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A Comprehensive Metabolomic Investigation in Urine of Mice Exposed to Strontium-90

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INTRODUCTION

During a nuclear or a radiologic disaster, the population is exposed to different types of radiation through inhalation, external exposure and ingestion of contaminated food and water sources. In the case of a nuclear bomb explosion or damage to a nuclear reactor core such as in the Chernobyl and Fukushima accidents, radionuclides are released into the environment. The most feared and environmentally persistent radionuclides are Cesium-137 (137Cs) and Strontium-90 (90Sr). The effects of exposure to 137Cs in serum and urine are the topic of a previous metabolomic study in mice, in which we established a metabolomic signature for exposure to 137Cs in serum and urine (1). In continuation of our efforts to develop unique metabolomic signatures for exposure to internal emitters, the current study explored the biological effects following exposure to 90Sr in mice using a similar metabolomics workflow.

Strontium-90, like 137Cs, is a fission product of uranium and plutonium with a half-life of 28.8 years. Although 90Sr is less likely to be released after a nuclear event and is less volatile than 137Cs, it still poses a serious health risk. The most common source of 90Sr exposure is through consumption of contaminated food and water after an event. Being a Group II element in the periodic table, 90Sr has biochemical properties similar to calcium and after ingestion it can become incorporated into bone and bone marrow. Thus, 90Sr is considered a “bone seeker”, which can result in bone cancer, leukemia and cancers associated with the surrounding soft tissue. 90Sr decays by beta emission into Yttrium-90, another beta emitter, which can further contribute to the radiation dose in bone and surrounding tissue (2). Due to the health effects associated with 90Sr, it is important to identify the primary cellular targets of 90Sr and determine a robust signature of exposure in easily obtainable biofluids to quickly and accurately triage patients while still in the field after a radiological disaster.

In the current study, we focused on determining a urinary metabolomic signature in mice after exposure to 90Sr at different time points, and cumulative doses over the course of 30 days via ultra-performance liquid chromatography coupled with mass spectrometry (UPLCMS). The sensitivity and accuracy of UPLCMS combined with our powerful statistical software allowed for the detection of subtle changes in the urinary metabolomic profile. The measured
absorbed doses to the skeleton ranged from 1.2–5.2 Gy over 30 days. The initial dose rate in this study was at 0.21 mGy/min while by the end of the experiment the dose rate had dropped to 0.12 mGy/min. The effects of changes in dose rate on the measured end points, although well recognized by the authors, could not be addressed in this study, since it is not possible to determine the effects of dose, dose rate and decreasing dose rate in a single study. More importantly, as in the case with $^{137}$Cs, this radiation model continues to irradiate the cells at risk during the entire experimental period, compared to a relatively instantaneous dose delivery from an external radiation source such as X rays. The goal of this study was to establish a robust metabolomic response to $^{90}$Sr in urine of mice and compare the results to known markers of external beam gamma irradiation and $^{137}$Cs exposure.

**MATERIALS AND METHODS**

Debrisoquine sulfate, 4-nitrobenzoic acid (4-NBA) and UPLC-grade solvents such as acetonitrile, water and isopropanol were purchased from Fisher Scientific (Hanover Park, IL), 4-Guanidinobutyric acid, riboflavin, retinoic acid, (−)-epinephrine, hippuric acid, nicotinic acid, S-(5′-adenosyl)-L-homocysteine, taurine, uric acid, homovanillic acid, acetyl carnitine and carnitine were purchased from Sigma-Aldrich®, (St. Louis, MO). The METLIN® (3) database (La Jolla, CA) was used for tandem MS (MS/MS) validations of 4-aminobutanoate, 3-hydroxybutanoate and quinolinic acid.

**Animal Irradiation and Sample Collection**

This study was conducted in accordance with applicable federal and state guidelines and was approved by the Institutional Animal Care and Use Committee of the Lovelace Biomedical and Environmental Research Institute (LBERI). C57Bl/6 mice (approximately 10–12 weeks old, 25–30 g) were received from Charles River Laboratories (Frederick, MD) and were quarantined for 14 days prior to group assignment by body weight stratification for randomization onto the study.

