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Liquid Chromatography–Mass Spectrometry-Based Metabolomics of Nonhuman Primates after 4 Gy Total Body Radiation Exposure: Global Effects and Targeted Panels

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Abstract

Rapid assessment of radiation signatures in non-invasive biofluids may aid in assigning proper medical treatments for acute radiation syndrome (ARS) and delegating limited resources after a nuclear disaster. Metabolomic platforms allow for rapid screening of biofluid signatures and show promise in differentiating radiation quality and time postexposure. Here, we use global metabolomics to differentiate temporal effects (1–60 d) found in nonhuman primate (NHP) urine and serum small molecule signatures after a 4 Gy total body irradiation. Random Forests analysis differentially classifies biofluid signatures according to days post 4 Gy exposure. Eight compounds involved in protein metabolism, fatty acid β oxidation, DNA base deamination, and general energy metabolism were identified in each urine and serum sample and validated through tandem MS. The greatest perturbations were seen at 1 d in urine and 1–21 d in serum.

Furthermore, we developed a targeted liquid chromatography tandem mass spectrometry (LC–MS/MS) with multiple reaction monitoring (MRM) method to quantify a six compound panel (hypoxanthine, carnitine, acetylcarnitine, proline, taurine, and citrulline) identified in a previous training cohort at 7 d after a 4 Gy exposure. The highest sensitivity and specificity for classifying exposure at 7 d after a 4 Gy exposure included carnitine and acetylcarnitine in urine and taurine, carnitine, and hypoxanthine in serum. Receiver operator characteristic (ROC) curve analysis using

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These mass spectrometry data have been deposited to the NIH data repository via Metabolomics Workbench with the data set identifier epannkuk_20190227_104940 (untargeted urine), epannkuk_20190228_060422 (untargeted serum), epannkuk_20190227_122530 (targeted urine), epannkuk_20190228_054732 (targeted serum).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.jproteome.9b00101](https://doi.org/10.1021/acs.jproteome.9b00101). Figures S1–S3, Tables S1–S3 (PDF)

combined compounds show excellent sensitivity and specificity in urine (area under the curve [AUC] = 0.99) and serum (AUC = 0.95). These results highlight the utility of MS platforms to differentiate time postexposure and acquire reliable quantitative biomarker panels for classifying exposed individuals.

Graphical Abstract

Keywords

targeted metabolomics; ionizing radiation; nonhuman primates; biodosimetry

1. INTRODUCTION

Due to threats ranging from malicious use of radioactive materials against the public and increasing presence from industrial waste (e.g., nuclear power plants, medical devices), several concerted efforts from international entities have focused on radiological countermeasures and recovery, such as radiation biodosimetry and mitigation (National Institute of Allergy and Infectious Diseases [NIAID]), standardized medical treatment protocols (METREPOL) (European Atomic Energy Community [EAEC]), among others, to minimize damage from large scale radiation exposures.^{1–3} Acute radiation syndrome (ARS) describes a plethora of symptoms after total body irradiation (TBI) or a significant partial body irradiation at doses >0.5 Gy.⁴ Symptoms manifest as a dynamic interplay from multiorgan damage, the most critical arising from dysfunction to hematopoietic, gastrointestinal (GI), neurovascular, and cutaneous organ systems.^{5,6} Mild levels of radiation exposure (<2 Gy) would require minor if any medical treatment (vomiting, headache, and fever would be present in some), as a full recovery would be expected (unless exacerbated by combined injury or chronic illness).

Higher exposure levels (2–4 Gy) exhibit increased severity of the above symptoms and onset of hematopoietic syndrome and the associated lymphopenia, thrombocytopenia, and granulopenia.⁷ This exposure range poses an important point of ARS, as possible mortality

could be prevented with proper supportive care (i.e., cytokine therapy), and high throughput dose estimates could aid in defining medical treatments. Another critical parameter is time of assessment after exposure, since casualties will probably arrive for triage and assessment at varying times after exposures. To address this issue, the NIAID Centers for Medical Countermeasures Against Radiation (CMCR) program chose a sublethal TBI dose that would allow the entire cohort to be assessed over a 60 d time span. Currently, the lack of rapid and reliable dose and time estimates (including exposure distribution and rate) leaves trained medical staff to rely solely on symptoms as an indicator of ARS.¹

