

# Rapidly assessing changes in bone mineral balance using natural stable calcium isotopes

Jennifer L. L. Morgan<sup>a,c</sup>, Joseph L. Skulan<sup>b,1</sup>, Gwyneth W. Gordon<sup>b</sup>, Stephen J. Romaniello<sup>b</sup>, Scott M. Smith<sup>c</sup>, and Ariel D. Anbar<sup>a,b</sup>

<sup>a</sup>Department of Chemistry and Biochemistry and <sup>b</sup>School of Earth and Space Exploration, Arizona State University, Tempe, AZ 85287; and <sup>c</sup>Human Adaptation and Countermeasures Division, National Aeronautics and Space Administration, Houston, TX 77058

Edited\* by Donald J. DePaolo, University of California, Berkeley, CA, and approved April 27, 2012 (received for review December 5, 2011)

The ability to rapidly detect changes in bone mineral balance (BMB) would be of great value in the early diagnosis and evaluation of therapies for metabolic bone diseases such as osteoporosis and some cancers. However, measurements of BMB are hampered by difficulties with using biochemical markers to quantify the relative rates of bone resorption and formation and the need to wait months to years for altered BMB to produce changes in bone mineral density large enough to resolve by X-ray densitometry. We show here that, in humans, the natural abundances of Ca isotopes in urine change rapidly in response to changes in BMB. In a bed rest experiment, use of high-precision isotope ratio MS allowed the onset of bone loss to be detected in Ca isotope data after about 1 wk, long before bone mineral density has changed enough to be detectable with densitometry. The physiological basis of the relationship between Ca isotopes and BMB is sufficiently understood to allow quantitative translation of changes in Ca isotope abundances to changes in bone mineral density using a simple model. The rate of change of bone mineral density inferred from Ca isotopes is consistent with the rate observed by densitometry in long-term bed rest studies. Ca isotopic analysis provides a powerful way to monitor bone loss, potentially making it possible to diagnose metabolic bone disease and track the impact of treatments more effectively than is currently possible.

osteopenia | biomarker | medical geology | biosignature | spaceflight

In humans and most other vertebrates, bone is continuously replaced through remodeling, resulting from the coupled actions of bone-forming cells (osteoblasts) and bone-resorbing cells (osteoclasts). In healthy adults, the rates of bone formation and resorption are equal, and therefore, net bone mineral balance (BMB) is near zero. Disruptions in BMB caused by diseases such as osteoporosis, multiple myeloma, and some metastatic cancers can have serious or even fatal consequences. Sensitive methods to directly measure BMB could benefit research and clinical practice in the study and treatment of bone disease.

The abundances of the naturally occurring isotopes of Ca in urine and soft tissue (blood and nonbone tissues) should be sensitive to changes in BMB. A large body of research establishes that these six isotopes (<sup>40</sup>Ca, <sup>42</sup>Ca, <sup>43</sup>Ca, <sup>44</sup>Ca, <sup>46</sup>Ca, and <sup>48</sup>Ca) react at different rates depending on atomic mass and that these differences fractionate the isotopes, producing variations in Ca isotope abundances in nature (1–3). In the body, changes in BMB should lead to variations in the abundances of Ca isotopes for two related reasons, one tied directly to bone metabolism and the other indirectly by the excretion of Ca through the kidneys. Both processes should shift Ca isotope abundances in urine in the same direction in response to changes in BMB.

First, in vertebrates, bone formation favors the lighter isotopes of Ca (4, 5), thus depleting soft tissue of light Ca isotopes. Bone resorption releases this isotopically light Ca back into soft tissue with little or no isotope selectivity. As a result of this difference in isotope fractionation, the Ca isotope abundances in soft tissue should shift to heavier Ca isotope values when BMB is positive (bone formation exceeds resorption) and lighter values when

BMB is negative (bone resorption exceeds formation) (4). Urinary Ca is derived from soft tissue. Therefore, shifts in soft tissue Ca isotopes should be reflected as correlated shifts in the Ca isotope composition of urine.

Second, Ca isotopes are also fractionated during excretion of Ca in the kidneys. Comparison of Ca in urine and soft tissue indicates that heavy isotopes are preferentially excreted (6). Although the mechanism of this renal Ca isotope effect is not well understood, Ca isotope abundances in soft tissue should shift to heavier Ca isotope values when BMB is positive and Ca excretion rate decreases and lighter values when BMB is negative and Ca excretion rate increases (see *Discussion*). As with the bone metabolism effect, such shifts in the Ca isotope composition of soft tissue should be reflected in the Ca isotope composition of urine. Consistent with this expectation, a significant inverse correlation has been observed between the Ca concentration of urine and its isotopic composition (7).

Two independent bed rest studies have confirmed that Ca isotope abundances in urine shift to lighter values when BMB is negative (7, 8), which was expected from the two processes described above. Bed rest induces bone loss because of skeletal unloading, and it is used to model the effects of space flight on human bone metabolism. In samples from four subjects enrolled in a 17-wk bed rest study, variations in Ca isotope abundances in urine were consistent with results from established collagen cross-link assays and X-ray densitometry indicating net bone resorption (8). In preliminary data from a 35-d bed rest study (7), similar variations in Ca isotope abundances in urine were observed in samples from seven subjects.