Animals were administered $^{90}$Sr intravenously by tail vein injection with 200 ± 0.3 kBq $^{90}$SrCl$_2$ solution in a volume of 50 µL. Strontium-85 ($^{85}$Sr) was used as a tracer for measuring strontium whole-body content. Strontium-85 comprised approximately 1% of the total strontium activity. After $^{85}$Sr/$^{90}$Sr administration, mice were housed individually in microisolator cages with lead shielding to prevent radiation exposure by cross irradiation from adjacent mice that were sources of radiation. All animals had unlimited access to Teklad Certified Global Rodent Diet 2016 (Harlan® Laboratories Inc., Madison, WI) and water except during dose administration and whole-body in vivo counting. No adverse effects were noted on the animals during the course of the study.

On scheduled necropsy days (4, 7, 9, 25 and 30 days after $^{85}$Sr/$^{90}$Sr administration), animals were euthanatized by intraperitoneal injection of Euthasol® (>150 mg/kg (390 mg/mL pentobarbital and 50 mg/mL phenytoin in sterile saline)) and weighed. Whole blood was collected by cardiac puncture in a sterile hood. When recoverable volumes were available, urine was collected directly from the bladder with a needle and syringe.

**Dosimetry of $^{90}$Sr in Mice**

Animals were measured for $^{85}$Sr/$^{90}$Sr whole-body content using the LBERI in vivo photon counting system described previously (4). Animals were placed in small containers with breathing holes, and daily measurements were taken to determine the amount of radioactivity present in each animal on days 0–7, then on day 9, 12, 16, 20, 25, 27 and 30 after $^{85}$Sr/$^{89}$Sr administration (until the time of necropsy). The measurement system was calibrated for different geometries; phantoms representing the animal body and biological samples were developed using a $^{85}$Sr NIST-traceable standard solution. Calibration was performed each day prior to measurement, and animals and samples were measured for 3 min.

The $^{89}$Sr whole-body retention profile was derived from whole-body measurements. The whole-body retention data from each mouse was fitted individually to negative exponential functions. The average values of the parameters of the whole-body retention equation are shown in Eq. (1),

$$R(t) = 52.1e^{-2.0t} + 20.7e^{-0.13t} + 27.2e^{-0.004t}$$

where $R(t)$ represents the whole-body $^{89}$Sr content at time [t] in days expressed as percentage of the injected $^{89}$Sr activity. The biological half-times were 0.3, 5.3 and 139 days, respectively.

To calculate the committed absorbed dose to the skeleton, the dose coefficient (Gy.Bq$^{-1}$ of administered activity) was derived using Eq. (2). The comparison between the whole-body activity and the $^{89}$Sr content in the skeleton at sacrifice time shows that about 95% of the whole-body activity was located in the skeleton for all time periods. Thus, the retention parameters of Eq. (1) were used to calculate the total number of nuclear transformations (Bq s) in the skeleton for each time period of the study. The $S$ value (Gy/Bq s) used in Eq. (2) was derived specifically for young adult mice and rats by Stabin et al. (5). The dose coefficients (Gy Bq$^{-1}$ of administered activity) for the various time periods used in this study are shown in Table 1.

The committed absorbed doses to the skeleton for each animal were calculated by multiplying the dose coefficient (Gy Bq$^{-1}$) related to the specific sacrifice time for each animal in the study by the administered activity (Bq). The average committed absorbed doses to the skeleton for each time period are shown in Table 2.

$$D_r = \int_{t_0}^{t_0+30} A(s) \times S(r_t \leftarrow r_s,t) \times \frac{Gy}{Bq}$$

where: $A(s)$ is the time-integrated activity (Bq s), equal to the total number of nuclear transformations in the source region (skeleton); $S(r_s \leftarrow r_t,t)$, in Gy per Bq s, is the $S$ value from $r_s$ to $r_t$ of $^{90}$Sr + $^{90}$Y, where the $S$ value for a given source ($r_s$)-target ($r_t$) pair is the mean absorbed dose to the target organ per $^{90}$Sr + $^{90}$Y total number of nuclear transformations in the source region.
Sample Preparation and Mass Spectrometry Analysis

