Urine}$ values in the morning urine following alcohol consumption (Figs 4 and 6). In Fig. 15, the data from all participants are shown as a function of the typical number of units of alcohol consumed per week. In line with the data from Participant 1, the few participants that reported consuming more than 6 units of alcohol per week show elevated $\delta^{44/42}Ca_{Urine}$ values relative to the other participants. This includes Participant 17, whose data displayed a much steeper $\delta^{44/42}Ca_{Urine}$ vs Ca/K slope than other participants (Fig. 10). Alcohol is known to lead to both increased urine flow and increased Ca loss in urine [66,67]. Since data from Participant 1 show no direct impact on the $\delta^{44/42}$Ca value of the urination directly following alcohol consumption (e.g. evening urine on day 9), but elevated $\delta^{44/42}Ca_{Urine}$ values in the morning urine following alcohol consumption (e.g. morning urine day 10), we hypothesize that the latter might reflect the body's response to depletion of Ca stores and higher retention of Ca during kidney function. More specifically, we propose the following succession in time. (i) Alcohol consumption triggers (ii) increased urine flow with typical Ca retention (~98%) by the kidney, leading to excess urinary Ca excretion without modification of the $\delta^{44/42}Ca_{Urine}$.

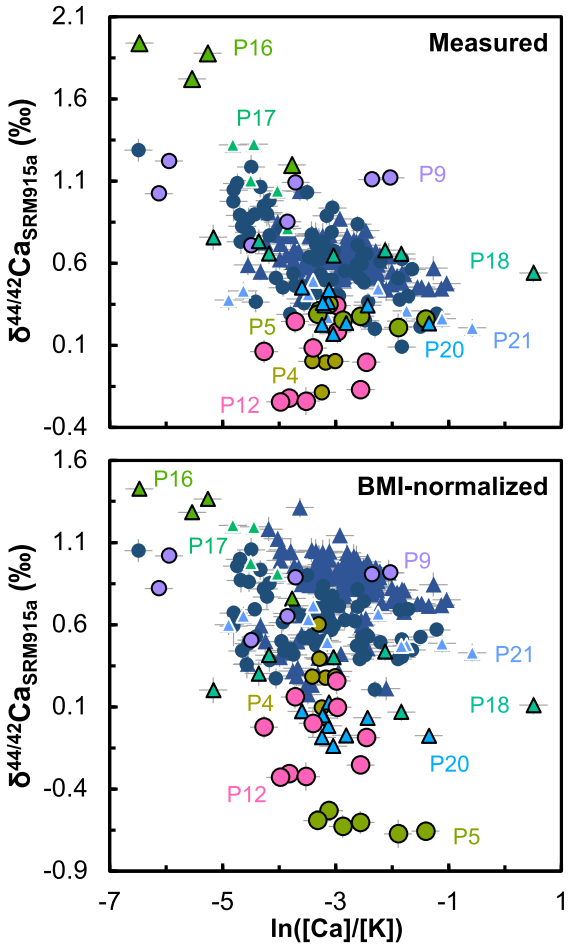

Figure 13. $\delta^{44/42}$Ca values in urine samples from all participants plotted against Ca/K ratios both before (top panel) and after (bottom panel) BMI-normalization (to an arbitrary value of 22). Symbols as in Fig. 10. Brightly colored symbols denote participants whose data deviate from the general trend defined by most participants. See text for details.

This increased Ca excretion in turn leads to (iii) increased renal Ca reabsorption, inducing an increase in $\delta^{44/42}Ca_{Urine}$ in the next urination (e.g. the morning urine on day 10 and 16 for Participant 1; Fig. 4).

