

Aptamers Evolved from Cultured Cancer Cells Reveal Molecular Differences of Cancer Cells in Patient Samples, *Dihua Shangguan,^{1,3} Zehui Charles Cao,^{1,3} Ying Li,^{2,3} and Weihong Tan^{1,3*}* (Departments of ¹Chemistry and ²Pathology and ³Shands Cancer Center, University of Florida, Gainesville, FL; * address correspondence to this author at: Department of Chemistry and Shands Cancer Center, University of Florida, Gainesville, FL 32611; fax 352-846-2410, e-mail tan@chem.ufl.edu)

Background: Molecular-level differentiation of neoplastic cells is essential for accurate and early diagnosis, but effective molecular probes for molecular analysis and profiling of neoplastic cells are not yet available. We recently developed a cell-based SELEX (systematic evolution of ligands by exponential enrichment) strategy to generate aptamers (designer DNA/RNA probes) as molecular probes to recognize neoplastic cells.

Methods: We tested 6 cell-SELEX-generated aptamers with equilibrium dissociation constants in the nanomolar to subnanomolar range: sgd5, selected from Toledo cells, a human diffuse large-cell lymphoma cell line (B-cell), and sgc8, sgc3, sgc4, sgd2, and sgd3 from CCRF-CEM cells, a human precursor T cell acute lymphoblastic leukemia (T-ALL) cell line. Aptamers were labeled with fluorescein isothiocyanate fluorophores and then used to recognize, by flow cytometric analysis, neoplastic cells in cultured hematopoietic cell lines and clinical samples.

Results: Aptamer sgd5 recognized only its target cells. Aptamers sgc3, sgd2, sgd3, sgc4, and sgc8, selected from a T-cell leukemia cell line, identified all of the cultured T-cell leukemia cell lines with relatively high fluorescence intensity. Aptamers sgc8, sgc3, and sgd3 showed good selectivity toward T-ALL cells and almost no binding to normal hematopoietic cells or lymphoma and myeloma cells. Selected aptamers also detected targets on the cell membranes of neoplastic cells in patient samples.

Conclusions: Aptamers selected against cultured neoplastic cells can effectively be used as molecular probes for recognition of neoplastic cells in patient samples. Cell-based aptamer selection can be used to generate aptamer probes to obtain molecular signatures of neoplastic cells in patient samples.

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Complex genetic and proteomic alterations reveal the molecular heterogeneity within individual cancer diagnostic categories. Identification and understanding of the molecular basis of diseased cells should provide the most reliable approach toward effective diagnosis and treatments. Given the complexity and diversity of cancers, even within similar categories, multiple cancer-specific molecular probes are needed to delineate unique fingerprints of tumor cells. Molecular-level differences exist between any 2 given types of cells, even 2 individual cases

of a same tumor type, but for most diseases no reliable molecular probes are specific enough to recognize these subtle molecular differences (1). A critically important task for molecular medicine is to identify these differences and then use them to further characterize and understand the molecular basis of diseases. Despite the variety of clinical variables used to classify human malignancies, most neoplastic diseases cannot be defined or classified according to abnormal molecular disease processes because of the lack of molecular probes that can be used to define these processes. Thus, patients receiving similar diagnoses can have markedly different clinical courses and responses to treatments (2).

Currently, the diagnosis of leukemia is commonly based on morphologic evaluation supplemented by immunophenotype analysis by flow cytometry with monoclonal antibodies of CD antigens (3). These antigens are usually expressed on both normal and neoplastic cells, however, and thus cannot accurately reflect the molecular features of the cancer cells. Although many antibodies are available for phenotyping leukemia, they were not developed to enable comprehensive recognition of molecular features of specific disease cells but were individually developed at different times for various purposes. Systematic production of a panel of antibodies for molecular differentiation of cancer cells would be very difficult because of the technical difficulties involved in systematic development of antibodies for unknown surface biomarkers. Novel approaches are therefore needed to systematically generate panels of new probes that recognize molecular signatures of cancers.