Urine samples were prepared by diluting each sample 1:4 in a 50% acetonitrile solution containing the internal standards, 30 μM of 4-nitrobenzoic acid and 2 μM of debrisoquine. The samples were centrifuged at 18,000 rcf to precipitate out the proteins. A 5 μL aliquot of the recovered supernatant was then injected into a reverse-phase 5 μm long mobile phase gradient started with aqueous solvent (98% MA) coupled to a time-of-flight mass spectrometry (TOFMS). The 13 min long mobile phase gradient started with aqueous solvent (98% water; 2% acetonitrile with 0.1% formic acid) and switched to 100% organic (acetonitrile) at a flow rate of 0.5 mL/min. The Q-TOF Premier™ (Waters Corp.) mass spectrometer was operated in positive (ESI+) and negative (ESI-) electrospray ionization modes. The acquired centroid mass spectrometer data was then processed using MassLynx™ software (Waters Corp.). Furthermore, twofold dilutions were performed using the internal standards at the initial concentration of 150 μg/mL to the final concentration of 0.59 μg/mL. The internal standards were also spiked into pooled control urine samples and processed every 7 injection intervals. The calculated standard curve for each internal standard was used to determine the relative abundance of different metabolites in each ionization mode.

Statistical Analysis

As described in our previous study (6) MarkerLynx™ software (Waters Corp.) was used to extract spectral features from raw chromatograms into a data matrix consisting of the retention time, mass-to-charge ratio (m/z) and abundance value for each ion. To determine the peak areas of internal standards, QuanLynx™ (Waters Corp.) was used. RandomForest was then used, initially to explore the overall urinary metabolomic signatures of the two dose/time points compared to that of the control mice. Subsequently, MetaboLyzer was used for in-depth statistical analysis of the data and for assigning putative identities to each ion (7). As described in our previous studies (4), MetaboLyzer was used to select ions that were present in at least 70% of the samples in all study groups with nonzero abundance values (complete-presence ions). The data were then log transformed and analyzed using the nonparametric Mann-Whitney U test for statistical significance (P < 0.05). The log transformed significantly complete-presence ion data were further utilized for principal component analysis (PCA) via singular value decomposition for the purpose of data visualization. Statistical significance testing for ions with nonzero abundance values in at least 70% of the samples in only one group (referred to as partial-presence ions) were analyzed as categorical variables for presence status (i.e., nonzero abundance) via Fisher’s exact test (P < 0.05).

Metabolic Pathway Analysis

The putative identities of all statistically significant ions (both complete- and partial-presence) were used for pathway mapping in MetaboLyzer via the Human Metabolome Database (HMDB), LipidMaps and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (8). The maximum m/z tolerance was set at 20 parts per million (ppm), while accounting for possible adducts, H+, Na+ and/or NH4+ in the ESI+ mode, and H+ and Cl- in the ESI- mode. The KEGG annotated pathways associated with these putative metabolites were also identified. Later, tandem mass spectrometry was used to generate fragmentation patterns for the metabolites of interest. These MS/MS fragmentations were then compared against those of matching pure chemicals or published MS/MS spectra on METLIN (3, 9) and HMDB for validation purposes.

RESULTS

RandomForest was initially used to assess the overall urinary metabolomic signatures of mice 7 and 9 days after 90Sr exposure (average cumulative dose of 2.0 Gy), and 25 and 30 days after 90Sr exposure (average cumulative dose of 5.0 Gy) compared to control mice. Supplementary Fig. S1A (http://dx.doi.org/10.1667/RR14011.1.S1) shows that the overall metabolomic signature of the 90Sr-exposed mice from both time point groups are distinctly separate from that of control mice based on the normalized urinary levels of 25 most variable ions. While the urinary metabolomic profile of the exposed mice is clearly separate from the controls, the heatmap in Supplementary Fig. S1B (http://dx.doi.org/10.1667/RR14011.1.S1) shows a persistent decrease in the levels of 64 highest ranked variable ions across the two dose/time points (ESI+ mode data). Additional in-depth statistical analyses were performed with the MetaboLyzer, which was employed to determine the statistical significance of individual ions (Mann-Whitney, P < 0.05) and their putative identity and pathway associations in both ESI modes.