Diet

The diet is the source of Ca to the body and fluctuations in its isotopic composition will directly impact the blood/urine composition. In healthy adults, for whom bone mass is essentially at steady state, changes in the Ca isotopic composition of the diet should be rapidly reflected in the urine $\delta^{44/42}$Ca values. Our data provide at least two clear lines of evidence supporting this expectation. (i) The first comes from the 30-day monitoring of Participant 1, and specifically day 14. On that day, the 24-h pooled fraction shows a $\delta^{44/42}Ca_{Urine}$ value ~0.2–0.3‰ lower compared to any other day in a 2-week period. The participant reported consuming excessive food and non-alcoholic drinks, and the 24-h value appears to be dominated by the evening urination (first post-dinner urination). (ii) The second comes from Participant 10, who provided a 24-h sample, which was measured multiple times and can be used to anchor her $\delta^{44/42}Ca_{Urine}$ to the value of +0.58 ± 0.03‰. Two months later, Participant 10 also collected a sample after 5 days of vegan diet, which displays an elevated

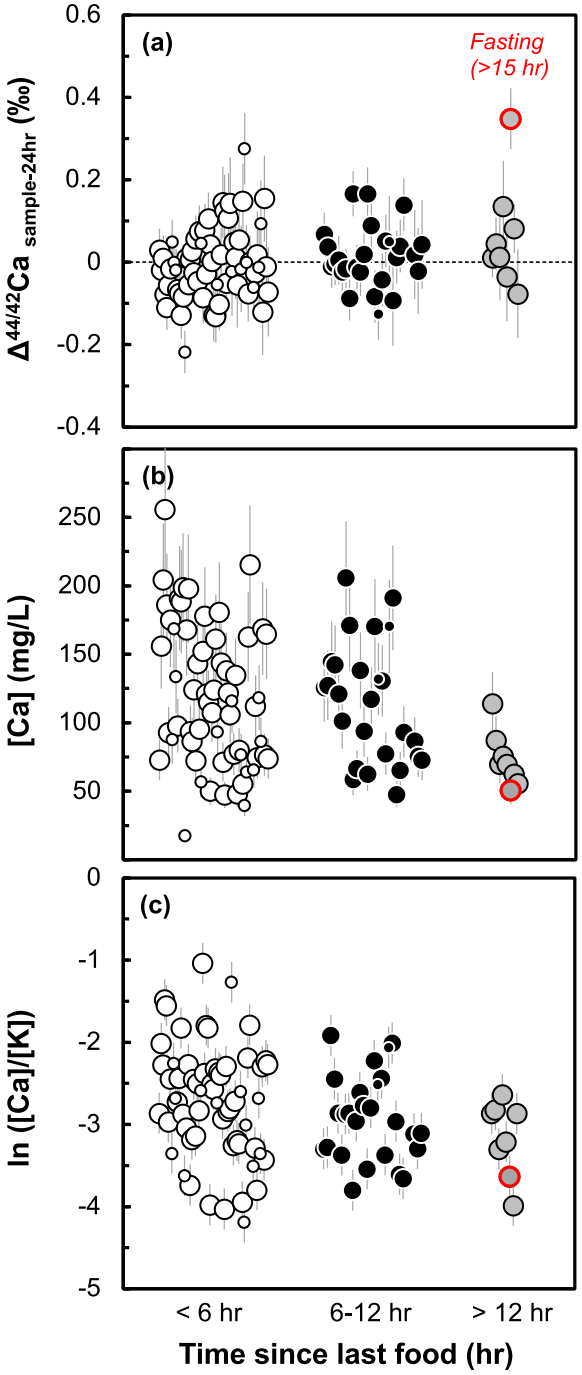

Figure 14. Urine Ca isotope composition (a), concentration (b) and Ca/K ratio (c) for individual urine samples from Participant 1 plotted against the time since last food (in hours). Panel (a) shows the difference between a given sample and its corresponding 24-h pooled fraction to highlight departures from the daily average value. Samples collected less than 4 h since the last urination (and thus more likely to be affected by short term fluctuations in kidney function) are shown as smaller symbols. Samples collected more than 12 h after eating tend to have low [Ca] and Ca/K ratios. The one sample collected more than 15 h after eating (labeled *Fasting*) displays both low Ca content and a significantly elevated $\delta^{44/42}$Ca value.

