direct evidence for the subtle molecular differences among the same type of cancers. It is well known that responses to specific treatments may differ among diseases of the same category *(1, 2)*, but confirmation of dissimilarities at the molecular level has been hindered by technical difficulties and lack of specific molecular probes. The cell-SELEX method *(4)* may provide a simple, fast, and low-cost way to generate panels of molecular probes and reveal subtle differences even before specific disease biomarkers are known.

In conclusion, with aptamers directly evolved from T-ALL cells we were able to identify leukemia cells in patient samples and detect subtle molecular differences among individual samples from leukemia patients in the same category. Our results demonstrate that cell-based aptamer selection can be a valuable approach for generating aptamer probes to obtain molecular signatures of individual patient samples. Although the molecular profile or signature may not necessarily indicate the detailed molecular mechanism of a disease, it may be the first step toward understanding the molecular basis of a disease. Because of features such as chemical-synthesis–based production, low molecular weight, easy modification, and long-term stability after modification, aptamers selected from cancer cells may be effective molecular probes for cancer diagnosis.

Table 1. Aptamer profiling of cancer cells in patients' samples.[a]

	sgc8	sgc3	sgc4	sgd2	sgd3	sgd5
T ALL 1	++	+++	+++	+++	+++	ND
T ALL 2	++	+	+++	++	+	0
T ALL 3	+	+	++++	+++	+	0
T ALL 4	+	+	++	+++	+	0
T ALL 5	+	+	++	+	+	0
T ALL 6	0	0	+	+	0	0
T ALL 7	0	0	++	++	0	0
TALL 8	+	+	++	++	+	0
TALL 9	+	0	+	+	0	0
TALL10	0	+	+	0	+	0
B ALL 1	0	0	++	++	0	0
B ALL 2	0	0	++	++	0	+
B ALL 3	++	0	++	++	0	+
B-ALL 4	0	0	+	+	0	0
AML 1	+	+	++	+	0	0
AML 2	+	0	++	+	0	0
AML 3	+	0	+	+	0	0
AML 4	0	0	++++	++++	0	0
AML 5	0	0	+	0	0	0
AML 6	+	0	0	0	0	0
AML 7	+	0	0	0	0	0
AML 8	+	0	+++	+++	0	0

[a] In the flow cytometry analysis, a threshold based on fluorescence intensity of FITC was chosen so that 99% percent of cells incubated with the FITC-labeled unselected DNA library would have fluorescence intensity below it. When FITC-labeled aptamer was allowed to interact with the cells, the percentage of the cells with fluorescence above the set threshold was used to evaluate the binding capacity of the aptamer to the cells. 0, <10%; +, 10%—35%; ++, 35%—60%; +++, 60%—85%; ++++, >85%.

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Natural Calcium Isotopic Composition of Urine as a Marker of Bone Mineral Balance, *Joseph Skulan,*[1*] *Thomas Bullen,*[2] *Ariel D. Anbar,*[3] *J. Edward Puzas,*[4] *Linda Shackelford,*[5] *Adrian LeBlanc,*[6] *and Scott M. Smith*[5] ([1] Department of Geology and Geophysics, University of Wisconsin-Madison, Madison, WI; [2] Branch of Regional Research, Water Resources Discipline, US Geological Survey, Menlo Park, CA; [3] School of Earth and Space Exploration and Department of Chemistry and Biochemistry, Arizona State University, Tempe, AZ; [4] Department of Orthopaedics, University of Rochester School of Medicine and Dentistry, Rochester, NY; [5] Human Adaptation and Countermeasures Division, NASA Johnson Space Center, Houston, TX; [6] Department of Medicine, Baylor College of Medicine, Houston, TX; [*] address correspondence to this author at: Department of Geology and Geophysics, University of Wisconsin-Madison, 1215 W. Dayton St., Madison, WI 53706; fax 608-262-0693, e-mail jlskulan@geology.wisc.edu)

Background: **We investigated whether changes in the natural isotopic composition of calcium in human urine track changes in net bone mineral balance, as predicted by a model of calcium isotopic behavior in vertebrates. If so, isotopic analysis of natural urine or blood calcium could be used to monitor short-term changes in bone mineral balance that cannot be detected with other techniques.**