At necropsy, urine could not be collected from the bladder of some of the mice at several time points due to the mice completely evacuating their bladders while still in their home cages. To increase the statistical power during analysis, we combined the results obtained from urine samples collected at day 7 and 9 after exposure, and averaged the skeletal doses. The average skeletal dose for these samples was 2.0 ± 0.28 Gy and the total number of samples in this dose group was 7, with 9 samples in the control group. The same approach was taken towards the urine samples collected at day 25 and 30 after exposure with the average dose of 5.0 ± 0.62 Gy. In addition all the available control samples were grouped for the statistical analyses in MetaboLyzer. Table 3 summarizes the number of samples per each dose and time point group and the matched controls. This table also contains the relative concentration of creatinine in each study group. The levels

<table>
<thead>
<tr>
<th>Study group</th>
<th>Necropsy (days after exposure)</th>
<th>Number of mice per study group</th>
<th>Internal dose (skeleton dose, Gy)</th>
<th>Creatinine (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7, 9</td>
<td>9</td>
<td>NA</td>
<td>1.3 ± 0.091</td>
</tr>
<tr>
<td>Control</td>
<td>25, 30</td>
<td>4</td>
<td>NA</td>
<td>1.4 ± 0.099</td>
</tr>
<tr>
<td>90Sr exposed</td>
<td>7, 9</td>
<td>7</td>
<td>2.0 ± 0.28</td>
<td>1.2 ± 0.069</td>
</tr>
<tr>
<td>90Sr exposed</td>
<td>25, 30</td>
<td>6</td>
<td>5.0 ± 0.62</td>
<td>1.2 ± 0.077</td>
</tr>
</tbody>
</table>

Note. The average levels of creatinine in urine for each study group are listed in terms of concentration (μM).

Average Cumulative Doses for Each Study Group (Gy) and the Time of Necropsy is Listed in Days after 90Sr Exposure

<table>
<thead>
<tr>
<th>Necropsy (days after exposure)</th>
<th>Number of mice per study group</th>
<th>Internal dose (skeleton dose, Gy)</th>
<th>Creatinine (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7, 9</td>
<td>9</td>
<td>NA</td>
</tr>
<tr>
<td>Control</td>
<td>25, 30</td>
<td>4</td>
<td>NA</td>
</tr>
<tr>
<td>90Sr exposed</td>
<td>7, 9</td>
<td>7</td>
<td>2.0 ± 0.28</td>
</tr>
<tr>
<td>90Sr exposed</td>
<td>25, 30</td>
<td>6</td>
<td>5.0 ± 0.62</td>
</tr>
</tbody>
</table>

Note. The average levels of creatinine in urine for each study group are listed in terms of concentration (μM).
of creatinine remained relatively unchanged throughout the study for the control and the exposed groups. We therefore normalized the abundance values of each ion to creatinine in our data matrix. The normalized data was then used in MetaboLyzer to establish a unique urinary metabolomic profile for exposure by $^{90}$Sr at each of the doses/time points with respect to the control urine samples.

The principal component analysis plot in Fig. 1A shows a clear separation between the overall metabolomic profiles of control and $^{90}$Sr-exposed urine samples at day 7 and 9 after exposure. The same separation in urinary metabolomic profiles was observed at the average dose of 5.0 ± 0.62 Gy when compared to the controls (Fig. 2A). Figure 2B shows a volcano plot of the $P$ value ($-1^{*}\log_{10}$) versus the fold change (log2) for all the complete-presence urinary spectral features at the average dose of 2.0 ± 0.28 Gy, and highlights the most statistically significant changes in their urinary abundances after exposure (red circles). The red circles to the right of the zero on this axis are the statistically significant spectral features whose urinary abundances increase significantly after exposure, and to the left of the zero are those whose urinary abundances decreased significantly as a result of $^{90}$Sr exposure. It is evident from this plot that more urinary ions showed a decrease in their abundances after $^{90}$Sr exposure, and the bottom one-third of the plot shows ions with increased (red) levels after $^{90}$Sr exposure at day 7 and 9.

