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A Serum Small Molecule Biosignature of Radiation Exposure from Total Body Irradiated Patients

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Abstract

The potential for radiological accidents and nuclear terrorism has increased the need for the development of new rapid biodosimetry methods. In addition, in a clinical setting the issue of an individual's radiosensitivity should be taken into consideration during radiotherapy. We utilized metabolomics and lipidomics to investigate changes of metabolites in serum samples following exposure to total body ionizing radiation in humans. Serum was collected prior to irradiation, at 3–

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Author Contributions

E.C.L., C.A.B., and A.J.F. were responsible for designing the experiment for sample acquisition. C.A.B. and M.L. acquired and organized the samples at MSKCC. E.C.L., E.L.P., Y.-W.W., and S.K.C. performed sample preparation and MS analysis. E.C.L. and E.L.P. analyzed the MS data. T.M. was involved with the statistical analysis. E.C.L. and G.A. interpreted the MS data. E.C.L. wrote the manuscript, and all authors read, provided comments, and approved the final version of the manuscript.

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteo-me.7b00468.

Untargeted metabolomic profiling and pathway annotation (Figure S1); examples of lipidomic profiles in positive and negative ionization modes (Figure S2); analysis of markers based on sex (Figure S3); targeted analysis of anti-inflammatory eicosanoids (Figure S4); individual responses for palmitic acid (Figure S5); demographic information of patients (Table S1); specifications on chromatography and MS (Table S2); metabolomic analysis (Table S3); lipidomic analysis (Table S4); mean and SEM values of significant ions and metabolites (Table S5); GC–MS analysis (Table S6); mean and SEM of eicosanoids (Table S7) (PDF)

Graphical abstract

INTRODUCTION

Metabolomics and lipidomics have gained significant momentum in research, clinical areas, and population studies.⁴⁻⁶

Although identification of water-soluble metabolites and annotation of existing libraries have appreciably progressed, lipidomics is still in its early stages due to the complexity of the molecules and the high number of species and isomers. Current methodologies provide limited structural identification of lipids, and a great majority of lipids still remain functionally uncharacterized. Nonetheless, the lipid class and degree of unsaturation of individual species can provide important information on the levels of oxidative stress and physiological conditions of an organism. Additionally, targeted quantitative approaches have contributed to the rapid dissection of changes in specific metabolic pathways or quantitation of representative key metabolites of choice. Taken together, results from untargeted and targeted approaches can provide important insights into the metabolic status of an individual with a disease or after exposure(s) to environmental and genotoxic agents, such as IR.

However, due to limitations of obtaining samples from human populations and ethical restrictions on research on such populations, understanding systemic responses to radiation at the metabolic level remains limited. Regardless, limited studies utilizing cancer patients have been analyzed in order to address such questions. Partial body exposure for glioblastoma,^{7,8} lung cancer,⁹ and head and neck cancer¹⁰ to name a few has concentrated on treatment efficacy for outcome analysis through metabolomic analysis at the end of a long radiation regimen with high cumulative doses. Jelonek et al. also recently reviewed such studies and further concluded that proteomic and metabolomic responses correlate to the extent of normal tissue exposure.¹¹ Studies addressing the more pertinent questions of early responses to radiation for radiotherapy monitoring and biodosimetry are still severely limited. In addition to the limitation of cancer patients enrolled in such studies, concurrent administration of radiation and chemotherapy in multiple clinical protocols further limits the pool of available volunteers.

We previously reported on urine analysis through metabolomics of total body irradiated (TBI) cancer patients¹² and identified significant effects at 6 h post irradiation. Here, we extend on our previous research and analyze the serum of a similar cohort in order to generate radiation signatures of TBI patients within the first 24 h post IR with untargeted and targeted approaches utilizing liquid chromatography (LC) mass spectrometry (MS) and gas chromatography (GC) MS. Results demonstrated significantly higher overall perturbations at the latter time point (~24 h post IR), whereas markers specifically associated with inflammatory processes through a targeted eicosanoids assay peaked at the earlier time point (6 h post IR). Such studies can aid in monitoring radiation responses and generating blood signatures that can be utilized in a radiological event in order to triage affected populations.