We previously developed panels of DNA aptamers, single-stranded oligonucleotides, directly from live tumor cells with a process called SELEX (systematic evolution of ligands by exponential enrichment) (4–6). These aptamers recognized surface targets of cancer cells with high affinity and specificity. Selected aptamers can bind to target molecules by folding into well-defined 3-dimensional structures (5–7). Unlike antibodies, aptamers, once the sequence is known, can be synthesized reproducibly by a DNA synthesizer at a very low cost (8–13). In addition, aptamers have low molecular weight, fast tissue penetration, and low toxicity. They can be specifically labeled with radioscopic, fluorescent, or other reporters for molecular recognition. Moreover, aptamers are stable during long-term storage, can be transported at ambient temperature, and sustain reversible denaturation. Despite their advantages and unique properties as molecular probes, however, aptamers have been used sparingly for medical applications because of the limited number of available aptamers that have medical relevance. Acquisition of aptamers directly from diseased cells is expected to link aptamers more closely to real medical problems and to greatly reduce the time gap between laboratory research and clinical applications.

We report a group of new aptamers selected directly from cancer cells (4) for the recognition of molecular differences among leukemia patient samples. These aptamers have high affinity and specificity for surface

targets of neoplastic cells in clinical samples, and bind with clinical samples to form distinct recognition patterns. Thus, these aptamers may be useful for both disease diagnosis and efficient personalized therapy for individual patients.

Aptamers are usually selected for single target molecules. In contrast, cell-SELEX elects aptamers by use of complex whole cells as targets (4). A counterselection strategy is used to isolate aptamer sequences that interact only with the target cells and not with the control cells. Through this process, a group of cell-specific aptamers can be selected in a relatively short period (4–8 weeks) even if it is not known which target molecules are present on the cell surface and which membrane molecules might play the most important role in cancer development. This feature is the most important difference between cell-SELEX and other current methods of molecular probe development, and enables cell-SELEX to generate multiple molecular probes to recognize biomarkers in their native states, producing molecular signatures of diseases.

We used the cell-SELEX to obtain many aptamers with high affinity and specificity to surface molecules on target cancer cells. We then chose 6 aptamers with equilibrium dissociation constants in the nanomolar to subnanomolar range: sgd5—selected from Toledo cells, a human diffuse large-cell lymphoma cell line (B-cell)—and sgc3, sgd2, sgd3, sgc4, and sgc8 from CCRF-CEM cells, a human T precursor T cell acute lymphoblastic leukemia (T-ALL) cell line.

The 6 selected aptamers were first conjugated with fluorescein isothiocyanate (FITC) for recognition of different kinds of cells. We then used flow cytometric analysis to monitor the binding of aptamers to cells from 4 T-cell leukemia cell lines; 8 B-cell lymphoma, leukemia, or myeloma cell lines; and normal human bone marrow aspirates (see the methods and materials in the Data Supplement that accompanies the online version of this Technical Brief at www.clinchem.org/content/vol53/issue6). Subpopulations of bone marrow cells were identified in the flow cytometric analysis by their side-scatter properties and the expression of CD3, CD7, CD10, CD19, and CD45. The following cell types were identified: mature B cells, immature B cells, CD3(+) T cells, monocytes, granulocytes, and nucleated erythrocytes. The dot plot of a typical flow cytometry analysis is shown in Fig. 1. The FITC-labeled DNA library was measured as the background binding, and a threshold was determined based on the background fluorescence. Cells recognized by aptamers were expressed as percentage of cells with fluorescence higher than the background threshold. The results (see Table 1 in the online Data Supplement) showed that aptamer sgd5 recognized only its target cells. All of the cultured T-cell leukemia cell lines were identified with relatively high fluorescence intensity by aptamers sgc3, sgd2, sgd3, sgc4, and sgc8, which was expected because they were selected from a T-cell leukemia cell line. Aptamers sgc8, sgc3, and sgd3 showed good selectivity toward T-ALL cells and almost no binding to normal hematopoietic cells in the human bone marrow

samples or lymphoma and myeloma cells. Further inspection showed that aptamers sgc4 and sgd2 recognized many different cell samples, including some normal bone marrow cells, indicating the presence of common binding entities on these cells. Combination of selected aptamers produced distinct patterns for different tumor cells, suggesting that aptamers may be used to define molecular signatures of tumors.