The principal component analysis plot in Fig. 1A shows a clear separation between the overall metabolomic profiles of control and $^{90}$Sr-exposed urine samples at day 7 and 9 after exposure. The same separation in urinary metabolomic profiles was observed at the average dose of 5.0 ± 0.62 Gy when compared to the controls (Fig. 2A). Figure 2B shows a volcano plot of the $P$ value ($-1^{*}\log_{10}$) versus the fold change (log2) for all the complete-presence urinary spectral features at the average dose of 2.0 ± 0.28 Gy, and highlights the most statistically significant changes in their urinary abundances after exposure (red circles). The red circles to the right of the zero on this axis are the statistically significant spectral features whose urinary abundances increase significantly after exposure, and to the left of the zero are those whose urinary abundances decreased significantly as a result of $^{90}$Sr exposure. It is evident from this plot that more urinary ions showed a decrease in their abundances after exposure at an average skeletal dose of 5.0 ± 0.62 Gy (Fig. 2B and C). Since the water consumption and the urine output, as well as the weight of the mice did not
change significantly throughout the course of the study, this observation may indicate perturbations in the metabolic pathways associated with these statistically significant urinary ions. Thus, the next phase of the study was focused on identifying individual urinary ions with significantly changed levels after exposure, and determining their association with various metabolic pathways.

We initially focused on urinary radiation exposure markers, which have been reported in the literature and our previous work with external gamma exposure and another internal emitter, Cesium-137 (4, 6, 10, 11). These urinary metabolite markers are listed in Table 4 along with the direction of change in their abundances after exposure to different types of radiation. We confirmed the identities of these markers in the urine samples studied here via MS/MS using authentic chemical standards. The relative abundances of these ions in the urine of mice exposed to $^{90}$Sr at both average doses of 2.0 and 5.0 Gy were established using standard curves for each metabolite and shown as down arrows for decreased post-exposure urinary abundance, and up arrows for increased post-exposure urinary abundances.

Xanthurenic acid, tiglylcarnitine and hexanoylcarnitine were detected at lower levels in urine of mice after exposure to $^{90}$Sr, which is similar to what we observed in the urine of mice after exposure to $^{137}$Cs and X rays at low dose rate (3 mGy/min) (4, 10). However, no changes were observed in the urinary excretion of these metabolites after X irradiation at the high dose rate of 1 Gy/min. There are also no reports of attenuation in the urinary levels of these metabolites after 1 Gy gamma irradiation in the literature. Therefore, these metabolites may be specific to exposure to low dose rates from either an external beam source or an internal emitter such as $^{137}$Cs or $^{90}$Sr. The metabolites that have shown robust changes in their urinary excretion levels after exposure to internal emitters, low dose rate and high dose rate X and gamma irradiations are hippuric acid, citrate and uric acid. These metabolites may serve as general radiation exposure markers, while the former may serve as dose-rate specific markers.

Finally, we focused on determining the identities of urinary ions, which displayed the most statistically significant changes after internal exposure to $^{90}$Sr. Among
these ions, we were able to confirm the identities of pantothenic acid and riboflavin via tandem mass spectrometry. The urinary levels of these vitamin B metabolites showed more than a threefold decrease after exposure at both 2.0 and 5.0 Gy as shown in Fig. 3 and Table 5. The levels of these two metabolites did not recover by the end of the experiment. Because there was no change in diet and food intake of the mice throughout the study, the decrease in the levels of these two metabolites are indicative of prolonged internal exposure to $^{90}$Sr. Cofactors derived from

### TABLE 4

<table>
<thead>
<tr>
<th>Robust Metabolite Markers Common among Different Exposure Types</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>m/z</strong></td>
</tr>
<tr>
<td>------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
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<td>5</td>
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<tr>
<td>6</td>
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<td>7</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>9</td>
</tr>
</tbody>
</table>

