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Radiation Metabolomics. 5. Identification of Urinary Biomarkers of Ionizing Radiation Exposure in Nonhuman Primates by Mass Spectrometry-Based Metabolomics

Caroline H. Johnson^{a,1}, Andrew D. Patterson^b, Kristopher W. Krausz^a, John F. Kalinich^c, John B. Tyburski^d, Dong Wook Kang^{e,f}, Hans Luecke^e, Frank J. Gonzalez^a, William F. Blakely^c, and Jeffrey R. Idle^{g,3}

^aLaboratory of Metabolism, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland ^bDepartment of Veterinary and Biomedical Sciences and The Center for Molecular Toxicology and Carcinogenesis, The Pennsylvania State University, University Park, Pennsylvania ^cArmed Forces Radiobiology Research Institute, Uniformed Services University of the Health Sciences, Bethesda, Maryland ^dDepartment of Biochemistry and Molecular & Cellular Biology, Georgetown University Medical Center, Washington, DC

^eLaboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland ^fDepartment of Pharmaceutical Science and Technology, College of Health and Medical Science, Catholic University of Deagu, 13-13 Hayang-ro, Hayang-eup, Gyeongsan-si, Gyeongsangbuk-do, 712-702, South Korea

^gHepatology Research Group, Department of Clinical Research, University of Bern, 3010 Bern, Switzerland

Abstract

Mass spectrometry-based metabolomics has previously demonstrated utility for identifying biomarkers of ionizing radiation exposure in cellular, mouse and rat *in vivo* radiation models. To provide a valuable link from small laboratory rodents to humans, γ -radiation-induced urinary biomarkers were investigated using a nonhuman primate total-body-irradiation model. Mass spectrometry-based metabolomics approaches were applied to determine whether biomarkers could be identified, as well as the previously discovered rodent biomarkers of γ radiation. Ultra-performance liquid chromatography-electrospray ionization quadrupole time-of-flight mass spectrometry analysis was carried out on a time course of clean-catch urine samples collected from nonhuman primates ($n = 6$ per cohort) exposed to sham, 1.0, 3.5, 6.5 or 8.5 Gy doses of ⁶⁰Co γ ray (~0.55 Gy/min) ionizing radiation. By multivariate data analysis, 13 biomarkers of radiation were discovered: *N*-acetyltaurine, isethionic acid, taurine, xanthine, hypoxanthine, uric acid, creatine, creatinine, tyrosol sulfate, 3-hydroxytyrosol sulfate, tyramine sulfate, *N*-acetylserotonin sulfate, and adipic acid. *N*-Acetyltaurine, isethionic acid, and taurine had previously been identified in rats, and taurine and xanthine in mice after ionizing radiation exposure. Mass spectrometry-based metabolomics has thus successfully revealed and verified urinary biomarkers of ionizing radiation exposure in the nonhuman primate for the first time, which indicates possible mechanisms for ionizing radiation injury.

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³Address for correspondence: Hepatology Research Group, Department of Clinical Research, University of Bern, 3010 Bern, Switzerland; jeff.idle@ikp.unibe.ch..

¹Current address: Department of Chemistry and Molecular Biology, Center for Metabolomics, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037.

INTRODUCTION

Current radiological threats have prompted a recent renaissance of research investigations to enhance radiological medical countermeasures and biodosimetry capabilities by filling identified and critical gaps. One major gap is the ability to rapidly identify individuals exposed to potentially life-threatening radiation doses from those exposed to lower or no doses of radiation (1). Biodosimetry devices for this purpose should be useful for triage applications involving mass-casualty exposure incidents and to obtain the necessary approvals by governmental regulatory agencies [i.e., Food and Drug Administration (FDA)] (2).

Approval by the FDA for use of medical radiological countermeasure drugs involves demonstration of safety and efficacy, ideally in human. In cases where human data is not accessible, relevant animal models can be used. No biodosimetry devices are currently approved by the FDA, and approaches to obtain approval for candidate devices will likely be based on initial studies using small rodent (i.e., mice and rats) models along with results from human patients undergoing radiation therapy for cancer and limited radiation accidents. Use of a relevant non-rodent animal model for human use that permits characterizing complete dose- and time-course responses are needed for biodosimetry device validation.

Nonhuman primates provide the bridge in scientific research from small laboratory rodents to humans. Nonhuman primates are physiologically and genetically similar to humans and can be managed to control for non-genetic potential confounders that can cause large inter-individual variability in human populations. Human inter-individual variability can result from genetic, environmental (diet, stress, xenobiotics and disease) and gut microflora influences (3). In a laboratory setting, these variables can be decreased through maintaining uniform variables such as identical diet and housing, and a strict light-dark cycle. Nonhuman primate laboratory models can thus exhibit much less inter-individual variation compared to individuals in a human population. Urinary biomarkers for ionizing radiation exposure could possibly be identified in nonhuman primates and would indicate the potential for biodosimetry development in humans.

Mass spectrometry (MS)-based metabolomics has become an indispensable tool for the discovery of biomarkers of ionizing radiation exposure. Biomarkers have been identified in cells (4), mouse urine (5, 6) and more recently in rat urine (7, 8). A number of these metabolites are cross-species biomarkers of ionizing radiation such as taurine, 2'-deoxyxanthosine, 2'-deoxyuridine, thymidine and *N*-hex-anoylglycine, while others are species-specific. These studies employed both ultra-performance liquid chromatography coupled to electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOFMS) and gas chromatography mass spectrometry (GCMS), which allowed for comprehensive coverage of the urinary metabolome, revealing similar dose-responsive urinary metabolites. Biomarkers of ionizing radiation exposure have typically been those pertaining to DNA damage (9, 10), inflammation and tissue damage (11, 12), and gene expression changes (13-16). However, low-molecular weight metabolites provide a more rapid and accurate means for radiation exposure assessment. These metabolites reveal perturbations to metabolic pathways that could indicate potential health consequences, and therefore may be targets for therapeutic intervention after ionizing radiation exposure.