MATERIALS AND METHODS

Subjects

Subjects were recruited through the Department of Radiation Oncology at Memorial Sloan-Kettering Cancer Center (MSKCC). Those planning to undergo TBI prior to hematopoietic stem cell transplantation (HSCT) were targeted for participation in this institutional review board approved study (#07–158). After giving informed consent to participate, subjects underwent biospecimen (serum) collection at three time points before and during

fractionated TBI. The first collection was on the day of the first dose of TBI, prior to treatment. The second collection was 3–8 h (averaged to ~6 h) after the first fraction of TBI with 1.25–2 Gy. The third collection was 24 h after the first specimen was collected, after a total of 1 fraction of 2 Gy, or 2–3 fractions of 1.25 Gy with a 4–6 h interfraction interval (2–3.75 Gy total) (final collection was performed at 20–22 h after the first fraction). All individual doses and times of irradiation are shown in Table S1. As part of routine patient care, combined blood count with differential and metabolic function was assessed by blood sampling every 24 h (Table 1), and statistically significant changes ($p < 0.05$) were determined through a Wilcoxon matched-pairs signed rank test. Irradiation was performed as previously described.¹² A total of 15 patients (7 male and 8 female), diagnosed with diseases including acute myelogenous leukemia, chronic myelogenous leukemia, acute lymphocytic leukemia, Hodgkin's lymphoma, non-Hodgkin's lymphoma, and myelodysplastic syndrome, participated in the study (Table S1). All patients were in remission, and the majority did not have chemotherapy treatment for at least 1 month prior to the start of the radiation regimen. Patients had no specific dietary restrictions during the first 2 days of TBI, with most consuming the first meal of the day after the first dose of TBI.

Chemicals

All chemicals used were of the highest purity and were LC–MS grade when applicable. Debrisoquine sulfate, 4-nitrobenzoic acid (4-NBA), oleic acid, linoleic acid, guanine, mevalonic acid, L-alpha-glycerophosphorylcholine (glycerophosphocholine), hydroxykynurenine, α -linolenic acid (ALA), ubiquinone Q2, phenylalanine, oxalic acid, palmitic acid, urease type III, anhydrous sodium sulfate, C4–C24 FAMES, methyl nonanoate, and alkane standard mix (C10–C40) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Standards for lipidomics included monoacylglyceride MG(17:1), diacylglyceride DG(20:1/20:1), and triacylglyceride TG(19:1/19:1/19:1) from Nu-Chek Prep Inc. (Elysian MN, USA) and phosphatidic acid PA(14:0/14:0), phosphatidylcholine PC(14:0/14:0), phosphatidylethanolamine PE(14:0/14:0), and phosphatidylserine PS(14:0/14:0) from Avanti Polar Lipids Inc. (Alabaster AL, USA). Glutamic acid- d_5 , citric acid- d_4 , D-sorbitol- $^{13}C_1$, and L-leucine- d_3 utilized in gas chromatography mass spectrometry (GC-MS) were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). *N*-Methyl-*N*-[trimethylsilyl]trifluoroacetamide (MSTFA) with 1% (v/v) trimethylchlorosilane (TMCS) was acquired from Thermo Scientific (Waltham, MA, USA). For the targeted lipidomics assay, (d_{11}) 14,15-DiHETrE, (d_4) 15-deoxy- 12,14-PGJ2, (d_4) 9(S)-HODE, (d_4) LTB4, (d_4) PGD2, (d_4) PGE2, (d_4) ($\pm$)12,13-DiHOME, (d_4) ($\pm$)9,10-DiHOME, (d_6) 20-HETE, (d_8) 12(S)-HETE, (d_8) 5(S)-HETE, (d_7) 5-OxoETE, (d_5) LTE4, (d_4) 6-keto PGF1 α , PGF2 α , and (d_4) TXB2 were all obtained from Cayman Chemical Company (Ann Arbor, MI, USA). 12-[(Cyclohexylamino)carbonyl]amino]-dodecanoic acid (CUDA) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Global Metabolomic Profiling

Total metabolites were extracted from serum samples by diluting 25 μ L in 175 μ L of 40% acetonitrile/25% methanol/35% water with internal standards (2 μ M debrisoquine sulfate and 30 μ M 4-NBA). After vortexing and 15 min of incubation on ice, the supernatant was collected following a 20 min centrifugation (13 000 rpm at 4 °C). 200 μ L of 100%