Methods: **Calcium isotopic compositions are expressed as δ^{44}Ca, or the difference in parts per thousand between the ^{44}Ca/^{40}Ca of a sample and the ^{44}Ca/^{40}Ca of a standard reference material. δ^{44}Ca was measured in urine samples from 10 persons who participated in a study of the effectiveness of countermeasures to bone loss in spaceflight, in which 17 weeks of bed rest was used to induce bone loss. Study participants were assigned to 1 of 3 treatment groups: controls received no treatment, one treatment group received alendronate, and another group performed resistive exercise. Measurements were made on urine samples collected before, at 2 or 3 points during, and after bed rest.**

Results: **Urine δ^{44}Ca values during bed rest were lower in controls than in individuals treated with alendronate ($P < 0.05$, ANOVA) or exercise ($P < 0.05$), and lower than the control group baseline ($P < 0.05$, *t*-test). Results were consistent with the model and with biochemical and bone mineral density data.**

Conclusion: **Results confirm the predicted relationship between bone mineral balance and calcium isotopes, suggesting that calcium isotopic analysis of urine might be refined into a clinical and research tool.**

An empirical model of the behavior of calcium isotopes in vertebrates *(1)* predicts that the natural calcium isotope composition of human soft tissue and urine is affected by changes in the relative rates of bone formation and resorption. Confirmation of this prediction would indicate that measurements of the calcium isotope composition of urine or blood might be a useful marker of net bone mineral balance. Such a marker, which would not require accelerator mass spectrometry or the administration of isotopic tracers, could provide information that cannot be gained from bone biochemical markers, and provide it more quickly than is possible with observed changes in bone mineral density (BMD).

Although measurements of calcium isotopes have a wide range of applications in research on bone and calcium metabolism, to date these applications have been limited to the use of artificially prepared calcium isotopic tracers such as ^{41}Ca *(2)*. The application described in this paper is fundamentally different from tracer studies in that it is based on measurements of natural, biologically induced variations in the calcium isotopic composition of urine. These variations are too small to be of interest in tracer studies, but they are biologically informative, and can be measured reliably with high-precision mass spectrometry.

Calcium has 6 naturally occurring isotopes (^{40}Ca, ^{42}Ca, ^{43}Ca, ^{44}Ca, ^{46}Ca, and ^{48}Ca). Because natural variations in calcium isotopic compositions are, with very rare exceptions *(3)*, strictly dependent on the relative masses of the isotopes, it is possible to calculate the complete isotopic composition of a calcium sample from the ratio of any 2 isotopes *(4, 5)*. Calcium isotopic compositions are most commonly reported as δ^{44}Ca, or the difference in parts per thousand between the ^{44}Ca/^{40}Ca of a sample and the ^{44}Ca/^{40}Ca of a standard reference material (in this case seawater):

$$\delta^{44}\text{Ca}\ (‰) = \{[(^{44}\text{Ca}/^{40}\text{Ca})_{\text{sample}}/(^{44}\text{Ca}/^{40}\text{Ca}_{\text{standard}})] - 1\} \times 1000$$

Natural processes can fractionate (that is, alter the relative abundances of) calcium isotopes *(1, 4, 6)*. In nature, δ^{44}Ca ranges from approximately −1.6‰ to −0.4‰ in nonbiogenic minerals, and from approximately −4.0‰ to +1.0‰ in biological materials. Within organisms, there typically is ~2‰–4‰ difference between the highest and lowest δ^{44}Ca values measured in different tissues *(1)*. Fractionation mechanisms are not well understood, but calcium isotope fractionation during abiological and biological mineralization is well documented, and probably accounts for much of the observed natural variation in δ^{44}Ca *(1, 4, 6)*. Among a phylogenetically diverse group of nonhuman vertebrates, δ^{44}Ca of bone is ~1.3‰ lower than that of dietary and dissolved soft tissue calcium from which bone mineral precipitates *(1)*. This difference largely results from fractionation during bone formation. Bone resorption, however, being a bulk dissolution process, does not fractionate calcium isotopes. As a result, when the rates of bone formation and bone resorption are equal and dietary intake of δ^{44}Ca is constant, the difference in δ^{44}Ca between bone and soft tissue is expected to be constant.

A simple model of calcium isotope behavior predicts that positive bone mineral balance (during which the rate of bone formation exceeds the rate of bone resorption) will cause positive excursions in soft tissue δ^{44}Ca, and vice versa:

$$\delta_s = \Delta_b + [V_s(\delta_b - \delta_d) - V_b\Delta_b]/(V_d + V_s),$$

where δ_s, δ_d, and δ_b are δ^{44}Ca of soft tissue, dietary Ca, and bone, respectively; V_s, V_b, and V_d are the fluxes of calcium resorbed from bone, incorporated into bone, and absorbed from diet, respectively; and Δ_b is the Ca isotope fractionation during bone formation *(1)*.