In this study, we used UPLC-ESI-QTOFMS-based metabolomics to analyze clean-catch urine samples collected from γ -irradiated nonhuman primates subjected to sham, 1.0, 3.5, 6.5 or 8.5 Gy total-body (TBI) irradiation ($n = 6$ per dose cohort). These doses are considered to be equivalent to 0.76, 2.7, 5.0 and 6.5 Gy, respectively, in humans (17). Urine

was collected 3 days predose and 3 days postdose of γ irradiation. The aim was to uncover dose- and time-dependent biomarkers of ionizing radiation by global metabolomics and to use targeted metabolomics to mine for previously identified rat and mouse radiation biomarkers. The overall goal of our study is to aid in the development of a biodosimeter for screening and triage of γ -radiation exposed individuals.

MATERIALS AND METHODS

Compounds

Isethionic acid, creatine, creatinine, adipic acid, 2'-deoxyuridine, thymidine, taurine, chlorpropamide, hypoxanthine, xanthosine, uric acid, xanthine, 3-hydroxytyrosol, tyrosol, and tyramine were all obtained from Sigma Aldrich (St. Louis, MO). 2'-Deoxyinosine was obtained from Spectrum Chemical & Laboratory Products (New Brunswick, NJ). Serotonin *O*-sulfate was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). *N*-hexanoylglycine was obtained from the Metabolic Laboratory, Vrije Universiteit Medical Center, (Amsterdam, The Netherlands). All other chemicals were of the highest purity grade.

Chemical Synthesis

N-Acetyltaurine was synthesized in-house, as described by Johnson *et al.* (8). The synthesis of tyrosol 4-*O*-sulfate and *N*-acetylserotonin 5-*O*-sulfate was also carried out in-house and is shown in the supplemental data (see supplementary data: <http://dx.doi.org/10.667/RR2950.1.S1>).

Nonhuman Primate Model System, Radiation Dosing and Dosimetry

Twenty-eight adult male and female rhesus monkeys (*Macaca mulatta*) (~5.5 kg; ~4 years old) were housed in individual stainless steel cages in conventional holding rooms at the Armed Forces Radiobiology Research Institute's (AFRRI) Veterinary Sciences Department, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. Research was conducted according to the principles stated in the guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, National Research Council. All animal procedures were approved by the AFRRI Animal Care and Use Committee.

In vivo radiation exposure and dosimetry of the rhesus macaques were previously described (10-12). The animals were ketamine-anesthetized with Ketaset (10 mg kg⁻¹, i.m., Fort Dodge Laboratories, Fort Dodge, IN) and placed in a plexiglass restraint chairs. They were allowed to regain consciousness and were irradiated bilaterally with ⁶⁰Co γ irradiation at a nominal dose rate of ~60 cGy min⁻¹. TBI to midline tissue was at γ -radiation doses of sham, 1.0, 3.5, 6.5 or 8.5 Gy, with 6 nonhuman primates per cohort. Two sham-treated animals were reused at the 8.5 Gy exposure. Dosimetry was performed using an alanine/electron paramagnetic resonance system, with calibration factors traceable to the National Institutes of Standards and Technology, and was confirmed by an additional check against the national standard ⁶⁰Co source of the UK National Physics Laboratory. The LD_{50/60} for human spans from 2.5 to 4.5 Gy, however, nonhuman primates are approximately 1.5 times less sensitive to ionizing radiation than humans, therefore a dose range that spans the region of mortality was chosen for nonhuman primates. In addition a sham dose was included to take into account stress-related metabolites.

Urine Collection

Starting three days before radiation exposure, clean-catch urines were collected in metal pans each day and stored at -80°C. On the day of γ -radiation exposure, a urine sample was

collected in the morning. After radiation exposure, nonhuman primates were placed back in their respective cages and clean-catch urine samples were collected in metal pans on the evening of dosing, and in the morning and evening for the next three days (12, 24, 36, 48, 60, 72 and 84 h times). Spot urine samples were collected at the following times for each dose; sham: predose ($n=6$), 12 h ($n=3$), 24 h ($n=4$), 36 h ($n=4$), 48 h ($n=3$) and 72 h ($n=3$); 1.0 Gy: predose ($n=6$), 12 h ($n=4$), 24 h ($n=2$), 36 h ($n=4$), 48 h ($n=3$), 72 h ($n=3$) and 84 h ($n=2$); 3.5 Gy: predose ($n=6$), 12 h ($n=6$), 48 h ($n=6$) and 60 h ($n=6$); 6.5 Gy: predose ($n=6$), 12 h ($n=3$), 24 h ($n=4$), 36 h ($n=5$), 60 h ($n=4$), 72 h ($n=5$) and 84 h ($n=3$); 8.5 Gy: predose ($n=6$), 24 h ($n=5$), 48 h ($n=5$) and 72 h ($n=5$). Spot urines could not be collected from all monkeys at every time due to infrequent urination. The samples were stored at -80°C . Specific gravity was measured before freezing urine samples. A digital refractometer (Model 300027, Kernco Instruments, El Paso, TX) was used according to the manufacturer's instructions. The instrument was first calibrated against distilled water and approximately 180 μl of urine was then placed on the instrument's prism window and the specific gravity measurement was taken. The prism window was cleaned with distilled water between each sample.

Sample Preparation for UPLC-ESI-QTOFMS

Urine samples were thawed and 50 μl was added to microcentrifuge tubes containing 50 μl acetonitrile:water (50:50 v/v) and 5 μM chlorpropamide at 4°C . The samples were vortexed for 1 min and centrifuged at 14,000g for 20 min at 4°C to remove proteins and particulates. The supernatants were transferred to UPLC vials. A pooled sample was also made for quality control that contained 5 μl of each sample.

UPLC-ESI-QTOFMS Analysis

The samples were randomized and analyzed by UPLC-ESI-QTOFMS, as described previously (5). The following mobile phase linear gradient consisting of 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B) was used with a flow rate of 0.5 ml/min: 98% (A) for 0.5 min to 80% (A) at 4.0 min to 95% (B) at 8 min. The column was washed with 100% (B) for 1 min and then equilibrated with 98% (A) for 1 min before subsequent injections. Samples were injected onto a reverse-phase 50 $\times$ 2.1 mm ACQUITY® 1.7 μm C18 column (Waters Corp, Milford, MA) using an ACQUITY® UPLC system (Waters). A water blank and pooled sample was injected after every five samples. Mass spectrometry was performed on a Waters® QToF-Premier™-MS operating in negative and positive ESI mode.