**FIG. 3.** Six selected urinary metabolite markers of exposure to $^{90}$Sr, which follow similar decreasing trends after exposure. The decrease in the urinary excretion levels of these metabolites is seen as early as day 7 and 9 after exposure at the average skeleton dose of 2.0 Gy and lasts through the course of the 30 day study at the average dose of 5.1 Gy by day 25 and 30. The y axis on each figure shows the urinary levels of the metabolite with respect to an appropriate internal standard in the units of concentration (µM) and the x axis specifies the dose point.
Glutamate (Cl– adduct) Negative 182.0461 ± 0.8448 5.21 0.66 0.65
Glutaconic acid Negative 129.0201 ± 0.5645 9.65 0.40 0.52
Indolelactic acid Negative 204.0673 ± 3.9967 7.72 0.67 0.69
NA Positive 369.1908 ± 3.4312 NA 1.35 1.76
NA Positive 202.0193 ± 2.9741 NA 1.62 1.57
Pantothenic acid Positive 220.1190 ± 1.6270 6.36 0.17 0.13
Malate (Cl– adduct) Negative 167.9827 ± 0.3577 8.96 0.70 0.59
Quinolinic acid Negative 166.0182 ± 0.3361 6.10 0.86 0.80
NA Negative 129.0201 ± 0.5645 9.65 0.40 0.52
NA Negative 339.1637 ± 2.8520 NA 3.43 3.78

**TABLE 5**

<table>
<thead>
<tr>
<th>Name</th>
<th>ESI mode</th>
<th>m/z_RT</th>
<th>Error (ppm)</th>
<th>Fold change²</th>
<th>2.0 ± 0.28 Gy</th>
<th>5.0 ± 0.62 Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pantothenic acid</td>
<td>Positive</td>
<td>220.1190_1.6270</td>
<td>6.36</td>
<td>1.00</td>
<td>0.17</td>
<td>0.13</td>
</tr>
<tr>
<td>NA</td>
<td>Positive</td>
<td>202.0193_2.9741</td>
<td>NA</td>
<td>1.00</td>
<td>1.62</td>
<td>1.57</td>
</tr>
<tr>
<td>NA</td>
<td>Positive</td>
<td>369.1908_3.4312</td>
<td>NA</td>
<td>1.00</td>
<td>1.35</td>
<td>1.76</td>
</tr>
<tr>
<td>Indolelactic acid</td>
<td>Negative</td>
<td>204.0673_3.9967</td>
<td>7.72</td>
<td>1.00</td>
<td>0.67</td>
<td>0.69</td>
</tr>
<tr>
<td>Glutaconic acid</td>
<td>Negative</td>
<td>129.0201_0.5645</td>
<td>9.65</td>
<td>1.00</td>
<td>0.40</td>
<td>0.52</td>
</tr>
<tr>
<td>Glutamate (Cl– adduct)</td>
<td>Negative</td>
<td>182.0461_0.8448</td>
<td>5.21</td>
<td>1.00</td>
<td>0.66</td>
<td>0.65</td>
</tr>
<tr>
<td>Quinolinic acid</td>
<td>Negative</td>
<td>166.0182_0.3361</td>
<td>6.10</td>
<td>1.00</td>
<td>0.86</td>
<td>0.80</td>
</tr>
<tr>
<td>Malate (Cl– adduct)</td>
<td>Negative</td>
<td>167.9827_0.3577</td>
<td>8.96</td>
<td>1.00</td>
<td>0.70</td>
<td>0.59</td>
</tr>
<tr>
<td>NA</td>
<td>Negative</td>
<td>339.1637_2.8520</td>
<td>NA</td>
<td>1.00</td>
<td>2.84</td>
<td>3.01</td>
</tr>
<tr>
<td>NA</td>
<td>Negative</td>
<td>128.9610_0.2692</td>
<td>NA</td>
<td>1.00</td>
<td>3.43</td>
<td>3.78</td>
</tr>
</tbody>
</table>

— Identities of ions with names were validated via MS/MS using METLIN and HMDB databases.

— Fold change was calculated by dividing the average peak area of ion at each dose group by that of a matched control group.