Recent efforts have explored the use of metabolomics (collective analysis of biomolecules <1 kDa) to provide quicker dose estimates of ARS by measuring perturbed metabolite levels in biofluids.⁸ Ionizing radiation can damage tissues and cellular targets by directly interacting with biomolecules or through free radical formation. The sum effect of this damage can produce small molecule “signatures” representative of ARS severity. For dose exposures in the 2–4 Gy range, metabolomic signatures have been more easily characterized in murine models (mice and rats) for various exposure types and time points (including external X-ray, ⁶⁰Co, and ¹³⁷Cs exposures and internal emitters [¹³⁷CsCl, ^{85/90}SrCl₂]). However, little has been explored for nonhuman primates (NHPs) and is limited to urine (12–72 h, 1 and 3.5 Gy; ⁶⁰Co exposure) or 7 d samples (2 and 4 Gy; ¹³⁷Cs exposure).^{8,9} While work on murine models is critical for initial discovery phase experiments, NHP models are paramount for comparison to human populations.^{10,11} Additionally, validation cohorts are required to assess repeatability and clinical utility.

In this study, we determined temporal (1–60 d) perturbed metabolite levels in NHP urine and serum using a liquid chromatography mass spectrometry (LC–MS) global metabolomics approach after 4 Gy TBI. We found a number of perturbed metabolites and were able to differentiate time points through multivariate analysis. In addition, we selected a panel of metabolites that were significantly altered in previous studies at 7 d post 4 Gy TBI^{12–14} and developed a rapid LC–MS/MS with multiple reaction monitoring (MRM) assay for simultaneous absolute quantification in biofluids and applied to both the previous and current cohort. While sample sizes are modest, here we refer to a previous cohort¹² ($n = 12$) as the training cohort and the current cohort ($n = 8$) as the validation cohort. A combination of three compounds (serum) and two compounds (urine) had excellent sensitivity and specificity for classifying 7 d post 4 Gy exposure. These results further highlight the dynamic state of metabolite flux after irradiation and the importance of timing for biodosimetry. Also, while NHP studies have remained “prohibitively” costly for designs with large sample sizes, validation of potential markers from previous studies is an absolute necessity for ultimate clinical use.

2. MATERIALS AND METHODS

2.1. Chemicals

All reagents were LC–MS optima grade (Fisher Scientific, Hanover Park, IL, USA) and all standards were of the highest purity available. Standards were acquired from Sigma-Aldrich (St. Louis, MO, USA) (hypoxanthine, L-carnitine, acetyl-L-carnitine, propionyl-L-carnitine, isobutyryl-L-carnitine, 2-methylbutyryl-L-carnitine, 7-methylguanine, creatine, xanthine,

asymmetric dimethylarginine [ADMA], guanine, oleamide, L-citrulline, L-proline, taurine, 4-nitrobenzoic acid, and debrisoquine sulfate), CDN isotopes (Pointe-Claire, QC, Canada) (carnitine- d_3 , acetyl- d_3 -carnitine, citrulline- d_7 , and proline- d_3), and Cambridge Isotope Laboratories, Inc. (Andover, MA, USA) (hypoxanthine- d_3 and taurine- $^{13}C_2$).

2.2. Nonhuman Primate System

Rhesus monkeys (*Macaca mulatta*) for the validation cohort were irradiated at the same AAALAC accredited facility as the previous training cohort^{12,3} and included 8 individuals (half male, half female, avg. 6.0 yrs. and 8.3 kg). NHPs were provided with commercial chow (Teklad certified hi-fiber primate diet #7195C, Envigo, Madison, WI, USA) twice daily, and fresh fruits, vegetables, or juice were intermittently provided. Animals were housed in an environment-controlled facility (temperature, 21 °C ± 3 °C; relative humidity, 50% ± 20%; 12 h light/dark cycles) individually for at least 7 d postexposure, and then healthy animals were paired or group housed. Food was removed the night before TBI, where NHPs were acclimated to the radiation facility and exposed to a total dose of 4 at 0.6 Gy/min with a ^{60}Co γ source over the course of 7 min (Theratron 1000, Best Theratronics, Ottawa, ON, CA) (½ exposure antero-posterior position, ½ exposure postero-anterior position). For this particular animal model, the LD_{50/60} is ~6.7 Gy and at 4 Gy severe weakness and recumbency is observed in ~40% of individuals if antiemetics are withheld. Radiation exposure was confirmed with a Farmer ionization chamber (PTW, Brooklyn, NY, USA) (primary) and two nanoDot dosimeters (Landauer Inc., Glenwood, IL, USA) (secondary, placed midplane of xiphoid and corresponding dorsal area). Animals received ondansetron (2 mg/mL, 1.5 mg/kg, IM) before (45–90 min) and after (30–45 min) irradiation for emesis. Clinical signs were recorded daily after irradiation, and detailed examinations were performed at 1, 3, 5, and 7 d and then weekly. Pre-exposure samples were collected at –8 and –3 d and postexposure samples collected at 1, 3, 5, 7, 15, 21, 28, and 60 d. Samples were frozen (–80 °C) and shipped to Georgetown University Medical Center (GUMC) at the end of the experiment. The study animals, experimental treatment, and biofluid collection for the previous training cohort have already been described.^{12–15} Briefly, NHPs were exposed to a single TBI of 4 Gy (exposure dose rate: 0.6 Gy/min, ^{60}Co γ source), and urine and serum were collected at 7 d and shipped to GUMC. Additional biofluids were collected from a separate unirradiated cohort and used as a control. All animal handling procedures were approved by the Institutional Animal Care and Use Committee.