Multivariate Data Analysis and Biomarker Identification

The mass spectral data were centroided, integrated and deconvoluted to generate a multivariate data matrix using MarkerLynx® (Waters). Peak picking, alignment, deisotoping and integration were performed automatically by the software with the following parameters: mass tolerance = 0.05 Da, peak width at 5% height = 1s, peak-to-peak baseline noise 10, intensity threshold = 100 counts, mass window=0.05 Da, retention time window=0.20 min and noise elimination level = 10. The data were also normalized to the total ion current (TIC) chromatogram by the MarkerLynx program. The raw data were then transformed into a multivariate matrix containing aligned peak areas with matched mass-to-charge ratios and retention times. The data were normalized to the peak area of the internal standard chlorpropamide, which appeared at a retention time of 5.3 min, 275.024 $[\text{M-H}]^{-}$ and 277.041 $[\text{M+H}]^{+}$ and was exported into SIMCA-P+ software (Umetrics, Kinnelon, NJ). The ESI+ and ESI- data were Pareto-scaled to increase the importance of low-abundance ions without significant amplification of noise and analyzed by principal components analysis (PCA), partial least squares discriminant analysis (PLS-DA) and orthogonal projection to latent structures discriminant analysis (OPLS-DA). For identification of

biomarkers specific to radiation, OPLS-DA models were constructed, comparing predose and sham urine samples (controls $y = 0$) to those taken after the four different doses of ionizing radiation (1, 3.5, 6.5 and 8.5 Gy), and seven potential postdose times (12, 24, 36, 48, 60, 72 and 84 h) ($y = 1$). Ions with a correlation (ρ_{corr}) above 0.8, and a peak area above 100, were subjected to tandem MS. Further confirmation of identity was then carried out by repeating the tandem MS fragmentation using authentic standards at 100 μM in water and urine.

Deconjugation of Sulfated Metabolites

A number of sulfated metabolites were seen in urine samples from nonhuman primates after ionizing radiation exposure. For each deconjugation reaction, 40 U sulfatase (from *Helix Pomotia*, Sigma Aldrich, St. Louis, MO) was dissolved in 50 μl 0.2% (w/v) sodium chloride solution and added to a 1.5 ml microcentrifuge tube containing 50 μl urine and 400 μL pH 5.0 sodium acetate buffer 200 mM. Control urine was also made in tandem without sulfatase. *p*-Nitrocatechol sulfate was used as a positive control for the deconjugation reaction. The mixtures were incubated overnight at 37°C. Acetonitrile (500 μl) was added, and samples were vortexed and centrifuged for 15 min at 14,000 rpm and 4°C. The supernatant was concentrated to a final volume of 250 μL for 3 h in a Savant Speedvac® (Thermo Scientific, Waltham, MA). Chlorpropamide (5 μM) was added as an internal standard, and 5 μl of each solution was injected onto the UPLC-ESI-QTOFMS for analysis by tandem MS fragmentation and comparison to standards where available.

Quantitation

Biomarkers were quantitated using an Acquity® UPLC H-class coupled to a XEVO®G2 QTOFMS with Quantof™ technology (Waters). Standard calibration curves of 0 to 35 μM were made for isethionic acid ($r^2=0.9930$), *N*-acetyltaurine ($r^2=0.9909$), creatine ($r^2 = 0.9606$), creatinine ($r^2 = 0.9595$), adipic acid ($r^2 = 0.9941$), 2'-deoxyuridine ($r^2 = 0.9989$), 2'-deoxyinosine ($r^2 = 0.9740$), thymidine ($r^2=0.9761$), N1-acetylspermidine ($r^2=0.8948$), taurine ($r^2=0.9651$), *N*-hexanoylglycine ($r^2 = 0.9954$), hypoxanthine ($r^2 = 0.9013$), xanthosine ($r^2 = 0.9978$), uric acid ($r^2 = 0.9722$), xanthine ($r^2 = 0.9775$), *N*-acetylserotonin sulfate ($r^2=0.9871$), tyrosine ($r^2=0.9920$), tyramine ($r^2 = 0.9698$), and tyrosol sulfate ($r^2 = 0.9929$) using authentic standards. Standards were not available for 3-hydroxytyrosol sulfate and tyramine sulfate, therefore, 3-hydroxytyrosol and tyramine were used respectively to calculate relative abundance. Urines were diluted 1:2 in water; an internal standard of chlorpropamide was added to each sample, with a final concentration 5 μM . The chromatographic conditions used were as listed above with the exception that an additional 2 min equilibration step was needed with the H-class system. The XEVO®G2 was operated in either ESI+ or ESI- with a capillary voltage of 3000 V and a sampling cone voltage of 30 V. The desolvation and cone gas flow were set to 850 and 50 L/H, respectively. The desolvation temperature was set to 450°C and the source temperature was set to 150°C. Data was acquired in centroid mode from 50 to 850 m/z . The samples were quantitated using TargetLynx® (Waters) software by integrating peak areas of extracted ion chromatograms. The concentration of each metabolite was calculated by comparison to a standard curve of an authentic standard. They were then normalized to the specific gravity of each sample and expressed in $\mu\text{mol/l}$.

Statistics

All concentrations were expressed as mean $\pm$ standard error of the mean (SEM) after two-tailed Mann-Whitney *U* test using GraphPad Prism 4 software (GraphPad Software, Inc., La Jolla, CA). Predose and sham were compared to 12, 24, 36, 48, 60, 72 and 84 h after irradiation by 1.0, 3.5, 6.5 and 8.5 Gy γ radiation. A comparison was carried out with $P < 0.05$ was considered statistically significant. Receiver operating characteristic (ROC)

analysis was carried out using Stata®/SE 11.0 software to assesses the robustness of biomarker classification for each dose and time point by comparing sham to postdose concentrations for each biomarker. The ROC area under the curve (AUC) demonstrates the sensitivity and specificity of the biomarkers from 0.5 (chance) up to 1.0 which shows perfect classification. Times that had less than three values >0 were not included in the analysis.