We report here a more detailed evaluation of Ca isotope variations from a 30-d bed rest study. This study was designed to assess how rapidly the Ca isotope signal appears and evaluate its potential as a quantitative measure of bone loss. The study included 12 participants monitored for 12 d before bed rest, 30 d during bed rest, and 7 d after bed rest. A 10-d diet rotation was controlled and consistent for all participants during the duration of the study. Urine samples were collected at time points throughout the study to examine short-term variations in Ca isotope abundances either from analyses of 24-h pooled samples or in some cases, on all individual voids throughout the day.

Author contributions: J.L.S., G.W.G., S.M.S., and A.D.A. designed research; J.L.L.M. and G.W.G. performed research; J.L.L.M. contributed new reagents/analytic tools; J.L.L.M., J.L.S., G.W.G., S.J.R., S.M.S., and A.D.A. analyzed data; and J.L.L.M., J.L.S., G.W.G., S.J.R., S.M.S., and A.D.A. wrote the paper.

Conflict of interest statement: Donald DePaolo served on the PhD dissertation committee for J.L.S. from 1997 to 2000, and J.L.S. and Donald DePaolo coauthored two papers on calcium isotopes (1997 and 1999). Some of the techniques used in this research are covered under patent claims being pursued by Arizona Technology Enterprises (AzTE), the technology transfer office of Arizona State University.

\*This Direct Submission article had a prearranged editor.

Freely available online through the PNAS open access option.

<sup>1</sup>To whom correspondence should be addressed. E-mail: jlskulan@geology.wisc.edu.

This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1119587109/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1119587109/-DCSupplemental).







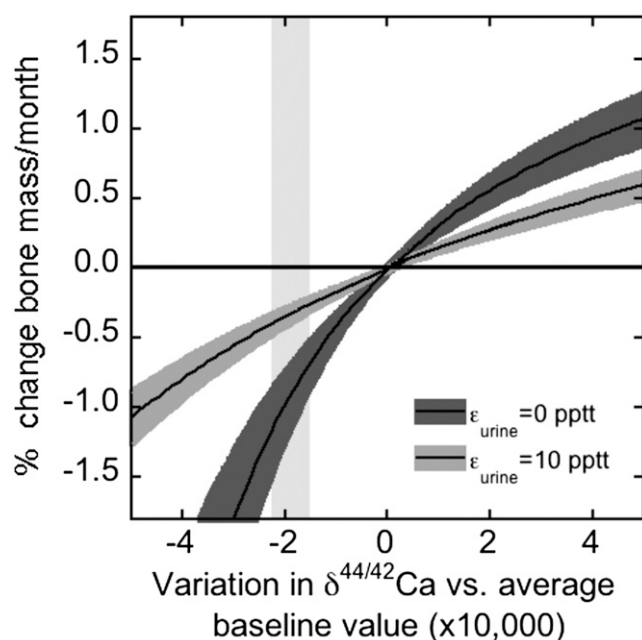
isotope fractionation factor ( $\epsilon^{44/42}\text{Ca}_{\text{urine}}$ ), the mass of skeletal Ca ( $M_{\text{bone}}$ ), the background rate of skeletal remodeling ( $F_{\text{bone}}$ ), and the rate of intestinal absorption of dietary Ca ( $F_{\text{diet}}$ ).

- $\epsilon^{44/42}\text{Ca}_{\text{bone}}$ . We estimated the fractionation factor of bone formation to be  $7.5 \pm 1$  pptt (1 SD) and based the estimate on the offset between bone and diet in terrestrial vertebrates (4, 5). This value seems to be similar among a phylogenetically wide range of vertebrates (4, 5) and is unlikely to vary between individuals.
- $\epsilon^{44/42}\text{Ca}_{\text{urine}}$ . Renal Ca isotope fractionation is an important but poorly constrained term (6). Soft tissue tend to have a Ca isotopic composition similar to the composition of diet, whereas urine tends to be isotopically heavy (4, 6, 8). For example,  $\delta^{44/42}\text{Ca}$  of all urine samples from a 17-wk bed rest study were higher than the dietary mean by an average of 9 pptt (8). In data from an animal model,  $\delta^{44/42}\text{Ca}$  of urine collected from a sow was 11.4 pptt higher than  $\delta^{44/42}\text{Ca}$  of simultaneously collected serum (Table S2). These values are nearly identical to the values estimated or measured in the work by Heuser and Eisenhauer (6) (9.6 pptt for humans and 10 pptt for sows). Together, these data indicate that the renal fractionation is almost certainly positive and that  $\epsilon^{44/42}\text{Ca}_{\text{urine}} \sim 10$  pptt.
- $M_{\text{bone}}$ . Assuming an average skeletal mineral mass of 3,000 g (29) and a Ca content in bone mineral of 32.2% (30), we estimate that  $\sim 1,000 \pm 100$  g Ca is stored in the average human skeleton.
- $F_{\text{bone}}$ . We estimated the background rate of skeletal remodeling to be  $500 \pm 100$  mg Ca/d based on two studies. In the