2.3. Sample Preparation and LC–MS Instrumentation

For global metabolic profiling, urine (20 μ L) was deproteinated with 80 μ L 50% cold acetonitrile with internal standards (2 μ M debrisoquine, 176.1188 [M + H]⁺; 30 μ M 4-nitrobenzoic acid, 166.0140 [M – H][–]), vortexed, placed on ice for 10 min, and centrifuged for 10 min (10 000g, 4 °C).¹² Serum (5 μ L) was deproteinated with 195 μ L 66% cold acetonitrile and prepared as above.¹³ Samples were injected (2 μ L) and analyzed by ultra performance (UP) LC (column: Acquity BEH C18 1.7 μ m, 2.1 × 50 mm [40 °C urine, 60 °C serum]) coupled to a Xevo G2 quadrupole time-of-flight (QTOF) MS (Waters, Milford, MA, USA). Both negative and positive electrospray ionization (ESI) data independent acquisition modes were run with leucine enkephalin (556.2771 [M + H]⁺ or 554.2615 [M – H][–]) used as

LockSpray. Compounds of interest were validated through tandem MS with a 5–50 V ramping collision energy and comparing fragmentation patterns and m/z to pure standards or online databases if standards were unavailable.^{16,17} Mobile phases consisted of water/0.1% formic acid (solvent A), acetonitrile/0.1% formic acid (solvent B), and isopropanol/acetonitrile (90:10)/0.1% formic acid (solvent C). For urine, the flow rate was 0.5 mL/min with a gradient of (solvent A and B) 4.0 min 5% B, 4.0 min 20% B, 5.1 min 95% B, and 1.9 min 5% B. Blanks and quality control (QC) samples were run after every 10 samples. For serum, the flow rate was 0.5 mL/min with a gradient of (solvent A, B, and C) 4.0 min 98:2 A:B, 4.0 min 40:60 A:B, 1.5 min 2:98 A:B, 2.0 min 2:98 A:C, 0.5 min 50:50 A:C, and 1.0 min 98:2 A:B. Blanks were run after every 5 samples, and QC samples run after every 10.

For targeted profiling, serum (5 μ L) was diluted with 200 μ L cold methanol:acetonitrile:water (80:15:5) spiked with internal standards (1.25 μ g/mL proline- d_3 ; 2.5 μ g/mL carnitine- d_3 , acetyl- d_3 -carnitine, citrulline- d_7 , hypoxanthine- d_3 , and taurine- $^{13}C_2$), vortexed, placed on ice for 10 min, and centrifuged for 10 min (10 000g, 4 °C). Calibration curves were prepared from 0.001–5000 ng/mL of standards spiked into charcoal stripped pooled human serum (Innovative Research, Novi, MI, USA) and QCs were prepared at 3 concentrations 5 (low), 50 (medium), and 500 (high) ng/mL. Urine (10 μ L) was prepared as above with calibration curves prepared in both solvent and pooled urine. Urine was diluted with an additional 600 μ L solvent for quantification of carnitine and taurine. Blanks, internal standards, QCs, samples, and calibration curves were injected (5 μ L) with samples into a Waters ACQUITY UPLC coupled to a Xevo tandem quadrupole (TQ-S) MS operating in MRM mode (column: Acquity BEH amide 1.7 μ m 2.1 $\times$ 100 mm) maintained at 30 °C. MRM transitions were obtained using the “IntelliStart” feature in MassLynx (Table 1) and limit of detection (LOD), limit of quantification (LOQ), range, and linearity of all compounds assessed (Table S1). Mobile phase consisted of water with 10 mM ammonium formate/0.2% formic acid (solvent A) and acetonitrile with 10 mM ammonium formate/0.2% formic acid (solvent B). The flow rate was 0.4 mL/min with a gradient of 2.1 min 100% B, 2.9 min 94% B, 1 min 82% B, 0.1 min 70% B, and 1.4 min 100% B.