acetonitrile was added to the supernatant, followed by a 15 min incubation on ice and further collection of the supernatant after a 20 min centrifugation (13 000 rpm at 4 °C). The samples were dried down using a speed vacuum with no heat and reconstituted in 200 μ L of 5% methanol/1% acetonitrile/94% water. Following a final round of centrifugation (13 000 rpm at 4 °C), samples were stored at –20 °C until further analysis. Metabolites were separated using an ACQUITY UPLC (Waters Corporation, Milford, MA, USA) fitted with a BEH C18, 2.1 $\times$ 50 mm maintained at 60 °C. The chromatographic conditions are outlined in Table S2. MS analysis was performed on a Synapt-G2 Si mass spectrometer (Waters Corporation, Wilmslow, UK), acquiring centroid data from 50 to 1200 Da in both positive and negative electrospray ionization modes with MS^E function. Accurate mass was obtained by intermittent injections of leucine enkephalin ($[M + H]^+ = 556.2771$ and $[M - H]^- = 554.2615$) introduced as Lockspray. A quality control sample was prepared by pooling all samples and was intermittently run every 11 injections for assessment of retention time drift and chromatographic integrity. Validations of candidate metabolites were conducted with tandem mass spectrometry (MS/MS), and fragmentation patterns were compared to those of pure chemicals for positive identification and/or to the METLIN library.¹³

Global Lipidomic Profiling

The global lipidomic preparation has been described in detail previously,^{14,15} utilizing chloroform/methanol extraction. Each sample was spiked with a mixture of lipid standards, as described earlier, for the relative quantification and identification of candidate lipids based on their m/z and retention time. Total lipid analysis was performed using an ACQUITY UPLC (Waters Corporation) with a CSH C18, 2.1 $\times$ 100 mm maintained at 65 °C, coupled to a Synapt-G2 Si mass spectrometer (Waters Corporation), operated in both positive and negative ionization modes, with MS^E functions. The chromatographic and MS conditions are presented in Table S2. A quality control sample was prepared by pooling all samples and was intermittently run every 11 injections for assessment of retention time drift and chromatographic integrity.

GC-MS Profiling

Serum (50 μ L) was aliquoted, and analytes were extracted using 1 mL of cold methanol with internal standards (4-NBA, glutamic acid- d_5 , citric acid- d_4 , D-sorbitol- $^{13}C_1$, and L-leucine- d_3 , final concentration 20 μ g/mL), vortexed, and centrifuged at 13 000 rpm for 10 min at 4 °C, as described in detail in ref 16. The serum extract was evaporated, placed in GC vials, and derivatized with 100 μ L of MSTFA with 1% TMCS (1 h at 60 °C) with rigorous shaking. Samples were injected (1.5 μ L) into a Leco Pegasus HT GC–TOF–MS (St. Joseph, MI) equipped with a Gerstel multipurpose sampler (Linthicum, MD) and a Rtx-5 (G27) column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m, length $\times$ internal diameter $\times$ film thickness, 5 m Integra-Guard column), and automated liner exchanges were programmed after every 10 samples. The temperature program was ramped from 70 °C (0.2 min hold) to 270 °C (10 °C/min, 4 min hold) to 320 °C (20 °C/min, 2 min hold to remove contaminants) with spectra collected from 200 to 1600 s at 30 spectra/s acquisition rate (m/z 40–600 scan range). The inlet was set at 220 °C, the transfer line was set at 270 °C, and the ion source was set at 200 °C, 1:20 split, with a 1.2 mL/min helium flow rate. Standard alkane and FAME mixes were run to establish retention indices.

Targeted Eicosanoid Profiling

Eicosanoid profiling was performed as previously described.^{14,17} A 250 μL aliquot of serum was mixed with antioxidants (0.2 mg of butylated hydroxytoluene/EDTA) immediately after thawing on ice and internal standards [50 nM concentration: (d_{11}) 14,15-DiHETrE, (d_4) 15-deoxy-12,14-PGJ2, (d_4) 9(*S*)-HODE, (d_4) LTB4, (d_4) PGD2, (d_4) PGE2, (d_4) ($\pm$)12,13-DiHOME, (d_4) ($\pm$)9,10-DiHOME, (d_6) 20-HETE, (d_8) 12(*S*)-HETE, (d_8) 5(*S*)-HETE, (d_7) 5-OxoETE; 100 nM concentration: (d_5) LTE4, (d_4) 6-keto PGF1 α (d_4), PGF2 α , (d_4) TXB2], and the volume was brought up to 1.3 mL with wash solution (water +0.1% formic acid/methanol, 95:5, v/v). A 100 nM quality marker (CUDA) was added to the samples. Samples were cleaned by solid-phase microextraction using Waters Oasis HLB 96-well plates, dried under a gentle nitrogen flow, and reconstituted in 100 μL of injection solvent (acetonitrile/methanol, 50:50, v/v). Samples were analyzed by using an ACQUITY UPLC (Waters Corporation) with a BEH C18 2.1 $\times$ 100 mm column coupled to a Xevo TQ-S MS (Waters Corporation, Wilmslow, UK). The LC method and MS conditions are described in detail in Table S2. A quality control sample was prepared by pooling all samples and was intermittently run every 10 injections. Eicosanoids were detected by multiple reaction monitoring transitions in negative ionization mode, which were previously optimized with synthetic standards. Subsequently, samples were diluted 1:1000, and free fatty acids were analyzed in single ion recording mode.