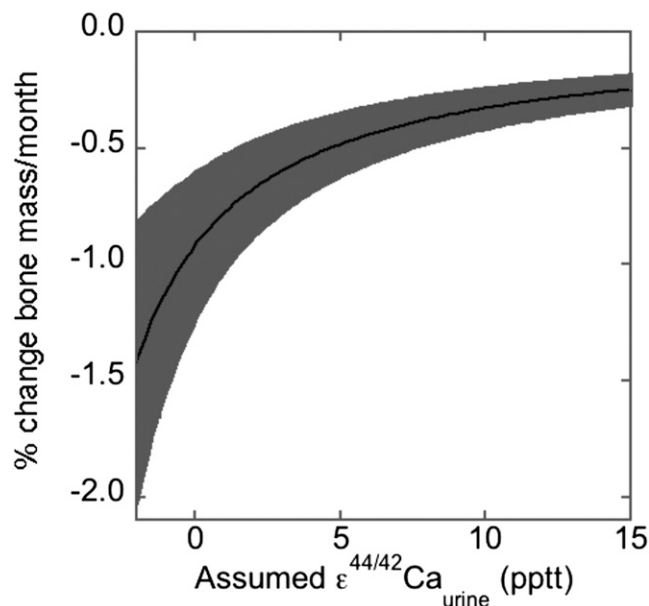
first study, Smith et al. (21) estimated rates of new bone formation of 190–635 mg Ca/d for male and female volunteers. In a follow-up study, the work by Smith et al. (22) estimated the rate of bone formation of 16 astronauts at  $490 \pm 153$  mg Ca/d before spaceflight and  $434 \pm 194$  mg Ca/d during spaceflight.

- $F_{\text{diet}}$ . The average measured dietary Ca intake during bed rest for all subjects in this study was  $1,450 \pm 170$  mg Ca/d. Accounting for the bioavailability of foods in the typical diet (31), we estimated that  $390 \pm 96$  mg Ca/d was absorbed in the intestines.

The model-derived relationships between the  $\delta^{44/42}\text{Ca}$  shift of urine during bed rest and BMB based on these parameters are shown in Fig. 3. The relationships permit a straightforward extrapolation of the rate of bone loss from a measured shift in  $\delta^{44/42}\text{Ca}$ . Because the exact value of  $\epsilon^{44/42}\text{Ca}_{\text{urine}}$  (the renal fractionation) has not yet been directly measured in humans, curves for  $\epsilon^{44/42}\text{Ca}_{\text{urine}} = 0$  and  $\epsilon^{44/42}\text{Ca}_{\text{urine}} = 10$  pptt are shown to give a sense of the parameter uncertainty. It is apparent that, as renal fractionation increases, a given shift in urinary  $\delta^{44/42}\text{Ca}$  corresponds to a smaller change in BMB. Hence, the net effect of renal fractionation is to amplify variations in urinary  $\delta^{44/42}\text{Ca}$  in response to changing BMB. This finding increases the sensitivity of the  $\delta^{44/42}\text{Ca}$  technique for detecting variations in BMB. This increase in sensitivity is greatest as renal fractionation rises from 0 to about 5 pptt. Greater increases add relatively little additional sensitivity, and therefore, uncertainty in the value of  $\epsilon^{44/42}\text{Ca}_{\text{urine}}$  adds minimal uncertainty to the calculation of BMB as long as renal fractionation in humans is above about 5 pptt (which is likely) (Fig. 4).



**Fig. 3.** Estimating changes in BMB from variations in  $\delta^{44/42}\text{Ca}$  using the Ca isotope model. The curved fields indicate predicted percentage bone loss per month for subjects as a function of the shift in  $\delta^{44/42}\text{Ca}$  of urine from the baseline value. Predictions are shown for two different values of renal fractionation factor ( $\epsilon^{44/42}\text{Ca}_{\text{urine}}$ ; abbreviated here as  $\epsilon_{\text{urine}}$ ): 0 and 10 pptt. The width of the shaded fields indicates the combined  $1\sigma$  uncertainty of the Ca isotope model. The gray vertical box indicates the observed mean baseline-normalized difference in abundance of  $\delta^{44/42}\text{Ca}$  during bed rest for all subjects in this study. The width of this box indicates 2 SE widths about the mean. The conversion from milligrams Ca per day to percentage bone loss is  $(\text{mg Ca/d}) \times (30 \text{ d/mo}) \times (1 \text{ g/1,000 mg}) / (1,000 \text{ g Ca/person}) \times 100\%$ .