Riboflavin (Fig. 3) and pantothenic acid (Table 5) are required for the conversion of pyruvate to acetyl-CoA as part of the energy production pathway. The resulting acetyl-CoA is then employed into adenosine triphosphate (ATP) production from the tricarboxylic acid (TCA) cycle. The decrease in the urinary levels of riboflavin and pantothenic acid is in line with a sharp decrease in the urinary excretion levels of TCA cycle intermediates, citrate and malate. In our previous study with internal exposure to 137Cs, we observed similar decreases in urinary excretion of citrate after exposure, with levels returning to those of pre-exposure by the end of the study. However, in the case of 90Sr exposure, the magnitude of attenuation in these two TCA cycle metabolites is greater and the levels do not recover to those of pre-exposure by the end of the 30-day period. Therefore, this persistent decrease in the urinary levels of ions appears to be unique to 90Sr exposure with more than 75% of the statistically significant urinary ions showing similar decreasing levels after exposure.

Among the identified and statistically significant metabolites were two dicarboxylic acids, glutaric acid and quinolinic acid (Table 5). Dicarboxylic acids are the result of fatty acid catabolism through β-oxidation. Therefore, dicarboxylic acids are part of the energy production pathway and changes in their urinary excretion may signal a shift in energy metabolism. The urinary excretion levels of these two dicarboxylic acids showed almost a twofold decrease after exposure throughout the course of the study. In addition, two intermediates in the tryptophan pathway metabolism, xanthurenic acid (Fig. 3) and indolelactic acid (Table 5) also showed significant decreases in their urinary excretion levels after exposure. The tryptophan pathway is also tied into the energy production machinery through acetyl-CoA. A decrease in its intermediates may result in a drop in the levels of free acetyl-CoA and slower energy production. Such decrease in the urinary excretion of metabolites associated with energy metabolism can point to a functional deficiency in mitochondria, which are the hub of energy production. Mitochondrial function and morphology are known to be affected under environmental stress and injury (12). Therefore, the results of this study along with our findings in our previous urinary metabolomics study of 137Cs exposure highlight the involvement of mitochondrial pathways, such as TCA cycle and lipid metabolism, in response to radiation exposure.

Moreover, the results of this study identified significant perturbations in butanoate metabolism, yet another mitochondrial pathway. Butanoate metabolism is linked directly to the TCA cycle through succinate and indirectly through pyruvate and acetyl-CoA (Fig. 4). It is also associated with alanine, aspartate and glutamate metabolism, which is further associated with TCA cycle through glutamate. We found the urinary levels of glutamate and subsequently 4-hydroxybutanoate to be significantly lower after exposure. Butanoate metabolism, like the TCA cycle, is a mitochondrial metabolic pathway, which is important for energy production, cell proliferation and protection against inflammatory factors in colonocytes. It has been shown that butanoate deficiency in colonocytes can lead to energy deprivation and ultimately autophagy (13). The decrease in the levels of butanoate post 90Sr exposure may signal a change in the energy production of colonocytes and a deficit in mitochondrial respiration. However, the majority of the dose was localized to the skeleton, so the target tissue for this drop in butanoate is uncertain. Interestingly, metabolites associated with butanoate metabolism were not found to be significantly perturbed in mice exposed to 137Cs or those exposed to external gamma and X rays. While 137Cs is primarily a gamma emitter, 137Cs/137Ba is also a beta-emitting radionuclide pair (1 beta per pair decay), so the unique 90Sr response is not beta-ray specific.
DISCUSSION

In this study we investigated the effects of exposure to internal $^{90}$Sr in mice through metabolomics. This study follows our earlier work on the effects of exposure to another internal emitter, $^{137}$Cs (4). Both $^{137}$Cs and $^{90}$Sr irradiate the organs and tissues of the mouse for the duration of the study. However, the spatial distribution of dose is different for the two radionuclides. About 95% of $^{137}$Cs decays by beta emission to $^{137m}$Ba, which decays emitting photon energy of 662 keV. Because of the high-energy gamma irradiation associated with the cesium deposition in the skeletal muscle tissue, the dose distribution in the body is uniform. However, $^{90}$Sr decays by beta emission to $^{90}$Y, which also decays by beta emission. Therefore, $^{90}$Sr + $^{90}$Y can be considered as pure beta emitters, with most of the committed dose delivered to skeleton tissue and bone marrow. Thus, the aim of the current study was to identify a unique metabolomic signature for $^{90}$Sr.