RESULTS

UPLC-ESI-QTOFMS-Based Metabolomics Analysis

Urine samples were analyzed by UPLC-ESI-QTOFMS in electrospray positive and negative ionization modes. After pre-processing, the data were subjected to multivariate data analysis (MDA). Unsupervised PCA models were made for each dose and time comparing predose and sham samples against postdose ionizing radiation exposure samples. No separation was seen between samples taken predose/sham and samples taken after 1.0 and 3.5 Gy radiation exposure, as seen in Fig. 1A and B. Separation was observed between samples taken predose/sham and samples taken after 6.5 and 8.5 Gy, as shown in Fig. 1C and D. Separation was seen up to 48 h postdose, but suspected gut microflora-related metabolites were more influential contributors to the variation between samples after this time. PLS-DA models were constructed revealing that at 1.0 Gy no separation was seen between the samples. Increasing the dose from 3.5 to 8.5 Gy caused samples to cluster in their pre- and postdose groupings at 24 h after ionizing radiation exposure. This clustering decreased however from 48 h after exposure. An example of this can be seen in Fig. S1 for the 8.5 Gy dose (see supplementary material: <http://dx.doi.org/10.667/RR2950.1.S1>). For urine samples collected 24 h after 8.5 Gy ionizing radiation exposure, upregulated radiation specific metabolites caused urine samples to cluster according to dose status (see supplementary material Fig. S1A: <http://dx.doi.org/10.667/RR2950.1.S1>), but at 48 and 72 h there is less clustering and outliers can be seen (see supplementary material Fig. S1B and C: <http://dx.doi.org/10.667/RR2950.1.S1>). Urine samples that were outliers were identified as being influenced by typical gut microbiota metabolites which had a larger abundance and correlation to the urinary metabolomes than the radiation markers at these times. Figure S1D (see supplementary material Fig. S1D: <http://dx.doi.org/10.667/RR2950.1.S1>) shows the PLS-DA loadings plot for urine samples collected 72 h after exposure and the gut-microbiota related metabolites, hippuric acid, *p*-cresol sulfate and indoxyl sulfate (18), which were confirmed after fragmentation by tandem MS. To identify the biomarkers of ionizing radiation exposure, OPLS-DA models were constructed. Sham and predose samples were compared to postdose samples for each dose and time. The ions with a correlation (pcorr) greater than 0.8 were fragmented by tandem MS and confirmed against authentic standards for identification. Confirmed biomarkers were quantitated by peak area integration of the extracted ion chromatogram (EIC) and were calculated using calibration curves of authentic standards. Two standards, tyrosol sulfate and *N*-acetylserotonin sulfate, were synthesized in-house after their structure was indicated by tandem MS fragmentation. Tyramine sulfate and 3-hydroxytyrosol sulfate were confirmed after tandem MS and comparison to standards of *p*-tyramine and 3-hydroxytyrosol. A neutral loss of 80.9646 and the fragment 79.9574 [M-H]⁻ (HSO₃)⁻ confirmed the presence of a sulfate group. Deconjugation assays were also carried out on all four sulfated metabolites and confirmed the loss of a sulfate moiety. Concentrations of tyramine sulfate and 3-hydroxytyrosol sulfate were calculated after calibration to standard curves of tyramine and tyrosol sulfate and are relative concentrations. In total, 13 metabolites were upregulated after ionizing radiation exposure with different specificities at different doses and times. Biomarkers included taurine, *N*-acetyltaurine, isethionic acid, hypoxanthine, uric acid, xanthine, adipic acid, 3-hydroxytyrosol sulfate, tyrosol sulfate, *N*-acetylserotonin sulfate, tyramine sulfate, creatine, and creatinine. These biomarkers are shown in Table 1 with their mass spectral retention times and ion masses.

N^1 -acetylspermidine was also identified as a biomarker by OPLS-DA with a correlation coefficient of 0.857 but was not significantly increased with respect to sham after quantitation.

Normalization of metabolites to creatinine is the gold standard for reporting metabolite concentrations in urine; creatinine, although not apparent as a correlated metabolite to ionizing radiation after MDA, was identified as a biomarker of ionizing radiation exposure after quantitation. Therefore, after TIC normalization each biomarker was quantitated by comparing its peak area to a concentration curve made from an authentic standard. It can be seen from Table 2, which shows the concentrations for each biomarker pre and post 8.5 Gy ionizing radiation, that creatinine is upregulated 3.1-fold ($P = 0.009$), 1.6-fold ($P = 0.052$) and 2.2-fold ($P = 0.065$) 24, 48 and 72 h after radiation exposure compared to predose. Therefore normalization to this metabolite would decrease the fold change of other radiation biomarkers. For example, isethionic acid, which is a biomarker for 8.5 Gy ionizing radiation 24 h postdose, has a fold change of 4.058 ($P = 0.004$) after normalization to creatinine compared to a fold change of 7.500 ($P = 0.002$) previously.

Dose Response

A trend could be seen between the upregulation of the urinary biomarkers at certain doses of ionizing radiation. The concentration of each biomarker at 12–36 h after radiation exposure to sham, 1.0, 3.5, 6.5 and 8.5 Gy ionizing radiation can be seen in Fig. 2. The times displayed represent the time at which the greatest number of nonhuman primates were represented.

Twenty-four hours after 8.5 Gy ionizing radiation, the following metabolites were statistically significant when compared to predose values ($P < 0.050$): isethionic acid (7.5-fold), *N*-acetyltaurine (4.6-fold), taurine (3.9-fold), adipic acid (54.6-fold), hypoxanthine (3.4-fold), uric acid (4.1-fold), xanthine (4.9-fold), 3-hydroxytyrosol sulfate (14.7-fold), *N*-acetylserotonin sulfate (14.9-fold), creatinine (3.1-fold), and tyramine sulfate (35.3-fold). Each averaged pre- and postdose concentration with fold change and P values can be seen in Table 2. Some markers were only statistically significant at the 8.5 Gy dose of ionizing radiation and not at any other dose, including adipic acid, xanthine and *N*-acetylserotonin sulfate.