2.4. Statistical Analysis

The global LC–MS analysis data was processed as previously described.¹³ After manual inspection in MassLynx v4.1, deconvolution and peak alignment was performed in Progenesis QI (Nonlinear Dynamics, Newcastle, UK) and the raw data file was normalized to the internal standard (debrisoquine [M + H]⁺, 4-nitrobenzoic acid [M – H][–]), and analyzed by unequal variances t tests (Welch’s t test, spectral features present >70%) or presence-absence models (Barnard’s test, spectral features present <70%) in the software MetaboLyzer.¹⁸ The MetaboLyzer data input was normalized as previously described including outlier removal and false discovery rate calculations for multiple testing corrections.¹³ MetaboLyzer was also used for generating putative identification and pathway analysis (± 10 ppm error) of spectral features through the Human Metabolome Database (HMDB), the Kyoto Encyclopedia of Genes and Genomes, BioCyc, and chemical entities of biological interest (ChEBI).^{18–23} Preexposure samples (–8 and –3 d) were averaged for the control group. The Random Forests (RF) machine learning algorithm through R

programming language was used to produce multidimensional scaling (MDS) plots and heatmaps.²⁴ Variable importance of metabolites was determined using the RF function and measuring increasing unbiased estimate of the classification error after permutation in MetaboAnalyst.^{22,25} For validated compounds, outliers were removed using robust regression and outlier removal and graphed in GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). For targeted LC–MS/MS analysis, raw data files were processed and analyzed using TargetLynx v4.1. The quantitative data output was then graphed and analyzed by a *t* test in GraphPad Prism 6.0 and combined ROC curves were generated in MetaboAnalyst using the linear support vector machine (SVM) model function.

3. RESULTS

3.1. Global LC–MS Metabolomics

3.1.1. Urine.—A RF algorithm was used to produce a MDS plot and heatmap of the top 100 ranked ions (ESI⁺) for visualization of the urine metabolomic data matrix (Figure 1). Observation of the MDS plot and heatmap showed the urinary metabolic signature deviates from pre-exposure samples mainly along dimension 2 at 1 d due to increased concentrations of several metabolites (Figure 1). The 3–7 d groups were more similar to pre-exposure at 4 Gy; however, better separation of NHPs 7 d post 4 Gy exposure has been observed through RF machine learning when only dose effect is considered.¹² A second cluster of spectral features (Figure 1B top) may influence 15–28 d groups to separate from pre-exposure samples along dimension 1, again returning at 60 d. Univariate analysis revealed significant increases in carnitine ($P < 0.001$, 208.4 fold change), acetylcarnitine ($P < 0.001$, 200.4 fold change), propionylcarnitine ($P < 0.001$, 107.7 fold change), creatine ($P < 0.001$, 18.5 fold change), 7-methylguanine ($P = 0.002$, 1.9 fold change), xanthine ($P = 0.028$, 1.4 fold change), and ADMA ($P < 0.001$, 2.1 fold change) in urine at 1 d (Table 2 and S2, Figure 2). Isobutyrylcarnitine or butyrylcarnitine also increased at 1 d in urine ($P = 0.012$, 2.9 fold change); however, we were unable to differentiate between isomers based on fragmentation pattern (represented as isobutyrylcarnitine/butyrylcarnitine). Most metabolites returned closer to basal levels; however, carnitine (3 d [$P = 0.004$, 27.8 fold change], 5 d [$P = 0.023$, 14.5 fold change], 7 d [$P = 0.012$, 11.7 fold change]), acetylcarnitine (7 d [$P = 0.005$, 8.0 fold change]), propionylcarnitine (3 d [$P = 0.015$, 11.5 fold change], 7 d [$P = 0.024$, 6.3 fold change]), and 7-methylguanine (28 d [$P = 0.030$, 1.5 fold change]) remain slightly higher in urine. Variable importance of validated metabolites was determined with a RF algorithm showing the highest rank for carnitine and acetylcarnitine (Figure S1).

3.1.2. Serum.—The heatmap produced for the serum data matrix (ESI⁺) showed general decreases in concentrations for compounds 5–60 d postexposure (Figure S2) and lower concentration variability as the urine data matrix. While metabolite levels remained relatively steady compared to urine, a higher dynamic flux across 60 d postexposure is observed.