Data Processing and Analysis

Peak Alignment and Deconvolution for Global Profiling—For global metabolomic and lipidomic profiling, peak alignment and deconvolution was conducted through the software Progenesis QI (Nonlinear Dynamics, Newcastle, UK) by utilizing a suitable quality control sample (QC from pooled samples) from the run as a reference for peak alignment. Peak normalization for metabolomics was conducted by normalizing to all compounds, a feature integrated within Progenesis QI, while internal standards were used for monitoring potential retention time drift and mass accuracy. Normalization for global lipidomic analysis was performed either to all compounds or to individual lipid classes' internal standards (MG, PC, PE, and TG), after abundance of each internal standard was extracted with the software TargetLynx (Waters Corporation). Data-independent acquisition allowed for fragmentation matching to theoretical fragmentation patterns through the databases LIPID Metabolites and Pathways Strategy (LIPID MAPS),¹⁸ The Human Metabolome Database (HMDB),¹⁹ and LipidBlast²⁰ with an error of <10 ppm.

Metabolomic Analysis—Analysis for perturbations in the global serum metabolome was conducted via untargeted metabolomics. The in-house statistical software MetaboLyzer²¹ was utilized to obtain statistically significant ions. No data transformation was conducted prior to statistical analysis, and outliers were removed via 1.5 IQR filtering. Complete presence ions, which are defined as ions having a presence in at least 70% of the samples in both control and experimental groups, were analyzed via the nonparametric Mann-Whitney U test ($p < 0.05$ was considered statistically significant). Partial presence ions, which are defined as ions having a 70% presence in only one group, were analyzed categorically via Barnard's test ($p < 0.05$ was considered statistically significant). Putative identities were obtained through the databases KEGG,^{22,23} HMDB,¹⁹ and LIPID MAPS,¹⁸ with a ppm

error below 20, via neutral mass elucidation from the MS1 m/z values. Volcano plots ($-\log_{10}$ p -value vs $\log_2$ fold change) were constructed utilizing all complete presence ions analyzed, with the statistically significant ions highlighted. Furthermore, KEGG based metabolic pathway enrichment analysis was conducted utilizing an FDR corrected hypergeometric statistical test ($FDR < 0.25$).

Statistical significance for each potential marker within the three groups was conducted with the nonparametric Kruskal–Wallis test ($p < 0.05$ was considered significant) with Dunn’s multiple comparison test correction through Prism 6 (GraphPad Software Inc.). Subsequent univariate analysis within each male or female group was conducted with the nonparametric Mann–Whitney U test ($p < 0.05$ was considered significant).

Lipidomic Analysis—The deconvoluted data set was separated according to putative lipid classes, and normalization was applied as described above. Statistically significant ions were identified with the nonparametric Kruskal–Wallis test within the three groups with Dunn’s multiple test correction through Prism 6 (GraphPad Software, Inc.), whereas p -values between two groups were calculated by applying a nonparametric Mann–Whitney U test ($p < 0.05$ was considered significant for both tests). Putative identities (carbon and double bond content) were further confirmed through retention time and matching of theoretical fragments through LIPID MAPS¹⁸ and LipidBlast²⁰ (when available) with a ppm error of less than 10.

GC–MS Analysis—Data were acquired, deconvoluted, and aligned with the software ChromaTOF v. 4.51.6.0 and the statistical compare function (Leco, St. Joseph, MI). Data were deconvoluted with a baseline offset of 1 and a signal-to-noise ratio of 100, aligned with a max retention time 1 s, and identified with minimum similarity match of 600 and a mass threshold of 10 to the National Institute of Standards and Technology (NIST) 14 mass spectral library (<http://www.nist.gov/srd/nist1a.cfm>). A minimum of 50% of samples in a class containing the analyte was used for establishing the output, and analyte identification was further validated by comparing its retention index to FiehnLib.²⁴ Output was normalized to an internal standard, and statistical significance was calculated with a nonparametric Kruskal–Wallis test ($p < 0.05$ was considered significant) with Dunn’s multiple test correction through Prism 6 (GraphPad Software, Inc.).