To test the hypothesized relationship between soft tissue δ^{44}Ca and bone mineral balance in humans, we measured δ^{44}Ca of urine from a 10-person subset of participants in a previously published human study of the effectiveness of countermeasures to bone loss in spaceflight *(7)*, in which extended bed rest (17 weeks) was used

to induce bone loss and simulate weightlessness. Study participants were assigned to 1 of 3 treatment groups: no treatment (controls; n = 4), treatment with the antiresorptive drug alendronate (n = 3), or treatment with resistive exercise (n = 3). Study participants were fed identical diets; 2 samples of homogenized diet yielded the same δ^{44}Ca (−1.88‰ and −1.90‰), indicating that dietary δ^{44}Ca was approximately constant during the study. Data were collected on BMD and biochemical markers of bone resorption (n-telopeptide, NTX) and bone formation (bone-specific alkaline phosphatase, BSAP). δ^{44}Ca was measured for food samples and 1-week pooled urine samples (a soft tissue proxy) collected at intervals before, during, and after bed rest.

Analytical procedures for BMD and biochemical markers are given elsewhere *(7)*. δ^{44}Ca measurements were conducted at the US Geological Survey in Menlo Park, CA, using well-established and rigorously tested geochemical techniques, including a calcium isotope "double-spike" that corrects for matrix effects and instrumental isotope fractionation *(1, 5, 6, 8, 9)*. Reported δ^{44}Ca values are based on a minimum of 60 isotope ratio measurements, with an internal imprecision of 0.15‰ (2σ, 95% CI) or better. Total procedural replicates of 15 urine samples collected for this study yielded an mean external (intraassay) imprecision of ±0.154‰ (2σ, 95% CI); external imprecision was ±0.040‰ to ±0.154‰ on full procedural replicates on reference materials having δ^{44}Ca values of 0‰ to −2.01‰ (see the Technical Information in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue6).

No funding organization influenced the design or execution of these methods, or the presentation of our results.

Results (Table 1 and Fig. 1) were consistent with the hypothesized association between changes in soft tissue (e.g., urine) δ^{44}Ca and changes in bone calcium balance, and with published interpretations of the BMD and biochemical marker data *(7)*. Tested against a null hypothesis of no change in calcium isotope composition, mean urinary δ^{44}Ca values became positive in the alendronate group ($P < 0.05$, *t*-test), remained unchanged in the exercise group ($P > 0.50$), and became negative in the control group ($P < 0.05$), the group expected to have lost bone during bed rest (Fig. 1A, Table 1). No significant trends were observed in any group between weeks 4 and 17 ($P > 0.50$, ANOVA).

δ^{44}Ca was lower in the control group than in the alendronate ($P < 0.05$, ANOVA) and exercise groups ($P < 0.05$). There was no significant difference between the alendronate and exercise groups ($P = 0.220$). Because analytical uncertainty in δ^{44}Ca measurements is low, interindividual variability within groups reflects real biological differences between individuals.

It is difficult to calculate bone mineral balance from biochemical markers of bone formation and resorption. These markers are of marginal clinical value *(10, 11)* and are not calibrated to the mass of bone gained or lost from the skeleton. In many cases bone formation and resorption increase or decrease together. For example, bone fractures cause a large increase in the rate of bone

Table 1. Summary of data.[a]

Marker	Unit	Treatment group	Mean change	SD	n
δ^{44}Ca[b]	‰	Alendronate	0.32	0.28	3
δ^{44}Ca	‰	Exercise[c]	0.12	0.30	3
δ^{44}Ca	‰	Control	−0.48	0.63	4
NTX[b]	nmol/d	Alendronate	−63.5	72	3
NTX	nmol/d	Exercise	122	196	3
NTX	nmol/d	Control	256	119	4
BSAP[b]	U/L	Alendronate	−2.86	3.17	3
BSAP	U/L	Exercise	9.81	7.67	3
BSAP	U/L	Control[d]	−0.32	1.39	4

[a] Values are means and SD of differences between initial values and values during bed rest of all samples taken during bed rest, by group.

[b] Samples obtained from each study participant at weeks 0, 4, 10 or 12, and 16 or 17 of bed rest (data for some individuals are missing for some weeks). *P* values for differences in δ^{44}Ca between treatment groups are all <0.05 except for exercise vs alendronate. [c] $P = 0.222$ for exercise vs alendronate. [d] $P = 0.143$ for control vs alendronate.