Increases were observed in carnitine (1 d [$P < 0.001$, 1.8 fold change], 3 d [$P = 0.004$, 1.7 fold change], 5 d [$P = 0.015$, 1.6 fold change], 7 d [$P = 0.045$, 1.4 fold change]), and acylcarnitines including isobutyrylcarnitine/butyrylcarnitine (1 d [$P = 0.025$, 6.4 fold

change], 3 d [$P = 0.028$, 4.1 fold change], 7 d [$P = 0.036$, 4.9 fold change], 28 d [$P = 0.004$, 3.3 fold change]), propionylcarnitine (1 d [$P = 0.003$, 3.0 fold change], 3 d [$P = 0.028$, 1.3 fold change]), 2-methylbutyrylcarnitine (3 d [$P < 0.001$, 3.5 fold change], 7 d [$P = 0.022$, 2.4 fold change], 15 d [$P = 0.044$, 3.8 fold change]), and 3-dehydroxycarnitine (1 d [$P < 0.001$, 1.6 fold change], 3 d [$P < 0.001$, 1.8 fold change], 5 d [$P = 0.017$, 1.3 fold change]), (Table 2, Figure 3). Oleamide also slightly increased after 2 weeks (15 d [$P = 0.012$, 2.5 fold change], 21 d [$P = 0.002$, 2.2 fold change]). Decreases were observed in hypoxanthine (1 d [$P < 0.001$, 0.8 fold change], 5 d [$P = 0.012$, 0.7 fold change], 7 d [$P = 0.001$, 0.8 fold change], 15 d [$P < 0.001$, 0.3 fold change], 21 d [$P < 0.001$, 0.1 fold change], 28 d [$P = 0.005$, 0.3 fold change], 60 d [$P = 0.043$, 0.7 fold change]), and guanine (7 d [$P = 0.005$, 0.5 fold change], 15 d [$P < 0.001$, 0.1 fold change], 21 d [$P < 0.001$, 0.1 fold change], 28 d [$P = 0.030$, 0.5 fold change]). Variable importance of validated metabolites showed the highest rank for guanine followed by hypoxanthine, carnitine/acylcarnitines, and oleamide (Figure S1).

3.2. Targeted LC–MS/MS Metabolomics

As previous research has been conducted by our group on NHPs at 7 d postexposure at multiple doses, we developed a quantitative targeted LC–MS/MS with MRM assay for previously identified significant metabolites (training cohort [$n = 12$]; 7 d post 4 Gy exposure) (Figure S3) and utilized the current cohort ($n = 8$) as a validation set to evaluate classification performance (method validation parameters Table 1 and S1; validated compounds [training cohort] are provided in Table S3).^{12–15} Biomarker selection was based on metabolites from the training cohort with excellent (citrulline [$P = 0.004$, AUC = 1.00]), good (carnitine [$P = 0.006$, AUC = 0.89], acetylcarnitine [$P = 0.007$, AUC = 0.87]), to fair (taurine [$P = 0.068$, AUC = 0.75]) sensitivity and specificity in urine (Figure S3). Likewise, serum markers showed excellent (hypoxanthine [$P < 0.001$, AUC = 0.98], taurine [$P < 0.001$, AUC = 0.99], citrulline [$P = 0.001$, AUC = 0.95], proline [$P < 0.001$, AUC = 0.94]) to fair (carnitine [$P = 0.038$, AUC = 0.74]) sensitivity and specificity (Figure S3).

In the validation cohort, both carnitine ($P = 0.022$, AUC = 0.80) and acetylcarnitine ($P = 0.004$, AUC = 0.91) showed significant increases in urine (Figure 4); however, taurine ($P = 0.823$, AUC = 0.51) and citrulline ($P = 0.705$, AUC = 0.52) did not significantly change. In serum, carnitine ($P = 0.021$, AUC = 0.77), hypoxanthine ($P < 0.001$, AUC = 0.95), and taurine ($P = 0.001$, AUC = 0.85) significantly changed (Figure 4). Proline ($P = 0.397$, AUC = 0.57) and citrulline ($P = 0.147$, AUC = 0.72) slightly decreased, but not significantly. AUC values for combined compounds have excellent sensitivity and specificity for both urine (AUC = 0.99) and serum (AUC = 0.95).