Twenty-four to thirty-six hours after 6.5 Gy of ionizing radiation exposure, the following six metabolites were statistically significant when compared to predose values ($P < 0.050$, at 24 h postdose): isethionic acid (3.1-fold, $P < 0.019$), *N*-acetyltaurine (5.0-fold, $P = 0.019$), taurine (3.4-fold, $P = 0.038$), hypoxanthine (2.5-fold, $P = 0.009$), uric acid (1.7-fold, $P = 0.038$), and creatinine (2.0-fold, $P = 0.038$). These six metabolites are thus biomarkers for 6.5–8.5 Gy radiation exposure. Creatine was only significant at the 6.5 Gy dose 24 h after radiation (11.2-fold, $P = 0.032$). At 3.5 Gy, hypoxanthine was significantly upregulated 48 h postdose, 2.1-fold ($P = 0.032$). It is the only metabolite that increases over the entire 3.5–8.5 Gy dose range of ionizing radiation. Tyrosol sulfate was upregulated 48 h after 3.5 Gy radiation with a 15.8-fold increase ($P = 0.032$), and 3-hydroxytyrosol sulfate was increased 11.4-fold ($P = 0.032$) at 60 h. There were two isomers seen for tyramine sulfate that were differentially upregulated at different doses. One isomer had a retention time of 0.83 min and was only present in the 8.5 Gy 24 h postdose samples, another isomer that had a retention time of 0.65 min was present in all samples but upregulated only after 3.5 Gy, 48–60 h after radiation [9.4 and 3.4-fold increase, respectively ($P = 0.009, 0.030$)]. As an authentic standard was not available for this metabolite, the true identity of the isomer could not be ascertained but either isomer could result from an *O*- or *N*-sulfation to meta-, para- or ortho-tyramine, although the most abundant is likely to be tyramine 4-*O*-sulfate, a common

urinary metabolite of tyrosine. After sham and 1.0 Gy ionizing radiation exposure, there were no significant biomarkers seen.

Temporal Effects

The doses that resulted in urinary ionizing radiation specific biomarkers were primarily seen 24–36 h after exposure, with some seen at 12 h and 48–72 h. Figure 3 shows the concentration of each urinary biomarker for the nonhuman primates exposed to 8.5 Gy at predose, 24, 48 and 72 h after ionizing radiation. The plots show the individual concentrations of the biomarkers for each nonhuman primate and thus the variability seen between them, significant differences can however be seen between times. It can also be seen that isethionic acid is significantly upregulated for the full 72 h after ionizing radiation exposure.

Assessment of Biomarker Robustness

ROC analysis was carried out to determine the predictive ability of biomarkers discovered by MDA. Curves were constructed comparing sham urines to all other doses 24 h postdose. Other times were not chosen as the number of urines collected from the sham dose was 3. For ROC analysis, a biomarker with an AUC score of >0.8 was designated as a robust marker of ionizing radiation exposure. Figure 4 shows the ROC analysis for each urinary biomarker of ionizing radiation 24 h after 8.5 Gy of ionizing radiation. Seven of the biomarkers had perfect predictive ability with AUCs of 1.0, most had AUCs >0.8 with the exception of tyrosol sulfate, which had an AUC of 0.7000 and creatine, which had an AUC of 0.7917. At the 6.5 Gy dose, there were seven biomarkers of radiation seen at 24 h which had AUCs ranging from 0.8333 to 1.0000 including, taurine, isethionic acid, *N*-acetyltaurine, uric acid, creatine, hypoxanthine and xanthine. At 3.5 Gy 12 h postdose, tyrosol sulfate had an AUC of 1.000 and 3-hydroxytyrosol had an AUC of 0.7778. There were no other biomarkers with a high AUC value. The optimal cut-off points at which the biomarker concentrations that are predictive of ionizing radiation exposure are as follows: taurine; 9.67 $\mu\text{mol/l}$, *N*-acetyltaurine; 0.91 $\mu\text{mol/l}$, isethionic acid; 1.50 $\mu\text{mol/l}$, hypoxanthine; 27.97 $\mu\text{mol/l}$, xanthine; 8.44 $\mu\text{mol/l}$, creatinine; 11.30 $\mu\text{mol/l}$, creatine; 9.547 $\mu\text{mol/l}$, uric acid; 5.86 $\mu\text{mol/l}$, tyrosol sulfate; 0.72 $\mu\text{mol/l}$, adipic acid; 40.4 $\mu\text{mol/l}$ and *N*-acetylserotonin sulfate; 0.12 $\mu\text{mol/l}$. 3-Hydroxytyrosol sulfate and tyramine sulfate were calculated as relative concentrations against calibration curves of tyrosol sulfate and tyramine so absolute concentrations could not be ascertained.

Targeted Metabolomics on Previously Discovered Rat and Mouse Biomarkers

A targeted metabolomics approach was carried out to quantitate urinary biomarkers of ionizing radiation previously discovered in rats (8) and mice (5, 6), which were not revealed by global metabolomics and MDA. These biomarkers included xanthosine, thymidine, 2'-deoxyuridine and *N*-hexanoylglycine. There was no change between predose and postdose metabolite concentrations for xanthosine, thymidine or 2'-deoxyuridine. *N*-hexanoylglycine increased in concentration after ionizing radiation exposure, but not in all nonhuman primates. Therefore, due to the large variability between the metabolite responses in the nonhuman primates, the markers in this model were not found to be significant or robust bio-indicators of ionizing radiation exposure.

A targeted analysis was also carried out on tyrosine and tyramine because a number of metabolites were upregulated in the same metabolic pathway as this metabolite. These metabolites were not significantly upregulated after ionizing radiation exposure.

DISCUSSION

UPLC-ESI-QTOFMS metabolomics was used to identify novel metabolites of ionizing radiation exposure in nonhuman primates and to quantitate already established biomarkers previously identified in mice and rats by UPLC-ESI-QTOFMS (5, 6) and GCMS (7, 8). The upregulated metabolites observed due to ionizing radiation exposure included *N*-acetyltaurine, isethionic acid, taurine, xanthine, hypoxanthine, uric acid, creatine, creatinine, tyrosol sulfate, 3-hydroxytyrosol sulfate, tyramine sulfate, *N*-acetylserotonin sulfate, and adipic acid.

The presence of elevated xanthine, hypoxanthine and uric acid in urine indicates the potential damaging effect of ionizing radiation to DNA. All three metabolites were significantly upregulated after 8.5 Gy radiation exposure. Uric acid and hypoxanthine were significant at 6.5 Gy, as was hypoxanthine at 3.5 Gy. These three metabolites are part of the purine catabolism pathway. Xanthine and hypoxanthine result from the deamination of guanine and adenine. Xanthine can also be formed directly from hypoxanthine and is a reversible reaction. Uric acid is the final product of purine metabolism formed from xanthine. 2'-Deoxyinosine, the nucleoside of hypoxanthine, was observed to be upregulated by 6.5 Gy radiation exposure when analyzed by OPLS-DA, but after quantitation, it was not seen to be a significant metabolite due to variation in concentrations between the nonhuman primates.