$\delta^{44/42}Ca_{Urine}$ value of +1.04 ± 0.04‰. This increase in $\delta^{44/42}$Ca values is consistent with the absence of dairy products, which are isotopically light, from the participant's diet. In fact, the magnitude of the shift seen in Participant 10's $\delta^{44/42}Ca_{Urine}$ value (+0.46‰), is in excellent agreement with (i) the ~0.40‰ increase in food

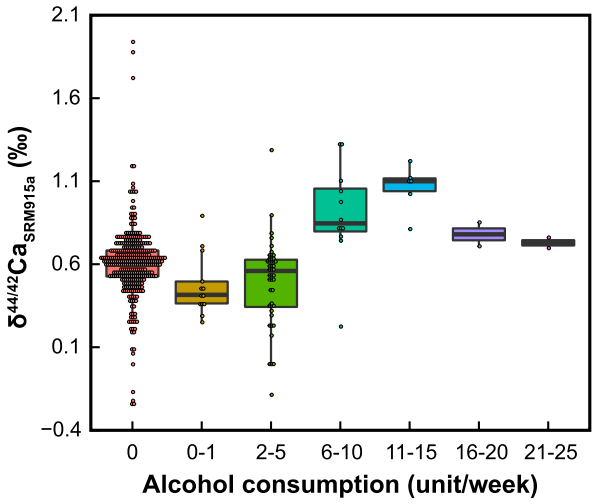

Figure 15. Boxplot showing the $\delta^{44/42}Ca_{Urine}$ values from all 22 participants as a function of units of alcohol consumed per week. To assess this number, participants were provided the following guideline: "For reference, a pint of beer = 3 units, a regular glass of wine = 2 units, 1.5 fl oz of spirits = 1 unit." Participants that reported consuming >6 units of alcohol per week show elevated $\delta^{44/42}Ca_{Urine}$ values relative to other participants.

$\delta^{44/42}$Ca expected for someone switching from a typical European diet to a vegan diet [13], as well as (ii) previously reported effect of dairy consumption on bodily Ca isotope compositions [63]. Some additional support can be found also in the data from participants that reported consuming dairy products 5–7 days a week, as several of them display low $\delta^{44/42}Ca_{Urine}$ values and/or $\delta^{44/42}Ca_{Urine}$ vs Ca/K relationship with shallower slopes than other participants (Participants 18, 20, and 21).

Physical exercise and sweating

While the data from all participants did not reveal a systematic difference between those who exercised regularly and those who did not, the impact of physical exertion and/or sweating is clearly visible in one sample from Participant 1 (labeled *workout* in Figs 4, 5, and 7). Collected shortly after the previous urine sample and immediately after a 1.5-h intense workout (squash game) during which the participant only hydrated with water, this sample displays an anomalously low Ca concentration and isotopic composition. The position of this sample in the Ca isotope vs Ca excretion space (Fig. 7b and d) cannot be explained by normal kidney regulation function. Considering the very limited time interval captured by the *workout* sample (2.5 h), and the fact that the participant underwent essentially one singular activity during that time, we hypothesize that the low Ca content and isotopic composition of that sample is due to loss of the complementary, isotopically heavy Ca via perspiration. This hypothesis builds on the observation that perspiration during exercise can lead to significant dermal calcium loss, on the order of 60–120 mg of Ca per hour [68,69]. Future experiments, ideally collecting both urine and sweat, would be needed to confirm this hypothesis.

Nonresolvable impact of other parameters

The controlling factors discussed above are but a subset of the information provided by the participants. While we systematically investigated all parameters, our data did not reveal any significant link between the Ca isotope composition of urine and the following: gender, race, ethnicity, consumption of multivitamin, punctual consumption of antacid tablets, daily water consumption, water sources, or smoking. This is not to say that some of these parameters have no impact on the Ca isotope composition of humans. For instance, smoking has been shown to cause imbalances in the mechanisms of bone turnover resulting, both directly (e.g. decrease in osteogenesis) and indirectly (e.g. suppression of appetite, increase in free radicals promoting bone resorption), in lower bone mass and bone mineral density [70]. Instead, we simply state that, given the still limited size of the population studied here, our data do not allow us to resolve the impact of these potential factors on the participants $\delta^{44/42}Ca_{Urine}$ values.