The urine from mice exposed to $^{90}$Sr at two cumulative doses, 2.0 ± 0.28 Gy and 5.0 ± 0.62 Gy, was collected in the course of this 30 day study. The overall urinary metabolomic signature of the control mice was distinctly different from that of mice after 2.0 and 5.0 Gy exposure based on the top 25 variable ions as ranked by Random-Forest in Supplementary Fig. S1A (http://dx.doi.org/10.1667/RR14011.1.S1). Although the overall metabolomic signatures of the two dose/time points are also distinguishable, there is a persistent decrease in the urinary levels of the top ranked ions after exposure as shown in Supplementary Fig. S2B (http://dx.doi.org/10.1667/RR14011.1.S1). The results of this preliminary statistical analysis in Random-Forest showed attenuation in the levels of many metabolites after exposure, with a few metabolites showing an increase in their urinary excretion levels after exposure. This is also evident from subsequent dose-specific analysis in MetaboLyzer, as shown in the Fig. 1 volcano plot. These results are in contrast to what we observed in the urine of mice

![Diagram of metabolic pathways](http://dx.doi.org/10.1667/RR14011.1.S1)
exposed to $^{137}$Cs. However, a closer look at individual urinary ions revealed that these two internal emitters affect the levels of several ions similarly. For instance, the levels of xanthurenic acid, tiglylcarnitine, hexanoylcarnitine, hippuric acid, citrate and uric acid showed similar perturbations after exposure to $^{90}$Sr and $^{137}$Cs (Table 4). These ions have also been reported in studies with external beam irradiation (Table 4). While these ions may be common to external beam irradiation and internal emitter exposure ($^{137}$Cs and $^{90}$Sr), each radiation type produces a unique metabolomic signature, which may be used to determine the type of exposure in a biological sample. For example, $^{90}$Sr exposure resulted in persistent attenuations in majority of the statistically significant ions, while $^{137}$Cs exposure resulted in increases in more than half the detected urinary ions. The attenuation in the urinary levels of ions after $^{137}$Cs exposure were dose specific and levels reverted back to those of pre-exposure by the end of the 30 day study (4). Therefore, the widespread persistent decreases in levels of urinary ions is unique to $^{90}$Sr exposure.

Furthermore, pathway analysis of the statistically significant ions mapped 3-hydroxybutanoate, acetoacetate, and 4-aminobutanoate, to butanoate metabolism. The urinary levels of these metabolites decreased significantly after $^{90}$Sr exposure. Butanoate metabolism is a mitochondrial pathway and is central to energy production in colonocytes. This pathway is crosslinked to TCA cycle, glycolysis, vitamin B metabolism, fatty acid oxidation, and glutamate metabolism (Fig. 4). We have identified at least two members of each of these pathways showing uniform significant decreases after $^{90}$Sr exposure, which may be unique to exposure to beta-particle emission of $^{90}$Sr. Furthermore, butanoate metabolism and TCA cycle are two important mitochondrial pathways involved in energy production. Therefore, any shift in the levels of metabolites associated with these pathways may signal a change in mitochondrial function and morphology.

Although $^{90}$Sr is referred to as a “bone seeker” due to having similar physical and chemical properties as calcium, we were not able to find any markers of injury to bone or surrounding soft tissue in the urine of mice in this study. This is probably because leukemia and soft tissue injury have been shown to occur after long-term chronic exposure to higher doses of $^{90}$Sr than those reported in this study (4).

**CONCLUSION**

In this study, we established a robust and unique metabolomic response to $^{90}$Sr exposure in mice using urine, an easily accessible biofluid. Butanoate metabolism demonstrated the most significant perturbations in its associated metabolites. In addition, metabolites associated with TCA cycle, and vitamin B metabolism displayed significant changes in their urinary excretion levels. Although much work remains to establish a panel of field-deployable $^{90}$Sr-exposure markers, butanoate metabolism appears to be a unique and promising target.

**SUPPLEMENTARY INFORMATION**

**Fig. S1.** The overall urinary metabolomic signature of mice exposed to $^{90}$Sr at two average dose points (2.0 and 5.0 Gy) is clearly distinguishable from that of control mice. **Fig. S2.** The overall metabolomic signatures of the two dose/time points are also distinguishable, there is a persistent decrease in the urinary levels of the top ranked ions after exposure.

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