Previously, upregulation of xanthine, xanthosine, thymidine, 2'-deoxyuridine and 2'-deoxyxanthosine were reported in mice and rats after various doses of radiation. In the mouse, xanthine was increased ~4-fold at 3 Gy (equivalent to approximately a 2.2 Gy dose in nonhuman primates). In comparison, xanthine increased 4.9-fold at 8.5 Gy in nonhuman primates. Hypoxanthine was not seen in the rodent models, but can be formed by a reversible reaction with xanthine. The larger fold increase in xanthine in nonhuman primates could cause an increase in both hypoxanthine and uric acid as a means to eliminate this metabolite. The formation of xanthine and hypoxanthine can have biological consequences, as they are mutagenic lesions. When guanine and adenine are deaminated, a transition mutation can occur and hypoxanthine can pair with cytosine, and xanthine with thymine. In addition, xanthine and hypoxanthine can undergo depurination to form an abasic site resulting in a transversion mutation (19). Thymidine, 2'-deoxyuridine and xanthosine were not identified as significant markers by multivariate analysis or after a targeted metabolomics approach. The presence of thymidine and 2'-deoxyuridine in rodent urinary metabolomes could be explained by hydrolytic or enzymatic deamination of 2'-deoxycytidine, 2'-deoxyguanosine and 5'-methylcytosine. The pyrimidine bases in DNA are known to be more susceptible to spontaneous hydrolytic deamination than are purine bases (20, 21), but purines are more prone to nitrosative deamination (22). Therefore, the presence of only purine derivatives here (xanthine and hypoxanthine) indicates that nitrous anhydride/reactive oxygen species (ROS) interaction generated by ionizing radiation was the prevalent route of deamination (23, 24).

Another set of related metabolites are those belonging to taurine metabolism, taurine, *N*-acetyltaurine and isethionic acid. These biomarkers were elevated after 6.5 and 8.5 Gy of ionizing radiation exposure. Taurine increased 3.9-fold (6.5 Gy 36 h and 8.5 Gy 24 h). In mice, taurine was increased 1.2-fold (8.0 Gy 24 h, equivalent to 6.1 Gy in nonhuman primates) (5), and 2.5-fold in rats (2.8 Gy 24 h, equivalent to 2.2 Gy in nonhuman primates) (8). *N*-Acetyltaurine was increased 4.3-fold (6.5 Gy up to 36 h) and 4.6-fold (8.5 Gy up to 36 h) compared to 1.6-fold (2.8 Gy 24 h) in rats. Isethionic acid was upregulated 2.9-fold (6.5 Gy up to 36 h) and 6.4-fold (8.5 Gy up to 72 h) in nonhuman primates, however it could not previously be quantitated in rats. Taurine is a well known urinary metabolite of radiation

exposure, but the precise cause for its elevation in response to radiation is unknown. Its physiological roles include bile acid conjugation, antioxidation and protection of the body by inhibiting ROS (5). It can also facilitate kidney-level sulfur homeostasis. It is possible that tissue injury results in a higher level of circulating sulfur-containing amino acids and that the excess taurine seen is a means of excreting sulfur in the urine. Another proposed mechanism is through radiation-induced destruction of circulating lymphocytes (25). The gastrointestinal mucosa show severe injury after exposure to intermediate dose levels of 5.0 to 12.0 Gy or higher radiation in humans (6.5–16.0 Gy in nonhuman primates) (17). This mode of death in acute radiation syndrome or sickness is called the “gastrointestinal syndrome”. Indeed, taurine was increased in nonhuman primates at this dose range possibly to protect these cells against injury. In addition, isethionic acid, the hydroxyl analogue of taurine, is metabolized through actions of the gut microflora (26). *N*-Acetyltaurine is a novel metabolite previously discovered as a biomarker of ionizing radiation exposure in rats (8) and could potentially be formed through direct acetylation of taurine by *N*-acetyltransferases, and is thus another route for excess taurine elimination. Both isethionic acid and *N*-acetyltaurine are upregulated for up to 72 h after 8.5 Gy radiation exposure compared to only 24 h for taurine, which also shows that these metabolites are being produced for some time after exposure to eliminate the excess taurine.

Creatine and creatinine were also increased after radiation exposure. Creatine was validated as a biomarker 24 h after 6.5 Gy γ radiation with an 11.2-fold increase in concentration. Creatine has previously been reported as a biomarker of radiation in other species including nonhuman primates (*Macaca mulatta*) (27). Creatinine was increased 24 h after 8.5 Gy and 6.5 Gy after radiation exposure, 3.1-fold and 2.0-fold, respectively. Creatinine is formed from creatine and phosphocreatine, increases of both creatine and creatinine in the urine of nonhuman primates signifies an inability of irradiated muscle to use creatine (28).

There were a number of novel biomarkers of radiation exposure including adipic acid, tyrosol sulfate, 3-hydroxytyrosol sulfate, *N*-acetylserotonin sulfate and tyramine sulfate detected in our study. Adipic acid, a dicarboxylic acid, increased 54.6-fold 24 h after 8.5 Gy γ -radiation exposure. There are a number of human diseases that clinically show increased urinary excretion of adipic acid, including malonyl-CoA decarboxylase deficiency, medium-chain acyl-CoA dehydrogenase deficiency, and glutaric aciduria type II. Ethylmalonic-adipic aciduria patients constantly excrete 2-ethylmalonic acid, adipic acid and *N*-hexanoylglycine in their urine (29), the latter of which has previously been identified as an ionizing radiation biomarker in rats (8) and mice (5). Indeed, both adipic acid and *N*-hexanoylglycine are formed from hexanoic acid by ω -oxidation and glycine conjugation, respectively (30). These diseases are thought to arise from defects in the β -oxidation pathway, for example, inhibition of dehydrogenase enzymes such as hexanoyl-CoA dehydrogenase by toxins or genetic mutations. In contrast, a decrease in dicarboxylic acids was seen in rats exposed to ionizing radiation (8) and resulted from the increased production of succinyl-CoA through the β -oxidation pathway for gluconeogenesis during starvation. *N*-Acetylserotonin sulfate was upregulated 14.9-fold 24 h after 8.5 Gy γ radiation and at no other dose or time. *N*-Acetylserotonin sulfate is a metabolite of melatonin (31). A number of studies have shown that melatonin can protect DNA, lipids and proteins against radiation-induced oxidative stress (32). The increase in *N*-acetylserotonin sulfate could be due to an increase in melatonin metabolism. However, changes were not seen in other melatonin metabolites by multivariate data analysis. 3-Hydroxytyrosol sulfate was upregulated 14.7-fold (8.5 Gy 24 h) and 11.4-fold (3.5 Gy 60 h), and tyrosol sulfate was upregulated 15.8-fold 48 h after 3.5 Gy ionizing radiation exposure. Tyramine sulfate was present as two isomers: one increased 35.3-fold 24 h after 8.5 Gy radiation and the other increased 9.4 and 3.4-fold 48–60 h after 3.5 Gy radiation, respectively. Each isomer exhibited the same fragmentation pattern, but exact identification could not be carried out due to the lack of an authentic