**Fig. 4.** Sensitivity of the Ca isotope model to the value assumed for the renal fractionation factor ( $\epsilon^{44/42}\text{Ca}_{\text{urine}}$ ) for the conditions of our study. The black line represents the model output assuming the following: a Ca isotope shift during bed rest of  $-2.0 \pm 0.4$  pptt, a value for the renal fractionation given by the x axis, and all other values and uncertainties as given in the text. The gray shaded area represents the model uncertainty determined by formal propagation of the uncertainty in the model parameters and Ca isotope measurements using the partial derivatives of all model equations with respect to the input variables. Estimates of the Ca isotopic offset between urine and blood in humans (6) and measurements of this offset in sows reported here (Table S2) indicate that the most likely value for human renal fractionation is about 10 pptt. As long as this value is  $>5$  pptt, the exact value of this parameter does not significantly alter our estimates of bone loss during bed rest.

Using this model, we estimate that subjects lost on average  $0.25 \pm 0.07\%$  (1 SD) of their bone mass from days 7 to 30 of bed rest ( $\sim 109$  mg Ca/d), assuming a renal isotopic fractionation of 10 pptt. The estimated error includes all measurement and model uncertainty, except for the uncertainty in renal isotopic fractionation. This rate of bone loss extrapolates to a loss of  $1.0 \pm 0.3\%$  of skeletal mass over 90 d, which is equivalent, within error, to bone loss rates determined by X-ray densitometry scans in long-term bed rest studies (14, 22, 32–34). Thus, the Ca isotope model used here yields quantitative results that are consistent with the best existing measures of changes in BMD.

**Conclusion.** Measurement of naturally occurring Ca isotopes has the potential to reduce the duration of experimental studies of bone metabolism, accelerating the pace of discovery of new treatments for metabolic bone disease. The Ca isotope technique also promises to provide insights into the short-term dynamics of bone metabolism. Research into renal and hepatic Ca isotope fractionation will increase this use by refining our knowledge of the quantitative relationship between Ca isotopes and BMD. In the future, the Ca isotope technique may be useful in clinical settings to allow close monitoring of subjects at risk for bone loss and safe, rapid assessment of individual subjects' response to treatment.

## Materials and Methods

**Experimental Methods.** The bed rest study was conducted at the University of Texas Medical Branch at Galveston's Institute for Translational Sciences—Clinical Research Center. Bed rest conditions were rigorously controlled, including room temperature and participant sleep and wake cycles. Energy intake was adjusted so that each participant maintained a constant weight ( $\pm 3\%$  of weight on bed rest day 3). Apart from this adjustment, participants were fed the same diet consisting of the same meals rotated on a 10-d cycle. Because participants entered the study on different calendar days, the diet cycle does not correspond to different study days. During bed rest, participants were confined to a strict  $-6^\circ$  head-down tilt bed rest. They were monitored to ensure round-the-clock compliance. Details have been reported elsewhere (34–36). In this study, 12 subjects (8 male subjects and 4 female subjects) with an age range of 25–49 y and an average age of  $32 \pm 8$  y were enrolled. All participants were of normal health with body mass indices between 20 and 30. All of the women were premenopausal. Demographic information for each subject is presented in Table S3. Details of participant recruitment and exclusion criteria are given elsewhere (35). Subjects began the  $\sim 54$ -d study at different times throughout a 6-mo period. The Johnson Space Center Committee for the Protection of Human Subjects and the University of Texas Medical Branch Institutional Review Board approved the study protocol. All subjects provided written, informed consent before they were enrolled.

Urine and blood samples were collected from all subjects, and food samples were collected from the 10-d meal rotation. The urine was acidified with trace metal-grade 20%  $\text{HNO}_3$  and shipped to Arizona State University. Digestion, purification, and measurement of selected samples followed the method in the work by Morgan et al. (9). Details of chemical and mass spectrometric methods, and of the isotopic standard (ICP1) used in the present study, are provided in the [Supplemental Information](#).

**Mathematical Model.** We derived a quasi-steady-state solution for the rate of bone loss during bed rest as a function of the shift in the Ca isotopic composition of urine (Fig. 2). In addition to Ca isotope fractionation during bone formation, the model allows for additional isotope fractionation during renal and hepatic (bile) excretion of Ca, which was proposed in the work by Heuser and Eisenhauer (6).

In this model,  $F_{\text{diet}}$  is the flux of calcium absorbed from diet,  $F_{\text{bone}}$  and  $F_{\text{resorp}}$  are the bone formation and resorption fluxes, and  $F_{\text{urine}}$  and  $F_{\text{bile}}$  are the net excretion fluxes of calcium through urine and bile, respectively. For simplicity,  $\delta^{44/42}\text{Ca}$  is abbreviated to  $\delta$  in equations.  $\delta_{\text{soft}}$  and  $\delta_{\text{bone}}$  are the Ca isotopic compositions of the soft tissue and bone pools,  $\delta_{\text{diet}}$  and  $\delta_{\text{urine}}$  are the isotopic compositions of the fluxes of Ca absorbed from diet and excreted in urine, and  $\epsilon_{\text{bone}}$ ,  $\epsilon_{\text{urine}}$ , and  $\epsilon_{\text{bile}}$  are the isotopic fractionations associated with forming bone, urine, and bile from the soft-tissue pool. For example, the isotopic composition of urine is given by Eq. 1:

$$\delta_{\text{urine}} = \delta_{\text{soft}} + \epsilon_{\text{urine}}. \quad [1]$$