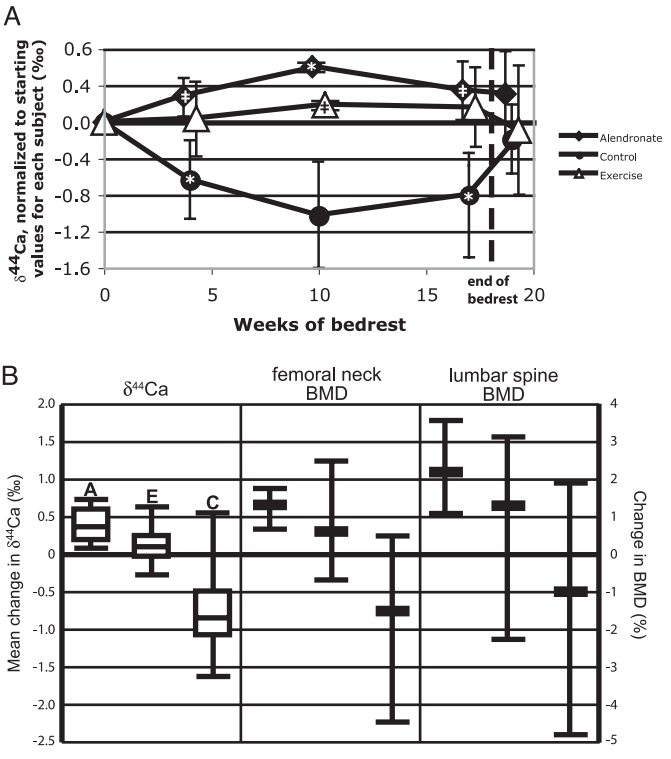

Fig. 1. (*A*), changes in δ^{44}Ca during bed rest, by treatment group, at weeks 4, 10, and 16 or 17, and after bed rest at week 19.

Symbols show mean values, ends of whiskers show extreme values. * $P < 0.05$. † $P < 0.10$. (*B*), mean change during bed rest from initial values of urinary δ^{44}Ca and BMD of the femoral neck and lumbar spine, for alendronate (*A*), exercise (*E*), and control (*C*) groups. For δ^{44}Ca, *dark lines* inside boxes show 50th percentiles, *ends of boxes* show 25th and 75th percentiles, and *ends of whiskers* show upper and lower extremes. For BMD data, *central bars* show means and *ends of whiskers* show extreme values.

remodeling and in the concentration of formation and resorption markers *(12)*. In this study both NTX and BSAP decreased in individuals treated with alendronate, and both increased in the exercise group (Table 1). In contrast, δ^{44}Ca is directly related to net change in skeletal mass and not to the individual rates of bone formation and resorption.

Large changes in δ^{44}Ca occur much more rapidly than in BMD (Fig. 1B). In the control group δ^{44}Ca had become negative by the 4th week of bed rest (the earliest point in the study from which urine samples were analyzed) and was trending positive in the alendronate group ($P < 0.05$ and 0.058, respectively), whereas 17 weeks were required to produce detectable changes in some BMD measures. In fact, extensive studies of calcium isotopic tracers *(13–15)* suggest that δ^{44}Ca responds to changes in bone mineral balance in much less than 4 weeks. The residence time of calcium in different nonskeletal pools varies, so a week or more may be required to achieve full equilibration between bone mineral balance and urine δ^{44}Ca *(16, 17)*, but changes in bone mineral balance should be detectable in urinary δ^{44}Ca within a few days. Long-term changes in bone mineral balance could be monitored by periodic sampling of urine calcium.

The calcium isotope data reported here constitute virtually all of the information we have on the natural behavior of calcium isotopes in humans, and more data are needed to validate the use of natural calcium isotopes as a biomarker. Although analytical variability in calcium isotope measurements is very low, we have no information on the background variation in urinary δ^{44}Ca in healthy humans outside of conditions designed to induce bone loss, and thus cannot accurately assess preanalytical variability *(18)* or establish a reference change value *(19)* in calcium isotope composition. Processes other than bone formation may fractionate calcium and produce isotopic differences between soft tissue calcium compartments. For example, δ^{44}Ca of all urine samples was higher than dietary δ^{44}Ca (see the online Data Supplement). Differences in calcium isotopic composition between soft tissue compartments must be investigated and explained.

Future research will focus on gaining a comprehensive understanding of the distribution of calcium isotopes in the human body. This research is warranted by the potential for measurement of δ^{44}Ca to be an important addition to the tools currently available for diagnosing, studying, and assessing treatments for metabolic bone disease in humans.

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