Targeted Eicosanoid Assay—Extraction of abundance levels for internal standards and within each sample was conducted with the software TargetLynx, which automatically calculated concentrations of each metabolite based on a standard curve. Statistically significant ions were identified with the non-parametric Kruskal–Wallis test ($p < 0.05$ was considered significant) within the three groups with Dunn’s multiple test correction through Prism 6 (GraphPad Software, Inc.), followed by pathway assignment. Limits of quantification were in the lower ng/mL range.

Fold Changes—Fold changes were calculated as Group 1 (e.g., 6 or 24 h, or male) divided by Group 2 (e.g., pre or female), applicable to metabolomics, lipidomics, GC–MS analysis, and eicosanoids assay. No trend alterations were considered when fold changes were calculated between the limits of 0.85 and 1.15.

RESULTS

Cancer patients undergoing HSCT at MSKCC were given TBI, and serum was collected immediately before and at two time points after IR. Hematologic parameters showed a significant decrease at ~24 h post IR (Table 1), as expected with TBI exposure. Untargeted (LC-MS and GC-MS) and targeted analyses were conducted as depicted in Figure 1. The overall metabolic differences showed higher responses with increased total dose and time compared to the pre-IR samples. This was evident from the construction of volcano plots (Figure S1), where assessment of *p*-values and fold changes showed a clear shift toward a profile of increased circulating ions in the later time point. Additionally, putative ion pathway enrichment through the KEGG database not only displayed an increase in pathways that were statistically significant, following a false discovery rate adjustment of 0.25, but also showed particular perturbations associated with biosynthesis of unsaturated fatty acid metabolism, linoleic acid metabolism, amino acid and purine metabolism, and taurine and hypotaurine metabolism. Nine ions were putatively identified, of which five (oleic acid, α -glycerophosphorylcholine, α -linolenic acid, phenylalanine, and ubiquinone Q2) were positively validated through MS/MS (Table S3), showing a role in cell signaling. Further analysis of each group to male and female comparisons revealed sex-dependent responses, with higher statistically significant perturbations in the female group. Figure 2 shows the normalized abundance of each ion based on sex. Six ions or metabolites through metabolomics exhibited statistically significant changes in the female population (oleic acid, glycerophosphocholine, 225.0875_8.39, α -linolenic acid, 279.2319_8.17, and phenylalanine), compared to only two in the male population (174.0388_0.34 and 149.0815_7.61). The unknown ions remain unverified, as their fragmentation patterns did not match the chemicals tested (linoleic acid, guanine, mevalonic acid, and hydroxykynurenine), as shown in Table S3. Although the circulating levels of each metabolite differed according to time point, the majority of the changes appeared in the later time point, as was observed by calculating fold changes (Table 2).

Global lipidomic analysis also revealed important perturbations of circulating lipid classes. As shown in Table S4, metabolites from four lipid classes [monoacylglycerides (MG), phosphatidylcholines (PC), phosphatidylglycerols (PG), and triacylglycerides (TG)] showed significant changes after IR. The classes were identified through appropriate matching of *m/z* and retention time with the use of internal standards and online database searches (LIPID MAPS, HMDB). Figure S2 depicts examples of chromatograms for both ionization modes of the major lipid classes, aiding in positive identification of individual candidates. Representative lipids of each class are shown in Figure 3A [PC(38:8), MG(18:1), TG(50:3), and PG(32:0)], with the predominant differences most evident at the later time point (~24 h) and higher total dose, similar to what was observed with global metabolomic profiling. In general, the MGs [MG(18:1) and MG(18:2)] and two TGs [TG(48:0) and TG(50:3)] showed increases in circulation, whereas the other lipids exhibited a progressive decrease in circulating levels with respect to pre-IR levels. Similar results were also observed through the GC-MS analysis, with increased levels in the circulation after exposure to IR. GC-MS provided the identities of four free fatty acids (FFAs) and a dicarboxylic acid (oxalic acid) (Tables 3, S5, and S6). Oleic acid was also identified with LC-MS, whereas linoleic acid,

palmitic acid, palmitoleic acid, and oxalic acid, shown in Figure 3B were not previously identified with LC-MS.