standard. Tyramine can be present in three isomeric forms with the hydroxy group at the meta, para or ortho position on the benzene ring. It is possible to have *O*-sulfation to any of these hydroxy groups and also *N*-sulfation at the amine group on the hydrocarbon arm. Tyramine sulfate, tyrosol sulfate and 3-hydroxytyrosol sulfate are all part of the tyrosine pathway as depicted in Fig. 5, and are excreted in the urine as a possible way to eliminate excess tyrosine produced after ionizing radiation exposure. Tyrosine concentration can be increased when phenylalanine undergoes hydroxylation by hydroxyl radicals and can indicate oxidative damage to proteins (33, 34).

Nonhuman primates can be valuable for scientific research as they bridge the gap between murine models and humans. In total, 24 biomarkers of ionizing radiation exposure have been identified in mice, rats and nonhuman primates and can be seen on Fig. 6, however, only four biomarkers overlap between the murine and primate species. There are many biological processes that differ between rodents and nonhuman primates, which may explain some of the differences between the biomarkers observed. However, it can be seen that the same metabolic pathways and processes are affected by ionizing radiation in all species, such as DNA damage and repair, β -oxidation and perturbations to the gut microbiota, but the metabolic end products are different. It is known that larger mammals are much more susceptible to hematopoietic damage from ionizing radiation than are smaller species. This can be reflected by the 50% lethal dose for mice, rats and monkeys, which is 7, 6.75 and 5.25 Gy, respectively. Numerous metabolism studies have also shown biochemical differences between nonhuman primates and murine models, in particular for drug metabolizing and conjugation enzymes. Therefore, it is not unexpected that the biological responses to radiation will be somewhat different.

At the highest doses, some variability was seen between the nonhuman primates. This variability increased with lower doses, showing the difference in metabolic response of each nonhuman primate to ionizing radiation that may reflect what would likely happen in a human population. Taurine, for example has a predose concentration range of 1.781 to 7.07 $\mu\text{mol/l}$ with an average of $3.613 \pm 0.907 \mu\text{mol/l}$ in 6 nonhuman primates. This value is consistent for all 30 nonhuman primates. Twenty-four hours after 8.5 Gy radiation exposure the concentration range was much wider, 8.238–21.312 $\mu\text{mol/l}$ with an average of $14.208 \pm 2.399 \mu\text{mol/l}$. At 36 h after 6.5 Gy radiation, the variability in fold change was higher, the concentration range was wider from 3.433 to 24.655 $\mu\text{mol/l}$. This wide variability was seen for other metabolites as well. One way to overcome this is to have a larger number of nonhuman primates so that the study is not underpowered, but due to the sensitive nature of the model used, it was not possible for this study.

CONCLUSIONS

This study has revealed 13 biomarkers of ionizing radiation exposure in the nonhuman primate model that indicate oxidative damage to proteins and DNA, mechanisms to protect against ROS and cellular injury, halting of biological pathways such as β -oxidation and the decrease in the usage of creatine by muscle. Of these biomarkers, four are unequivocal cross-species markers with rats and mice. In total, 24 biomarkers of ionizing radiation exposure have been collectively identified in mouse, rat and nonhuman primate urine, and are summarized in Fig. 6. Some of the markers are related in the same biological pathways between species, such as adipic acid seen in nonhuman primates and *N*-hexanoylglycine seen in mice and rats, or are created due to similar interactions, such as the gut microbial related metabolites. Further studies need to include validation of these biomarkers of ionizing radiation exposure in humans, with the ultimate goal of development of a radiation biodosimeter.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.

PCA scores plots from nonhuman primate (NHP) urine collected after exposure to ionizing radiation and analyzed by UPLC-ESI-QTOFMS ESI-mode. Panel A: 36 h after 1.0 Gy dose ($R^2=0.667$, $Q^2=0.122$); panel B: 12 h after 3.5 Gy dose ($R^2=0.545$, $Q^2=0.290$); panel C: 36 h after 6.5 Gy dose ($R^2=0.562$, $Q^2=0.219$) and panel D: 24 h after 8.5 Gy dose ($R^2=0.658$, $Q^2=0.442$). Labeling is as follows: $\square$ Sham and pre-dose, $\blacktriangle$ exposed to ionizing radiation.

FIG. 2.

Dose response of each biomarker after exposure to sham (24 h), 1.0 (36 h), 3.5 (12 h), 6.5 (36 h) and 8.5 (24 h) Gy ionizing radiation. Panel A: taurine; panel B: *N*-acetyltaurine; panel C: isethionic acid; panel D: hypoxanthine; panel E: uric acid; panel F: xanthine; panel G: adipic acid; panel H: *N*-acetylserotonin sulfate; panel I: 3-hydroxytyrosol sulfate; panel J: tyramine sulfate isomer seen at retention time 0.83 min; panel K: tyrosol sulfate and panel L: creatine. Error bars are SEM.

FIG. 3.

Temporal response of each biomarker at 8.5 Gy. Panel A: taurine; panel B: *N*-acetyltaurine; panel C: isethionic acid; panel D: hypoxanthine; panel E: uric acid; panel F: xanthine; panel G: adipic acid; panel H: *N*-acetylserotonin sulfate; panel I: 3-hydroxytyrosol sulfate; panel J: tyramine sulfate isomer seen at retention time 0.83 min; panel K: tyrosol sulfate and panel L: creatinine. Significance as determined by Mann-Whitney *U* test. *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$. NS = Not significant.