4. DISCUSSION

NHPs were exposed to 4 Gy TBI ($LD_{50/60}$ is ~6.7 Gy), and biofluids (urine and serum for the current study) were collected for a 60 d postexposure duration. A comparable dose in humans would be ~2.2 Gy ($LD_{50/60}$ is ~4.5 Gy) and would elicit minor hematopoietic syndrome.^{26,27} Generally, ARS levels can be classified as mild (<2 Gy), moderate (2–4 Gy), severe (4–6 Gy), very severe (6–8 Gy), to lethal (>8 Gy) exposures. While milder exposures

elicit less severe symptoms (e.g., headache and vomiting), the moderate exposure range may lead to potentially lethal levels that can be ameliorated with supportive care or more active measures.⁷ We performed global metabolomic analyses for assessment of overall temporal perturbations and found distinct separation of time points by multivariate analysis. Previously, we assessed dose effects (2, 4, 6, 7, and 10 Gy) on NHP urine and serum signatures 7 d postexposure utilizing LC and GC global profiling^{12,13,15} and demonstrated rapid analysis of a metabolite panel with targeted differential mobility spectrometry (DMS) MS.²⁸ Here, we selected an enriched panel of metabolites and established a targeted quantitative LC–MS/MS with MRM method to determine repeatability in a validation cohort, which is required to ultimately determine their utility in clinical settings. Of the previously identified metabolites tested, acetylcarnitine (urine), taurine (serum), hypoxanthine (serum), and carnitine (urine and serum) exhibited excellent to fair sensitivity and specificity.

Urinary metabolites showed the greatest fold changes 1 d postexposure, while serum levels exhibited a more dynamic flux state along the 60 d time course. Indicators of purine deamination included perturbed levels of guanine (serum), hypoxanthine (serum), xanthine (urine), and 7-methylguanine (urine) after 4 Gy exposure. Xanthine can be formed from hypoxanthine oxidation or deaminated guanine and has been identified in other radiation metabolomic studies including NHP,^{9,12} human,²⁹ and mouse urine.³⁰ Levels of xanthine fluctuate in urine with increases at 1 d and 28–60 d but decreased from 3–7 d, which was observed in the previous NHP cohort at 7 d.¹² Similarly, 7-methylguanine increased in urine at 1 and 28 d, and its presence has been implicated in DNA depurination, indicative of damage from endogenous processes.³¹ Another study found hypoxanthine formation through adenine deamination that may drive further downstream mutagenesis.³² Hypoxanthine has been found in lower levels in serum at 7 d¹³; however, here it was found at its lowest levels ~15–28 days along with guanine. These results indicate unique metabolomic signatures during the initial prodromal phase by possible increasing apoptosis or purine deamination, leading to increasing excretion rates in urine with levels returning to a perturbed state at a second phase ~15–28 days after exposure.

Carnitine and acylcarnitines are integral to fatty acid β oxidation and have been implicated in numerous metabolic disorders³³ as well as markers of radiation exposures (both external exposure and internal emitters) in biofluids from humans and multiple animal models.^{12–14,29,34–38} As circulating carnitine levels are maintained through proper renal function, the increasing concentrations that have been observed may be partly due to radiation-induced renal toxicity³⁹ in addition to fatty acid β oxidation disorders. Here we also found increases in two novel acylcarnitine metabolites in serum after irradiation, 3-dehydroxycarnitine and 2-methylbutyrylcarnitine. 2-Methylbutyrylcarnitine has been putatively identified in autosomal dominant polycystic kidney disease,⁴⁰ while 3-dehydroxycarnitine reductions are lowered in skeletal muscle after exercise in *db/db* mice (*Lepr^{db}*)⁴¹ and naturally higher in *ob/ob* mice (*Lepr^{ob}*) (mutations leading to obesity and diabetes) compared to wild type.⁴² Although little biological information is known for these two unusual and branched-chain acylcarnitines (as well as other acylcarnitines), other than their association with perturbed energy metabolism and certain enzyme dysfunctions, the

drastic increases across multiple animal models after IR exposure highlights their importance as robust radiation biomarkers of possible mitochondrial dysfunction.

Both dimethyl-L-arginine (ADMA) and creatine increased in urine at 24 h postirradiation then returned to basal levels. ADMA was previously identified as lower in NHP serum postexposure; however, concentration values of the previous study were below LOD and thus not reported.¹⁴ ADMA inhibits nitric oxide synthase and may lead to endothelial or cardiovascular dysfunction.⁴³ Previous reports show little change in urinary creatine levels at 0.5 d with a 3.5 Gy dose but increases at 1.5 d with a 6.5 Gy dose.⁹ Also, creatine was recently reported to increase in serum at 1 d after a 7.2 Gy exposure.⁴⁴ Possible sources of creatine levels from direct damage to muscle and subsequent atrophy may confound its use when attempting a dose level prediction.⁴⁵ Finally, serum levels of oleamide significantly increased 15–21 d post-exposure. Oleamide is a fatty amide thought to interact with multiple neurotransmitter systems, suppress inflammatory cytokines,⁴⁶ and act synergistically with radiation exposure in limiting tumor growth.⁴⁷ Possible roles for oleamide concentration fluctuations in response to radiation exposure remain to be explored.