We assume that subjects are in a state of long-term equilibrium before bed rest, such that there is no net change in bone mass and the isotopic composition of bone is in equilibrium with the soft tissue pool. The implication is that, before bed rest (as indicated by the superscript  $i$ ), the fluxes and isotopic composition of absorbed and excreted calcium must be equal (Eq. 2):

$$F_{\text{diet}} = F_{\text{urine}}^i + F_{\text{bile}}^i \quad [2]$$

and Eq. 3:

$$\delta_{\text{diet}} = \frac{F_{\text{urine}}^i \delta_{\text{urine}}^i + F_{\text{bile}}^i (\delta_{\text{urine}}^i - \epsilon_{\text{urine}} + \epsilon_{\text{bile}})}{F_{\text{urine}}^i + F_{\text{bile}}^i} = \frac{F_{\text{diet}} \delta_{\text{urine}}^i + F_{\text{bile}}^i (\epsilon_{\text{bile}} - \epsilon_{\text{urine}})}{F_{\text{diet}}} \quad [3]$$

Additionally, the isotopic composition of bone is given by Eq. 4:

$$\delta_{\text{bone}} = \delta_{\text{soft}} + \epsilon_{\text{bone}} = \delta_{\text{urine}}^i - \epsilon_{\text{urine}} + \epsilon_{\text{bone}} \quad [4]$$

When bed rest begins, we allow the rate of bone loss to increase, but we hold the dietary Ca flux and bone formation flux constant. Because the Ca content of soft tissue is tightly regulated, the Ca released from bone resorption during bed rest must be quickly excreted. This excretion happens primarily through the kidneys, where decreased Ca resorption during urine formation results in increased Ca excretion. We assume that the flux of hepatic Ca excretion flux via bile remains constant.

Because of the short residence time of Ca in soft tissue, the soft tissue Ca pool attains a state of quasiequilibrium after several days of bed rest. In this state, the mass and isotopic composition of soft tissue Ca is in equilibrium with the pool of bone Ca, which is slowly adjusting to bed rest on a timescale of years. During this period, we can write the following mass balance for soft tissue (Eq. 5):

$$0 = F_{\text{diet}} - F_{\text{bone}} + F_{\text{resorp}} - F_{\text{bile}} - F_{\text{urine}} \quad [5]$$

and Eq. 6:

$$0 = F_{\text{diet}} \delta_{\text{diet}} - F_{\text{bone}} (\delta_{\text{soft}} + \epsilon_{\text{bone}}) + F_{\text{resorp}} \delta_{\text{bone}} - F_{\text{bile}} (\delta_{\text{soft}} + \epsilon_{\text{bile}}) - F_{\text{urine}} (\delta_{\text{soft}} + \epsilon_{\text{urine}}). \quad [6]$$

When we substitute Eqs. 1–5 into Eq. 6 and solve for  $F_{\text{resorp}}$ , all of the terms involving excretion of Ca in bile cancel, and therefore, we obtain Eq. 7:

$$F_{\text{resorp}} = \frac{F_{\text{bone}} (\epsilon_{\text{bone}} - \epsilon_{\text{urine}}) + F_{\text{diet}} (\delta_{\text{urine}} - \delta_{\text{urine}}^i)}{\epsilon_{\text{bone}} - \epsilon_{\text{urine}} - (\delta_{\text{urine}} - \delta_{\text{urine}}^i)} = \frac{F_{\text{bone}} (\epsilon_{\text{bone}} - \epsilon_{\text{urine}}) + F_{\text{diet}} \Delta^{44/42}\text{Ca}_{\text{baseline}}}{\epsilon_{\text{bone}} - \epsilon_{\text{urine}} - \Delta^{44/42}\text{Ca}_{\text{baseline}}}, \quad [7]$$

where  $\Delta^{44/42}\text{Ca}_{\text{baseline}} = \delta_{\text{urine}} - \delta_{\text{urine}}^i$ . We report the relative rate of bone loss or gain relative to the mass of skeletal Ca,  $M_{\text{bone}}$ , as Eq. 8:

$$\text{BMB} = \frac{F_{\text{bone}} - F_{\text{resorp}}}{M_{\text{bone}}}. \quad [8]$$

We use equation Eq. 7 to assess the sensitivity of our BMB estimate to uncertainties in the renal fractionation over a range of possible values from  $\epsilon_{\text{urine}} = 0$  to +15 pptt, with the most probable values near +10 pptt (Fig. 4). Errors in the estimate of the renal fractionation factor have minimal effect on estimates of BMB as long as this fractionation factor is greater than about 5 pptt.