Implications for Ca isotopes as a medical tool

The promise of Ca isotopes as a potential biomarker is that of a noninvasive, natural tracer, which would provide from a single urine sample, a quantitative assessment of the global BMB of an individual. As discussed in the introduction, in such a framework, several key assumptions are being made, including (i) that individuals of similar age have a similar blood/serum $\delta^{44/42}$Ca value, and by extension, similar $\delta^{44/42}Ca_{Urine}$ value (assuming healthy kidney function) or (ii) that for a given individual, $\delta^{44/42}Ca_{Blood/Serum}$ is constant over short timescales (hours to months), and changes in $\delta^{44/42}Ca_{Urine}$ values are thus only driven by kidney functions and are independent of other potential drivers such as diet, demographic, or lifestyle. The results presented above provide some new insights into the validity of these assumptions, and below, we summarize them and present some practical recommendations for the use of Ca isotopes as a biomarker of BMB.

(i) Large inter-individual variability in $\delta^{44/42}Ca_{Urine}$ might be a severe hindrance to cross-sectional approaches of BMB monitoring with Ca isotopes. In line with the results on a large population reported in the OsteoLabs study [15], the range of natural variations in the $\delta^{44/42}Ca_{Urine}$ values of the 22 participants studied here spans nearly 2.2‰. This is particularly large considering that all 22 participants have lived for the last few months to years in the same general area, and therefore, geographic differences are not represented in our dataset. While our data show that some of the variability coming from kidney regulation and differences in BMI can be accounted for by, respectively, combining Ca excretion and Ca isotopes (Fig. 10), and normalizing to an arbitrary BMI value (Fig. 13), our current understanding of the causes of isotopic difference between individuals remains too limited to make cross-sectional approaches reliable at this point ('Age' section).

(ii) Limited intra-individual variability in $\delta^{44/42}Ca_{Urine}$ suggests that self-referencing approaches for BMB monitoring hold greater promise than cross-sectional ones. While the differences in $\delta^{44/42}Ca_{Urine}$ between participants span a 2.2‰ range, most participants display values stable within ±0.2–0.3‰ over timescales of 160 days, with Participant 1 extending this range to nearly 1 year (Figs 8 and 9). Independent results from the OsteoLabs study [15] show similar intra-individual stability in 63 individuals over periods of up to 2 years. Stability over such timescales is a key assumption of BMB monitoring using Ca isotopes in urine, which these data thus seem to validate. As such, monitoring of the at-risk population would most efficiently be done by following a 'baseline' approach [12], for instance, through the early establishment of a healthy reference value for each subject (ideally around the time of peak bone mass,

30–40 years old), followed by long-term episodic monitoring (every 6 months to a year) to detect early onset of changes in bone mass.

(iii) Morning urine is an excellent proxy for 24-h pooled urines. As shown by the data from Participant 1 (Figs 4 and 6), morning urines and 24-h pooled urines have nearly invariably identical Ca isotope compositions (within error). In contrast, samples taken during the day show 50% more variability in their $\delta^{44/42}$Ca values (Fig. 5), being more subject to rapid changes linked with diet and kidney function, as well as other processes like physical exercise. We therefore recommend that urine samples intended for Ca isotope analysis be collected in the morning, before the first food of the day, as these samples will give more robust/representative values. If morning urine cannot be obtained, a sample taken more than 4–6 h after the last urination is highly preferable.

(iv) Diet, lifestyle, and other medical procedures can influence $\delta^{44/42}Ca_{Urine}$. Our data show that changes in diet, even if punctual, can have a significant impact on urine Ca isotope composition. This includes consumption of alcohol or dairy products, or more drastic changes (e.g. from omnivorous to vegan) as well as intermittent/prolonged fasting. Similarly, the introduction in the human body of external tracers (such as an MRI contrast agent), can affect the analysis of Ca isotopes (Fig. 3). Values most representative of the long-term Ca homeostasis, and by extension, BMB of an individual will therefore be obtained on samples collected not directly after sudden changes in diet, consumption of alcohol, intake of unusual amounts of food (higher or lower), or other medical procedures.