While previous studies have identified numerous compounds that create unique molecular signatures after radiation exposure,⁸ validation cohorts are required to assess performance for future clinical utility. Validation cohorts for repeatability studies have been lacking for NHP studies due to ethical and financial factors. We established a quantitative method for a panel of metabolites (hypoxanthine, carnitine, acetylcarnitine, proline, taurine, and citrulline) due to their observed high-fold changes in the training NHP cohort and consistent identification in humans and other animal models. In the training cohort, carnitine and acetylcarnitine show increases in both urine and serum, with the greatest fold change in urinary acetylcarnitine levels (Figure S3). In the current validation cohort, urinary acetylcarnitine and serum carnitine have a combined excellent AUC value (0.99) indicating high sensitivity and specificity. Additionally, serum levels of taurine, hypoxanthine, and carnitine show high sensitivity and specificity in the validation cohort with an excellent AUC value (0.95). Serum acetylcarnitine levels were previously reported as slightly higher at 4 Gy,¹⁴ however, there was not a significant change observed in the current study ($P = 0.582$, 1.1 fold change) and this compound may only be useful at higher exposure levels that would elicit hematopoietic and GI syndrome. One robust marker of radiation induced GI syndrome, citrulline,^{48–50} showed high fold decreases and AUC values for both urine and serum in the previous cohort along with blood proline levels. The current cohort revealed similar trends in citrulline and proline levels but much lower fold changes. As circulating citrulline is released from enterocytes, their role at lower exposure levels with little GI damage may be small to negligible. These results highlight both the requirement of validation cohorts along the utility of multicomponent panels for classifying ARS severity from pre-exposure.

5. CONCLUSION

For MS based metabolomics to transition to practical clinical use for biodosimetry, a panel of diagnostic biomarkers must be assembled and validated to create ready-to-use “kits” that can be processed with available software and instrumentation. The use and repurposing of existing infrastructure and products are critical for health emergency preparedness, as

current budgets are limited and new technology development can be time-consuming.^{51,52} Commercially available MS platforms currently in clinical use could serve as a valuable tool for differentiating ARS severity, as they would be operational with trained staff. In this study, we found that urinary metabolic signatures are more appropriate for differentiating exposed vs nonexposed at earlier time points (1 d), while serum signatures are more dynamic and may be useful at later time points (e.g., 7 d) at 4 Gy. As single compounds are not sufficient to precisely determine radiation exposure in individuals without a priori knowledge, we developed a targeted LC–MS/MS MRM method in addition to our previous multiplex DMS MS assay^{28,53} to rapidly obtain quantitative values for a panel of metabolites and applied it to a validation NHP cohort. Combined metabolite ROC curve analysis showed excellent classification of minor ARS from pre-exposure samples to 7 d post 4 Gy exposure. Future efforts will incorporate additional compounds into our quantitative LC–MS/MS method to further refine dose estimates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Random Forests analysis of urine with (A) multidimensional scaling (MDS) plot and (B) heatmap. Both represent the top 100 ranked ions in positive mode. The 1 d postexposure samples separate from pre-exposure the most along dimension 2 in the MDS plot and the heatmap shows higher concentrations for many compounds at this time point. Postexposure groups are more similar to pre-exposure at 3–7 d, separate along dimension 1 from 15–28 d, then return to pre-exposure at 60 d.

Figure 2.

Urine metabolites perturbed in a nonhuman primate model after 4 Gy total body irradiation at 1, 3, 5, 7, 15, 21, 28, and 60 d. The primary perturbations to metabolites occur at 1 d as observed in all metabolites ($*P < 0.05$ determined by a Welch's t test between groups, mean $\pm$ S.E., pre-exposure samples [-8 and -3 d] were averaged for the control group).

Figure 3.