**ACKNOWLEDGMENTS.** We thank Carina Arrua for help with developing the Ca method and processing samples; Thomas Crenshaw for supplying sow urine and blood samples; Thomas Owens for measuring their Ca isotope composition; Michael Whitaker and Yu-Hui Chang for assistance with statistics; Jane Kraus for editorial assistance; and Michael Anbar for helpful discussions. The studies described here were funded by National Aeronautics and Space Administration Human Research Program Grants 07-HRP-2-0042 and NNX-08Aq38G and specifically, the Human Health and Countermeasures Element and the Flight Analogs Project. Bed rest studies were conducted at the University of Texas Medical Branch at Galveston's Institute for Translational Sciences—Clinical Research Center, and they were supported in part by National Center for Research Resources, National Institutes of Health Grant 1UL1RR029876-01.

1. DePaolo DJ (2004) Calcium isotopic variation produced by biological, kinetic, radiogenic and nucleosynthetic processes. *Geochemistry of Non-Traditional Stable Isotopes*, Reviews in Mineralogy and Geochemistry, eds Johnson CM, Beard BL, Albarede F (The Mineralogical Society of America, Washington DC), Vol 55, pp 255–288.
2. Nielson LC, Druhan JL, Yang W, Brown ST, DePaolo DJ (2011) Calcium isotopes as tracers of biogeochemical processes. *Handbook of Environmental Isotope Geochemistry*, ed Baskaran M (Springer, Berlin), Vol 1, pp 105–124.
3. Russell WA, Papanastassiou DA, Tombrello TA (1978) Ca isotope fractionation on the Earth and other solar system materials. *Geochim Cosmochim Acta* 42:1075–1090.
4. Skulan J, DePaolo DJ (1999) Calcium isotope fractionation between soft and mineralized tissues as a monitor of calcium use in vertebrates. *Proc Natl Acad Sci USA* 96:13709–13713.
5. Reynard LM, Henderson GM, Hedges REM (2010) Calcium isotope ratios in animal and human bone. *Geochim Cosmochim Acta* 74:3735–3750.
6. Heuser A, Eisenhauer A (2010) A pilot study on the use of natural calcium isotope ( $^{44}\text{Ca}/^{40}\text{Ca}$ ) fractionation in urine as a proxy for the human body calcium balance. *Bone* 46:889–896.
7. Heuser A, Frings-Meuthen P, Rittweger J, Galer SJG (2011) Calcium isotopes in human urine under simulated microgravity conditions. *Mineral Mag* 75:1019.
8. Skulan J, et al. (2007) Natural calcium isotopic composition of urine as a marker of bone mineral balance. *Clin Chem* 53:1155–1158.
9. Morgan JLL, et al. (2011) High-precision measurement of variations in calcium isotope ratios in urine by multiple collector inductively coupled plasma mass spectrometry. *Anal Chem* 83:6956–6962.
10. Coplen TB (2011) Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratio measurement results. *Rapid Commun Mass Spectrom* 25:2538–2560.
11. Brage M, et al. (2004) Osteoclastogenesis is decreased by cysteine proteinase inhibitors. *Bone* 34:412–424.
12. Baecker N, et al. (2003) Bone resorption is induced on the second day of bed rest: Results of a controlled crossover trial. *J Appl Physiol* 95:977–982.
13. Manolagas SC (2000) Birth and death of bone cells: Basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. *Endocr Rev* 21:115–137.
14. Shackelford LC, et al. (2004) Resistance exercise as a countermeasure to disuse-induced bone loss. *J Appl Physiol* 97:119–129.
15. Chu N-C, Henderson GM, Belshaw NS, Hedges REM (2006) Establishing the potential of Ca isotopes as proxy for consumption of dairy products. *Appl Geochem* 21:1656–1667.
16. Weaver CM, Proulx WR, Heaney R (1999) Choices for achieving adequate dietary calcium with a vegetarian diet. *Am J Clin Nutr* 70:543S–548S.
17. Weaver CM, Rothwell AP, Wood KV (2006) Measuring calcium absorption and utilization in humans. *Curr Opin Clin Nutr Metab Care* 9:568–574.
18. Wigertz K, et al. (2005) Racial differences in calcium retention in response to dietary salt in adolescent girls. *Am J Clin Nutr* 81:845–850.
19. Bonjour JP (2005) Dietary protein: An essential nutrient for bone health. *J Am Coll Nutr* 24(Suppl 6):S26S–S36S.
20. Heaney RP, Dowell MS, Hale CA, Bendich A (2003) Calcium absorption varies within the reference range for serum 25-hydroxyvitamin D. *J Am Coll Nutr* 22:142–146.
21. Smith SM, et al. (1996) Calcium kinetics with microgram stable isotope doses and saliva sampling. *J Mass Spectrom* 31:1265–1270.
22. Smith SM, et al. (2005) Bone markers, calcium metabolism, and calcium kinetics during extended-duration space flight on the mir space station. *J Bone Miner Res* 20:208–218.
23. Leeming DJ, et al. (2006) An update on biomarkers of bone turnover and their utility in biomedical research and clinical practice. *Eur J Clin Pharmacol* 62:781–792.
24. Sorensen MG, Henriksen K, Schaller S, Karsdal MA (2007) Biochemical markers in preclinical models of osteoporosis. *Biomarkers* 12:266–286.
25. Schafer AL, Vittinghoff E, Ramachandran R, Mahmoudi N, Bauer DC (2010) Laboratory reproducibility of biochemical markers of bone turnover in clinical practice. *Osteoporos Int* 21:439–445.
26. Wieser ME, Buhl D, Bouman C, Schwieters J (2004) High precision calcium isotope ratio measurements using a magnetic sector multiple collector inductively coupled plasma mass spectrometer. *J Anal At Spectrom* 19:844–851.
27. Watts NB (2004) Fundamentals and pitfalls of bone densitometry using dual-energy X-ray absorptiometry (DXA). *Osteoporos Int* 15:847–854.
28. Baim S, et al. (2005) Precision assessment and radiation safety for dual-energy X-ray absorptiometry: Position paper of the International Society for Clinical Densitometry. *J Clin Densitom* 8:371–378.
29. Mazess RB, Peppler WW, Gibbons M (1984) Total body composition by dual-photon ( $^{153}\text{Gd}$ ) absorptiometry. *Am J Clin Nutr* 40:834–839.
30. Martin AD, Bailey DA, McKay HA, Whiting S (1997) Bone mineral and calcium accretion during puberty. *Am J Clin Nutr* 66:611–615.
31. Guéguen L, Pointillart A (2000) The bioavailability of dietary calcium. *J Am Coll Nutr* 19(Suppl 2):119S–136S.
32. Smith SM, Uchakin PN, Tobin BW (2002) Space flight nutrition research: Platforms and analogs. *Nutrition* 18:926–929.
33. Whedon GD, Rambaut PC (2006) Effects of long-duration space flight on calcium metabolism: Review of human studies from Skylab to the present. *Acta Astronaut* 58:59–81.
34. Spector ER, Smith SM, Sibonga JD (2009) Skeletal effects of long-duration head-down bed rest. *Aviat Space Environ Med* 80(Suppl 5):A23–A28.
35. Meck JV, Dreyer SA, Warren LE (2009) Long-duration head-down bed rest: Project overview, vital signs, and fluid balance. *Aviat Space Environ Med* 80(Suppl 5):A1–A8.
36. Zwart SR, et al. (2009) Nutritional status assessment before, during, and after long-duration head-down bed rest. *Aviat Space Environ Med* 80(Suppl 5):A15–A22.