Selected aptamers also detected targets on the cell membranes of neoplastic cells in patient samples, including T-ALL, B-cell ALL, acute myeloid leukemia (AML), and lymphomas (Table 1). All lymphoma samples with mature T or B cells showed no or very low binding (see Table 1 in the online Data Supplement), indicating that recognition was not due to nonspecific interactions, in agreement with the fact that most of the aptamers were selected against a cultured precursor T-ALL cell line. As expected, the aptamers showed more binding with cells from T-ALL patients than with other cell samples. Aptamer binding patterns corresponded well with general categories of acute leukemia predefined by antibody immunophenotyping.

Despite the results showing that aptamers can selectively recognize cultured T-ALL cells, demonstrating the specificity of selected aptamers (Table 1), individual cases of clinical specimens may have quite different patterns even in the same disease category. Although the explanation for these differences remains unknown, they precisely reflect the complex nature of the disease. In addition to general categorization of the leukemia suggested by available antibodies, our aptamer analyses provide

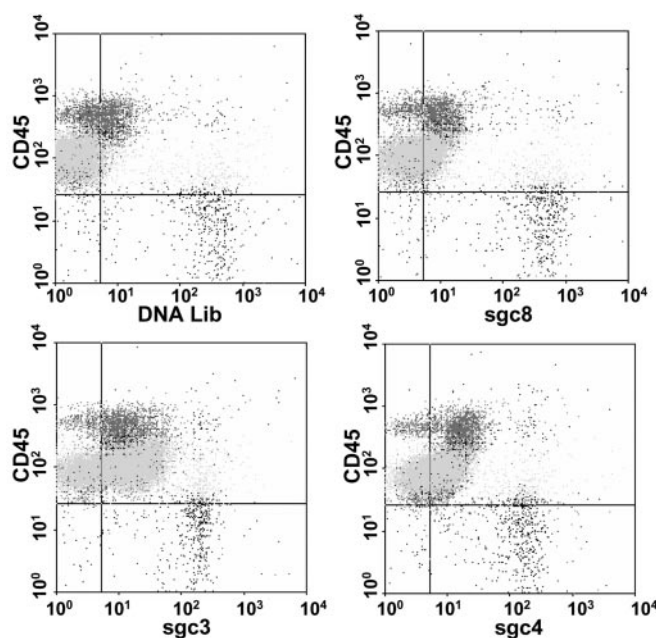


Fig. 1. Molecular recognition of T-ALL cells in patient bone marrow aspirates with FITC-labeled sgc8, sgc3, sgc4, and PerCP-labeled anti-CD45 antibody.

The background was measured by using FITC-labeled unselected library. The light gray dots represent T-ALL cells.

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
T ALL 8	+	+	++	++	+	0
T ALL 9	+	0	+	+	0	0
T ALL 10	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. Smith⁵ (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 $\delta^{44}\text{Ca}$, or the difference in parts per thousand between the $^{44}\text{Ca}/^{40}\text{Ca}$ of a sample and the $^{44}\text{Ca}/^{40}\text{Ca}$ of a standard reference material. $\delta^{44}\text{Ca}$ 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 $\delta^{44}\text{Ca}$ 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.

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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 $\delta^{44}\text{Ca}$, or the difference in parts per thousand between the $^{44}\text{Ca}/^{40}\text{Ca}$ of a sample and the $^{44}\text{Ca}/^{40}\text{Ca}$ of a standard reference material (in this case seawater):