The general conclusion from the global profiling experiments (Tables 2, 3, and S5 and Figures 2, 3, and S3) pointed to higher levels of biomarker circulation when time and dose increased compared specifically to pre-IR samples, with the exception of TGs. Addressing the inherent sex differences of various identified ions through fold changes at all three time points (pre, 6 h, and 24 h), it became apparent that females exhibited higher circulating levels of the majority of the ions compared to males following exposure to IR based on the results from Table 2 assessed through fold changes at each time point. At 6 h, females showed a 40% increase, compared to 13% for males, in the overall levels of the identified circulating ions shown in Table 2, and at 24 h, females had 50% increased levels of ions compared to 30% for males based on data from the same table. These percentages were calculated from the number of ions exhibiting an increased or decreased trend at each time point (fold change >1.15 of male/female or <0.85 of male/female) compared to the ions presented in Table 2. However, males showed increased levels of polyunsaturated lipids with higher carbon content [e.g., PC(40:10) and TG(50:6)], indicative of increased oxidative stress. Females, on the other hand, showed increased levels of two FFAs (α -linolenic acid and palmitoleic acid) and oxalic acid compared to males (Table 3). Overall, however, no statistically significant changes were observed between males and females within the same time points for each of the ions and metabolites presented in Tables 2 and 3.

Results from the assessment of the levels of eicosanoids with a targeted metabolomics approach with levels above the limits of quantification are reported in Table S7 and mapped on the corresponding metabolic pathways (Figures 4 and S4). Standard curves for quantification had an $R^2 = 0.99$. We observed a significant increase in the levels of the oxygenated metabolites of linoleic acid and arachidonic acid, which dissipated at the later time point (Figure 4). In particular, the levels of 13-KODE, 9-HODE, 9-HOTrE, 12(13)-EpOME, 9(10)-EpOME, 12,13-DiHODE, 9,10-DiHOME, arachidonic acid, 13,14-dihydro-15-keto PGE2, 12(*S*)-HpETE, 11,12-DiHETrE, and 14,15-DiHETrE were increased at 6 h post IR. Although linoleic acid levels were not shown as altered with this method, it was previously identified through GC-MS that they were indeed increased at both time points. However, some metabolites, such as thromboxanes and prostaglandins, were significantly downregulated even after IR. Less prominent perturbation were observed in the omega-3 fatty acid precursors (Figure S4). No differences in patterns of circulation were observed between males and females (data not shown).

DISCUSSION

Metabolomics and lipidomics, with untargeted or targeted approaches, have provided valuable insights in the effects of IR on overall metabolism. Significant biomarkers have been identified to generate signatures that can contribute to monitoring radiation responses in therapeutics or be used with great promise as biosignatures in emergency responses. To date, limited research has been conducted on human populations, particularly TBI patients, due to the limited availability of samples that do not include confounding factors from concurrent administration of chemotherapy drugs. The majority of radiation metabolomic

studies with TBI patients have focused on analysis at the end of the therapeutic regimen, when total doses far exceed the considerations set for biodosimetry. Here, we have investigated metabolic changes in serum at time points 24 h post IR. Clinical parameters were congruent with published reports on radiation-induced apoptosis.^{25,26}

Global profiling with metabolomics and lipidomics with LC-MS and GC-MS showed elevated metabolic dysregulation with the highest accumulated dose and time point.

TG lipids exhibited significant decreases relative to pre-IR samples; however, given that the majority have low degrees of unsaturation, their primary origin may be attributed to nutritional intake, which may also be responsible for decreased levels of phenylalanine. This is further corroborated by increases in MG(18:1) and MG(18:2). Regardless, it has been shown in other species, such as non-human primates, that these molecules can be informative of the oxidative stress status of an individual when molecules with high degrees of unsaturation are observed.¹⁵ Similarly, PC and PG lipids also decreased with increased dose and time. Decreases in PCs translate downstream to decreases in glycerophosphocholine, as that molecule is generated from PCs through enzymatic action of phospholipases.²⁷ Glycerophosphocholine is also a natural osmolyte and important in the integrity of renal function,^{28,29} and perturbations of this balance may be a contributor to radiation-induced renal injury^{30,31} that has also been seen in some TBI patients.