# Supporting Information

Morgan et al. 10.1073/pnas.1119587109

## SI Text

Analytical methods were previously validated (1). Urine samples were dissolved in a microwave digestion system (MARS system; CEM Corporation) using trace metal-grade concentrated  $\text{HNO}_3$  and 30%  $\text{H}_2\text{O}_2$ . Digestion continued in Teflon reaction vessels with alternating rounds of aqua regia and  $\text{HNO}_3/\text{H}_2\text{O}_2$  until high aqueous surface tension indicated that the samples were low in dissolved organics. Two-column purification procedures were performed. First, Biorad AG50-WX-12 200- to 400-mesh resin was used with a hydrobromic acid (HBr) based elution scheme (1) to remove major elements including K, Na, Mg, and Ti. Second, Eichrom Sr-specific resin was used to separate Ca and Sr. Quantitative chemical recovery is required to prevent laboratory-induced isotopic fractionation; yields were measured by quadrupole inductively coupled plasma MS (ICP-MS; X Series; Thermo Scientific), and all were greater than 85%. The process blank consistently contained less than 0.5  $\mu\text{g}$  Ca.

Isotope abundances were measured by multiple-collector ICP-MS (ThermoScientific) in the W. M. Keck Foundation Laboratory for Environmental Biogeochemistry using the method in the work by Wieser et al. (2). Samples were introduced into a 1,200-W plasma with a desolvating system (Apex) in medium-mass resolution. Ca concentrations were between 1.5 and 5 ppm depending on instrumental sensitivity, resulting in beam intensities of 0.5–1.5 V on  $^{42}\text{Ca}$ . Each analysis collected 30 measurement cycles; ratios of the ion beams  $^{44}\text{Ca}/^{42}\text{Ca}$ ,  $^{44}\text{Ca}/^{43}\text{Ca}$ ,  $^{48}\text{Ca}/^{42}\text{Ca}$ , and  $^{48}\text{Ca}/^{43}\text{Ca}$  were calculated and averaged into a single mea-