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

Natural processes can fractionate (that is, alter the relative abundances of) calcium isotopes (1, 4, 6). In nature, $\delta^{44}\text{Ca}$ ranges from approximately -1.6‰ to -0.4‰ in nonbiogenic minerals, and from approximately -4.0‰ to $+1.0\text{‰}$ in biological materials. Within organisms, there typically is $\sim 2\text{‰}$ – 4‰ difference between the highest and lowest $\delta^{44}\text{Ca}$ 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 $\delta^{44}\text{Ca}$ (1, 4, 6). Among a phylogenetically diverse group of nonhuman vertebrates, $\delta^{44}\text{Ca}$ of bone is $\sim 1.3\text{‰}$ 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 $\delta^{44}\text{Ca}$ is constant, the difference in $\delta^{44}\text{Ca}$ 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 $\delta^{44}\text{Ca}$, 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 $\delta^{44}\text{Ca}$ 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 $\delta^{44}\text{Ca}$ and bone mineral balance in humans, we measured $\delta^{44}\text{Ca}$ 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 $\delta^{44}\text{Ca}$ (-1.88‰ and -1.90‰), indicating that dietary $\delta^{44}\text{Ca}$ 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). $\delta^{44}\text{Ca}$ 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). $\delta^{44}\text{Ca}$ 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 $\delta^{44}\text{Ca}$ 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 $\pm 0.154\text{‰}$ (2σ , 95% CI); external imprecision was $\pm 0.040\text{‰}$ to $\pm 0.154\text{‰}$ on full procedural replicates on reference materials having $\delta^{44}\text{Ca}$ 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) $\delta^{44}\text{Ca}$ 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 $\delta^{44}\text{Ca}$ 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).

$\delta^{44}\text{Ca}$ 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 $\delta^{44}\text{Ca}$ 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

Marker	Unit	Treatment group	Mean change	SD	n
$\delta^{44}\text{Ca}^b$	‰	Alendronate	0.32	0.28	3
$\delta^{44}\text{Ca}$	‰	Exercise ^c	0.12	0.30	3
$\delta^{44}\text{Ca}$	‰	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 $\delta^{44}\text{Ca}$ 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.

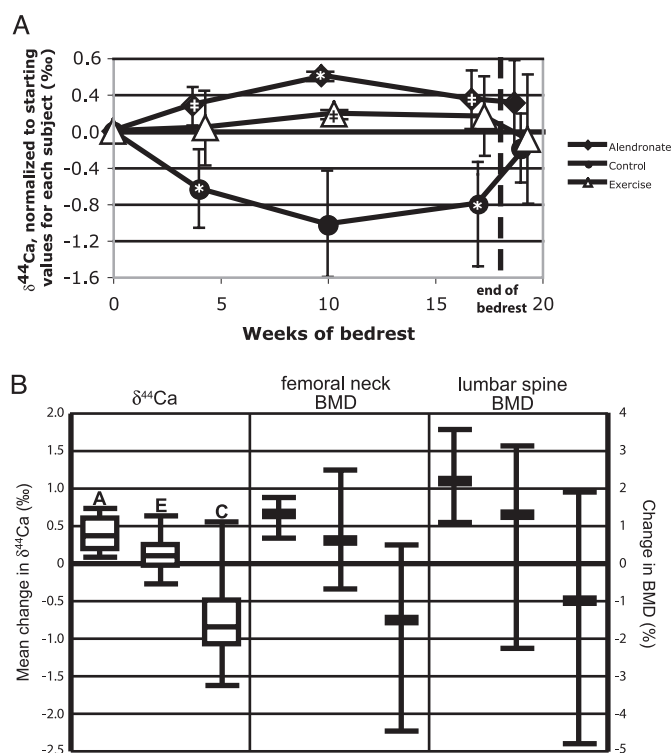


Fig. 1. (A), changes in $\delta^{44}\text{Ca}$ during bed rest, by treatment group, at weeks 4, 10, and 16 or 17, and after bed rest at week 19.

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 $\delta^{44}\text{Ca}$ and BMD of the femoral neck and lumbar spine, for alendronate (A), exercise (E), and control (C) groups. For $\delta^{44}\text{Ca}$, 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, $\delta^{44}\text{Ca}$ is directly related to net change in skeletal mass and not to the individual rates of bone formation and resorption.

Large changes in $\delta^{44}\text{Ca}$ occur much more rapidly than in BMD (Fig. 1B). In the control group $\delta^{44}\text{Ca}$ 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 $\delta^{44}\text{Ca}$ 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 $\delta^{44}\text{Ca}$ (16, 17), but changes in bone mineral balance should be detectable in urinary $\delta^{44}\text{Ca}$ 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 $\delta^{44}\text{Ca}$ 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, $\delta^{44}\text{Ca}$ of all urine samples was higher than dietary $\delta^{44}\text{Ca}$ (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 $\delta^{44}\text{Ca}$ 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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