Current limitations and open questions

Despite the large analytical undertaking that this study represents, the population size remains limited ($n = 22$). Some of the hypotheses presented in the study, such as the impact of alcohol or dairy consumption, or the potential loss of heavy Ca isotopes through perspiration, will need to be investigated more systematically in future studies for a statistically significant assessment to be done. Our data analysis also relies on the ability of the study participants to accurately fill in the questionnaire accompanying each sample submission. This is a potential concern for some categorical questions, such as the amount of water/dairy/meat/alcohol consumed per day/week, where each participant brings their own biases toward such self-assessments.

This study was limited to the investigation of Ca isotopes in urine samples, which are strongly influenced by the regulatory function of the kidney. While this approach allowed us to place novel constraints on the magnitude and timescales of Ca isotope variations in urine, additional insights into BMB would have undoubtedly been obtained by also studying blood samples from each participant. Most importantly, this would have allowed to clearly deconvolve the impact of kidney regulation from other drivers of Ca isotope variations.

Concluding remarks

The last two decades have seen an exciting expansion of *isotope metallomics*, a field which aims to leverage the natural isotopic variations of metabolically critical metals (e.g. Mg, Ca, Cu, Fe, Zn) to study the function (and dysfunction) of the human body. Of all the bio-relevant metals studied to date, Ca isotopes have shown what may arguably be the greatest potential to becoming a diagnostic tool, specifically of BMB. To fully realize this potential requires not only the study of the impact of diseases and conditions affecting the Ca homeostasis, but also establishment of a robust benchmark from healthy subjects, which has received much less attention.

Here, we thus investigated the Ca isotope composition of urine samples ($n = 300$) from 10 male and 12 female participants (age 19–60), over timescales ranging from days to months. Our data reveal large inter-individual $\delta^{44/42}Ca_{Urine}$ variability (up to 2.2‰). While some of this variability seems to stem from differences in anthropometric measurements between participants (BMI), our results overall bode unfavorably for cross-sectional approaches of BMB monitoring using Ca isotopes. In contrast, most participants displayed encouraging intra-individual $\delta^{44/42}Ca_{Urine}$ stability (within ~±0.2–0.3‰) over timescales >160 days, suggesting that self-reference approaches to BMB monitoring using Ca isotopes might be more effective.

At this writing, high-precision Ca isotope analyses remain a challenging task necessitating state-of-the-art specialty instruments (MC-ICPMS), and highly skilled operators. But as the automation of sample processing (e.g. prepFAST MC) and measurement continues, the barrier to performing high-precision Ca isotope analysis gets lower. As such, it is not unreasonable to imagine that Ca isotopes could become a routine test performed on annual (or more frequently collected) urine samples. Our data provide some important insights into how such monitoring could work. Since we found that morning urine is an excellent proxy for the 24-h pooled urine fraction, the patient to monitor would ideally collect a sample of morning urine (first urination of the day, pre-breakfast) to be sent for Ca isotope analysis. The sample would be taken on a day representative of the typical dietary, behavioral and physiological states of the patient, so as to avoid complications arising from other drivers of $\delta^{44/42}Ca_{Urine}$ variability, such as alcohol consumption, excessive food intake, intense physical activity, fasting, or injection of non-natural agents into the body (e.g. gadolinium during an MRI). Under such conditions, a significant drop (the magnitude of which remains to be established by future studies) in $\delta^{44/42}Ca_{Urine}$ from the established benchmark value of the patient could signal a potential disturbance of the patient's BMB, and a need for a more sustained medical examination.

As more studies continue to refine our understanding of the Ca isotope homeostasis of the human body, the exact modalities of such an approach to health monitoring will become clearer. These include studies of larger populations, both healthy and diseased, as well as more targeted studies aimed at assessing the impact of specific potential confounding factors (e.g. smoking), or unraveling the mechanistic drivers behind Ca isotope fractionation during bone formation, urine formation, and other important metabolic function affecting Ca.

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Supplementary data

Supplementary data is available at *Metallomics* online.

Conflict 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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