FIG. 4.

ROC analysis of each biomarker 24 h after 8.5 Gy irradiation compared to sham irradiation. Panel A: taurine; panel B: *N*-acetyltaurine; panel C: isethionic acid; panel D: hypoxanthine; panel E: uric acid; panel F: xanthine; panel G: adipic acid; panel H: *N*-acetylserotonin sulfate; panel I: 3-hydroxytyrosol sulfate; panel J: tyramine sulfate; panel K: tyrosol sulfate; panel L: creatinine and panel M: creatine.

FIG. 5.
Tyrosine metabolism.

FIG. 6.
Robust markers of ionizing radiation exposure in mice, rats and nonhuman primates (NHP).

TABLE 1

Urinary Biomarkers of Ionizing Radiation Exposure in Nonhuman Primates

Retention time (min)	Experimental ion mass	Calculated ion mass	Mass error (ppm)	Formula	Metabolite name
0.33	166.0170	166.0174	2.4	C ₄ H ₉ NO ₄ S ⁻	N-Acetyltaurine
0.33	124.9929	124.9909	16.0	C ₂ H ₅ O ₄ S ⁻	Isothionic acid
0.33	124.0078	124.0068	8.1	C ₂ H ₇ NO ₃ S ⁻	Taurine
0.43	151.0254	151.0256	1.3	C ₃ H ₄ N ₄ O ₂ ⁻	Xanthine
0.33	135.0290	135.0307	12.6	C ₃ H ₄ N ₄ O ⁻	Hypoxanthine
0.40	167.0208	167.0205	1.8	C ₃ H ₄ N ₄ O ₃ ⁻	Uric acid
0.33	132.0774	132.0773	0.8	C ₄ H ₇ N ₃ O ₂ ⁺	Creatine
0.30	114.0669	114.0667	1.8	C ₄ H ₇ N ₃ O ⁺	Creatinine
1.61	145.0508	145.0501	4.8	C ₆ H ₁₀ O ₄	Adipic acid
1.40	233.0117	233.0120	1.3	C ₈ H ₁₀ O ₆ S ⁻	3-Hydroxytyrosol sulfate
1.74	217.0162	217.0171	4.2	C ₈ H ₁₀ O ₅ S ⁻	Tyrosol sulfate
0.80	216.0331	216.0331	0.0	C ₈ H ₁₁ NO ₄ S ⁻	Tyramine sulfate
1.95	297.0550	297.0545	1.7	C ₁₂ H ₁₄ O ₅ S ⁻	N-Acetylserotonin sulfate

Urinary Biomarkers of Nonhuman Primate Radiation Exposure in the Nonhuman Primates Showing Concentrations and Fold Change Compared to Predose for 8.5 Gy Irradiated Nonhuman Primates

	Predose						24 h						48 h						72 h					
	Mean μmol		SEM	<i>P</i> value ^a	Fold change		Mean μmol		SEM	<i>P</i> value ^a	Fold change		Mean μmol		SEM	<i>P</i> value ^a	Fold change		Mean μmol		SEM	<i>P</i> value ^a	Fold change	
Taurine	3.594	0.903	N/A	N/A	N/A		14.143	2.386	0.0022	0.0022	3.935		8.562	1.787	0.0519	0.0519	2.382		9.307	2.818	0.1797	0.1797	2.590	
<i>N</i> -Acetyltaurine	0.449	0.187	N/A	N/A	N/A		2.079	0.298	0.0043	0.0043	4.630		2.108	0.876	0.0303	0.0303	4.695		1.650	0.608	0.0649	0.0649	3.675	
Isethionic acid	0.382	0.104	N/A	N/A	N/A		2.865	0.663	0.0022	0.0022	7.500		1.660	0.501	0.0087	0.0087	4.346		2.455	0.909	0.0152	0.0152	6.427	
Hypoxanthine	10.505	2.352	N/A	N/A	N/A		35.752	3.651	0.0022	0.0022	3.403		29.136	9.295	0.0519	0.0519	2.774		27.160	8.013	0.1797	0.1797	2.585	
Uric acid	1.976	0.0741	N/A	N/A	N/A		8.010	0.883	0.0022	0.0022	4.054		5.158	2.364	0.1775	0.1775	2.610		4.176	1.293	0.1797	0.1797	2.113	
Xanthine	3.294	1.235	N/A	N/A	N/A		16.158	4.385	0.0043	0.0043	4.905		17.863	12.4042	0.0952	0.0952	5.423		14.053	4.739	0.0823	0.0823	4.266	
Adipic acid	8.563	2.807	N/A	N/A	N/A		467.439	108.551	0.0043	0.0043	54.588		46.673	15.059	0.0095	0.0095	5.450		36.115	27.067	0.4762	0.4762	3.161	
<i>N</i> -Acetylserotonin sulfate	0.077	0.046	N/A	N/A	N/A		1.144	0.206	0.0075	0.0075	14.935		0.189	0.0776	0.0878	0.0878	2.481		0.026	0.0170	N/A	N/A	N/A	
3-Hydroxytyrosol sulfate	0.465	0.112	N/A	N/A	N/A		6.832	1.129	0.0043	0.0043	14.692		1.428	0.458	0.0381	0.0381	3.071		1.358	0.662	0.3290	0.3290	2.920	
Tyramine sulfate	1.515	0.6806	N/A	N/A	N/A		53.448	9.190	0.0079	0.0079	35.279		4.250	2.163	0.0449	0.0449	2.805		4.284	1.996	0.0746	0.0746	2.828	
Tyrosol sulfate	1.331	0.285	N/A	N/A	N/A		1.199	0.405	0.8413	0.8413	0.900		3.991	0.665	0.0159	0.0159	2.998		3.057	1.281	0.8413	0.8413	2.297	
Creatinine	5.109	0.706	N/A	N/A	N/A		15.656	2.772	0.0087	0.0087	3.064		7.953	0.713	0.0519	0.0519	1.557		11.210	3.415	0.0649	0.0649	2.194	

^aSignificance as determined by Mann-Whitney *U* test.