Serum metabolites perturbed in a nonhuman primate model after 4 Gy total body irradiation at 1, 3, 5, 7, 15, 21, 28, and 60 d. More variation is observed across 60 d postexposure compared to urine, as carnitine and acylcarnitines primarily fluctuate within 7 d, guanine and oleamide significantly change after 7 d postexposure, and hypoxanthine decreases through 60 d with the lowest values observed at 15–28 d ($*P < 0.05$ determined by a Welch's t test between groups, mean $\pm$ S.E., identification of 3-dehydroxycarnitine based on in silico MS/MS spectrum, pre-exposure samples [–8 and –3 d] were averaged for the control group).

Figure 4.

Targeted quantitative analysis and receiver operator characteristic curves of metabolites in nonhuman primate urine and serum at 7 d post 4 Gy exposure. (A) Training cohort urine, (B) validation cohort urine, (C) training cohort serum, (D) validation cohort serum (bar graphs generated in GraphPad Prism 6.0 and compared with a *t* test, mean $\pm$ S.E., and ROC curves generated in MetaboAnalyst, blue shading 95% confidence interval).

Table 1.
Multiple Reaction Monitoring Transitions and Parameters for Compounds and Internal Standards in ESI⁺

metabolite	parent ion (<i>m/z</i>)	daughter ion (<i>m/z</i>)	dwell time (sec)	cone (eV)	collision (eV)	retention time (min)
Hypoxanthine	137.110	109.930	0.009	44	20	0.93
Hypoxanthine- <i>d</i> ₃	138.831	111.859	0.025	100	20	0.94
Carnitine	162.260	84.990	0.025	34	20	1.08
Carnitine- <i>d</i> ₃	164.905	85.038	0.025	94	14	1.09
Acetyl/carnitine	203.956	85.077	0.010	72	10	0.90
Acetyl- <i>d</i> ₃ -carnitine	206.916	85.021	0.010	80	8	0.91
Proline	116.000	70.100	0.009	20	10	1.32
Proline- <i>d</i> ₃	119.000	73.100	0.009	20	10	1.33
Taurine	126.000	44.000	0.009	24	14	1.31
Taurine- ¹³ C ₂	128.126	46.192	0.009	14	12	1.32
Citrulline	176.000	69.900	0.009	16	20	1.98
Citrulline- <i>d</i> ₇	183.055	76.950	0.009	28	20	1.99

Table 2.

Validated Compounds in Nonhuman Primate Serum Perturbed after Total Body Irradiation

biofluid	metabolite name	RT (min)	experimental (m/z)	calculated (m/z)	mass error (ppm)	formula	HMDB ID
Urine	Carnitine	0.33	162.1131	162.1130	0.62	C ₇ H ₁₅ NO ₃	0000062
	Acetylcarnitine	0.35	204.1238	204.1236	0.98	C ₉ H ₁₇ NO ₄	0000201
	Propionylcarnitine	0.48	218.1392	218.1392	0.00	C ₁₀ H ₁₉ NO ₄	0000824
	Isobutyrylcarnitine/Butyrylcarnitine	0.92	232.1549	232.1549	0.00	C ₁₁ H ₂₁ NO ₄	–
	Creatine	0.33	132.0771	132.0773	–1.51	C ₄ H ₉ N ₃ O ₂	0000064
Serum	7-Methylguanine	0.35	166.0735	166.0729	3.61	C ₆ H ₇ N ₅ O	0000897
	Dimethyl-L-arginine	0.33	203.1491	203.1501	–4.92	C ₈ H ₁₈ N ₄ O ₂	0001539
	Xanthine ^a	0.37	151.0257	151.0256	0.66	C ₅ H ₄ N ₄ O ₂	0000292
	Carnitine	0.32	162.1130	162.1130	0.00	C ₇ H ₁₅ NO ₃	0000062
	Propionylcarnitine	0.48	218.1392	218.1392	0.00	C ₁₀ H ₁₉ NO ₄	0000824
	Isobutyrylcarnitine/Butyrylcarnitine	0.94	232.1546	232.1549	–1.29	C ₁₁ H ₂₁ NO ₄	–
	3-Dehydroxycarnitine	0.32	146.1179	146.1181	–1.36	C ₇ H ₁₅ NO ₂	0006831
	2-Methylbutyrylcarnitine	1.97	246.1705	246.1705	0.00	C ₁₂ H ₂₃ NO ₄	0000378
	Hypoxanthine	0.43	137.0463	137.0463	0.00	C ₅ H ₄ N ₄ O	0000157
	Guanine	0.41	152.0571	152.0572	–0.66	C ₅ H ₅ N ₅ O	0000132
	Oleamide	6.60	282.2796	282.2797	–0.35	C ₁₈ H ₃₅ NO	0002117

^aH[–] adducts, remaining H⁺ adducts