The most striking metabolic response is the increase in circulating FFAs in the later time point and higher total dose. Linoleic acid, palmitoleic acid, palmitic acid, oleic acid, and α -linolenic acid showed statistically significant increases. Biosynthesis of fatty acids and linoleic acid metabolism were also two of the most prominent pathways through pathway annotation from global profiling (Figure S1). Fatty acids, particularly polyunsaturated species, can be released from hydrolysis of complex lipids and can be converted to downstream products such as eicosanoids. Such species can further contribute to the perpetuation of oxidative stress and proinflammatory signaling (e.g., omega-6 pathway) or even counteract it through anti-inflammatory mechanisms (e.g., omega-3 pathway). However, increases in polyunsaturated fatty acids (PUFAs) are consistent with increased apoptosis and a proinflammatory phenotype associated with radiation responses.^{32–34} Indicative of this is the increased circulating levels of ubiquinone Q2, a precursor of ubiquinone Q10 synthesis. Ubiquinone Q10 plays a significant role in ATP production in mitochondria through the electron transport chain and possible reduction in oxidative stress.³⁵

As mentioned above, PUFAs can serve as the basis for the generation of eicosanoids that are primarily proinflammatory in nature (eicosanoids). Previous studies in irradiated mice identified a severe shift in balance toward an inflammatory phenotype at 24 h post-IR,¹⁴ with many products known to originate from neutrophil responses.³⁶ Similar results were seen in this study, although the inflammatory response was observed as early as 6 h post-IR (Figure 4). Although the linoleic acid derivatives returned to normal or below normal levels, derivatives of arachidonic acid showed persistently increased trends, primarily through the enzymatic action of lipoxygenase, that can be attributed to the effects of increased dose. Such low persistently increased levels may contribute to priming and activation of the

immune response or even increase cell signaling and metabolic responses that can lead to long-term radiation injury. In this case, it is unclear whether a fractionated dose scheme itself has a dampening effect on the responses, as previous studies in mice showed a clear statistically significant response at the same time point (24 h).¹⁴ The anti-inflammatory pathway on the other hand (omega-3) showed minimal response (Figure S4).

As in our previously published study on urine metabolomics from TBI patients,¹² sex-dependent differences were seen with a number of the metabolites in serum as well. In particular, both metabolomics and lipidomics showed differences in trends not only between males and females but also in statistical significance (Figure 2 and Tables 2 and 3) within each group. Unlike the urinary studies, where the majority of responses were seen in males, serum appears to show a higher metabolic response for females. Due to the small group number, however, no statistical significance was observed when comparing male and female responses at the same time point and dose. Sex-dependent differences were also not observed for results from the eicosanoids assay (data not shown), indicating that the inflammatory response may be sex-independent. Results such as these are painting a picture of the need for individualized cancer-related radiotherapy treatments, which will remain an active area of research as we increase the number of patients that are recruited.

Taking a look at the current results and bringing it into the context of biodosimetry and generation of biosignatures, it is possible to identify exposed individuals from their overall metabolic profile and to determine changes in specific circulating metabolites that can indicate the level of exposure. Although radiation studies in humans are limited (e.g., underlying conditions, therapeutic regimen), they are still powerful in providing markers that can directly be translated to the population. However, individual variability and responses remain a concern, particularly for nonresponders that may register as false negatives, as can be seen for palmitic acid in Figure S5 (patients 1, 7, 9, and 15). Such differences, however, were not evident for PG(32:2) (individual responses not shown). It is imperative therefore not to rely on a small number of metabolites but rather to develop a collection of metabolites that can indicate exposure to radiation. Individuals therefore that do not pass certain criteria, e.g., a percentage of positive metabolic hits, can subsequently be screened with methods such as cytogenetics that have long been considered the gold standard for radiation biodosimetry.

A significant level of cross-species validation has already been demonstrated in studies with rodents and more importantly nonhuman primates (current studies summarized by Pannkuk et al.³), providing confidence in metabolomic radiation responses. The potential to translate signatures from animal models to humans for various radiation scenarios will aid in enriching results obtained through metabolomics, as studies in humans are limited and biomarkers provided are specific only for the conditions used, such as in this study. Nevertheless, this particular cohort is unique and provides invaluable information, and continuing efforts to acquire new samples will provide validation of the identified biomarkers. In the event that thousands of individuals will be required to be screened in a rapid manner, metabolomics can effectively provide a method for biodosimetry. Finally, identifying responses in blood such as the ones we described may also benefit patients

receiving radiation therapy, as the effectiveness of such treatments can be monitored through a simple blood test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Experimental design of untargeted and targeted approaches. Serum was collected from patients undergoing total body irradiation as a precursor of allogeneic hematopoietic transplantation. Global metabolomic and lipidomic analyses were conducted with a Waters UPLC-Synapt G2 Si operated in MSE mode, and chromatographic data were deconvoluted with the software Progenesis Q1. Targeted eicosanoid profiling focused on quantification of omega-6 and omega-3 lipids, analyzed with multiple reaction monitoring on a Xevo TQ-S. Gas chromatography mass spectrometry complemented the liquid chromatography analysis and was performed with a Leco Pegasus HT.