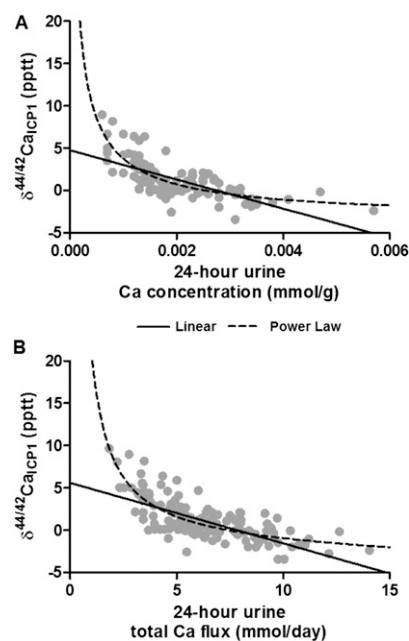
surement. Standard-sample-standard bracketing was used to correct for instrumental mass bias. The bracketing standards used for all samples and external standards were the in-house standard National Institute of Standards and Technology Ca ICP solution (National Institute of Standards and Technology lot no. X-10-39Ca; "ICP1"). For comparison with other literature values, the  $\delta^{44/42}\text{Ca}_{\text{ICP1}}$  values for International Association for the Physical Sciences of the Ocean standard seawater and standard reference material SRM 915a measured relative to ICP1 were  $7.6 \pm 1.5$  pptt and  $-1.5 \pm 1.3$  pptt, respectively. In this manuscript, all Ca isotope measurements are measured and reported relative to ICP1, except where values are reported relative to subjects' pre-bed rest baseline value.

The abundance ratio of  $\delta^{44/42}\text{Ca}$  can be measured using  $<25$   $\mu\text{g}$  Ca with a typical precision of  $\pm 2.0$  ( $\pm 2$  SD) pptt compared with a standard. Measurement accuracy was comparable with the measurement accuracy of other published methods and determined by measuring the Ca isotope ratios on selected urine samples using both multicollector inductively coupled plasma source MS and thermal ionization MS (the traditional instrument for measuring Ca isotopes). Measurement accuracy was within the measurement precision for the two instruments.

Sow urine and blood samples were obtained from Thomas Crenshaw (University of Wisconsin, Madison, WI). These samples were analyzed with thermal ionization MS at the Berkeley Center for Isotope Geochemistry in 1999 using a well-established isotope double-spike technique (3).

1. Morgan JLL, et al. (2011) High-precision measurement of variations in calcium isotope ratios in urine by multiple collector inductively coupled plasma mass spectrometry. *Anal Chem* 83:6956–6962.
2. Wieser ME, Buhl D, Bouman C, Schwieters J (2004) High precision calcium isotope ratio measurements using a magnetic sector multiple collector inductively coupled plasma mass spectrometer. *J Anal At Spectrom* 19:844–851.

3. Skulan J, et al. (2007) Natural calcium isotopic composition of urine as a marker of bone mineral balance. *Clin Chem* 53:1155–1158.



**Fig. S1.** Urinary  $\delta^{44/42}\text{Ca}$  plotted vs. (A) Ca concentration (mmol/g) from 24-h urine pools and (B) total daily Ca flux (mmol/d) per 24-h period. Ca excretion rate (Ca flux) was obtained by multiplying the Ca concentration of the 24-h pool by mass (weight in grams) of urine excreted per 24-h period. The negative correlation strengthens the interpretation that the shift to low  $\delta^{44/42}\text{Ca}$  reflects an influx of isotopically light Ca from bone resorption. Two trend lines are plotted for each graph. (A) Linear regression:  $r^2 = 0.45$ ,  $P < 0.0001$ ; power law:  $r^2 = 0.59$ . (B) Linear regression:  $r^2 = 0.48$ ,  $P < 0.0001$ ; power law:  $r^2 = 0.60$ .





**Table S2. Ca isotope composition of pig feed and urine and serum collected simultaneously from the same pig measured relative to calcium isotope standard 915a,  $\delta^{44/40}\text{Ca}$  (‰)  $\pm$  2 SD, and the same values converted to  $\delta^{44/42}\text{Ca}$  (pptt) relative to ICP1**

	$\delta^{44/40}\text{Ca}$ (‰), 915a	$\delta^{44/42}\text{Ca}$ (pptt), ICP1
Sow urine	$1.71 \pm 0.16$	$6.84 \pm 0.78$
Sow serum	$-0.57 \pm 0.08$	$-4.28 \pm 0.39$
Sow feed	$0.46 \pm 0.23$	$0.75 \pm 1.12$

Pig samples were supplied by Thomas Crenshaw (University of Wisconsin, Madison, WI).

**Table S3. Demographic data for subjects in 6° head-down bed rest study**

Subject	Age (years)	Sex	Height (cm)	Weight (kg)	BMI* (kg/m <sup>2</sup> )
P1	32	Male	176	71.3	22.9
P2	27	Male	172	67.3	22.7
P3	34	Female	171	80.4	27.6
P4	29	Female	155	55.3	22.9
P5	29	Female	177	57.5	18.5
P6	26	Male	172	78.3	26.4
P7	25	Male	186	87.0	25.1
P8	25	Male	176	73.5	23.7
P9	46	Male	176	89.3	29.0
P10	28	Male	177	79.5	25.3
P11	30	Male	170	78.5	27.1
P12	49	Female	169	66.5	23.3

\*Body mass index.