Figure 2.

Metabolites and ions exhibiting significant differences between time points and doses.

Normalized abundance values are presented for male and female responses in scatter plots.

Although similar trends exist between male and female for the majority of the ions presented, specific changes are more pronounced in either male (174.0388_0.34 and 149.0815_7.61) or female (remaining, except ubiquinone Q2), allowing for the generation of sex-specific signatures. The data are represented as the mean $\pm$ SEM; p -values between two groups were calculated with the Mann-Whitney U test, and $p < 0.05$ was considered statistically significant.

Figure 3.

(A) Untargeted lipidomic analysis results with representative molecules from four different classes of lipids (phosphatidylcholines, monoacylglycerides, phosphatidylglycerols, and triacylglycerides) as scatter plots. (B) Abundance levels of two free fatty acids and oxalic acid identified with GC-MS. For both A and B, the more pronounced differences are observed at the later time point (higher cumulative dose). The data are presented as mean $\pm$ SEM, and the p -values between two groups were calculated with the Mann-Whitney U test, with $p < 0.05$ considered statistically significant.

Figure 4.

Targeted analysis of proinflammatory eicosanoids. Omega-6 linoleic acid (LA) derived eicosanoids exhibit perturbations at both 6 and 24 h after irradiation. Dark red boxes indicate statistically significant metabolites, and lighter red boxes signify increased levels according to fold change, although statistical significance of $p < 0.05$ was not reached. Green boxes show decreased levels with respect to pre-irradiation levels. Primary enzymes catalyzing and contributing to these changes include lipoxygenase (LOX), enzymes of the cytochrome P450 (CYP450) family, and cyclooxygenase (COX). With increased time and dose, a shift occurs

from eicosanoids having linoleic acid as a precursor to eicosanoids with arachidonic acid as their precursor.

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Table 1
Blood Parameters Measured Prior to the First Fraction and Following the Last Fraction of Radiation

	pre-TBI			post-TBI			change after TBI	post TBI/ pre TBI	p-value ^d
	group mean (n = 15)	group standard deviation (n = 15)	group mean (n = 15)	group standard deviation (n = 15)	group mean (n = 15)	group standard deviation (n = 15)			
WBC (k/ μ L)	2.89	1.69	2.75	1.86	2.75	1.86	↓	0.95 ^b	0.1475
RBC (M/ μ L)	3.52	0.71	3.32	0.67	3.32	0.67	↓	0.94 ^b	0.0006
Hg (g/dL)	10.93	1.9	10.36	1.84	10.36	1.84	↓	0.95	0.0002
Hct (%)	32.71	5.71	30.67	5.56	30.67	5.56	↓	0.94	0.0008
Plt (10k/ μ L)	14.68	6.54	13.35	6.08	13.35	6.08	↓	0.91	0.0034
% neut	60.91	18.71	76.22	11.69	76.22	11.69	↑	1.25	0.0031
% lymph	23.28	14.97	10.49	7.78	10.49	7.78	↓↓	0.45 ^b	0.0012
% mono	7.24	4.71	7.38	4.79	7.38	4.79	↑	1.02	0.4548
% eos	5.82	4.36	3.89	2.61	3.89	2.61	↓↓	0.67	0.0134
% baso	0.74	0.61	0.56	0.63	0.56	0.63	↓	0.76 ^b	0.0134
Na	141.6	2.20	141.8	2.04	141.8	2.04		1.00	0.8652
K	3.9	0.26	3.9	0.29	3.9	0.29		0.99	0.8657
Cl	106.7	2.05	107.8	2.14	107.8	2.14	↑	1.01 ^b	0.0171
CO ₂	29.1	1.94	28.2	2.24	28.2	2.24		0.97	0.0938
BUN	11.8	2.68	9.5	2.70	9.5	2.70	↑	0.81	0.0003
Cr	0.7	0.19	0.7	0.22	0.7	0.22		1.02	0.6172
Ca	8.5	0.29	8.6	0.28	8.6	0.28		1.01	0.2646
Glu	96.8	14.18	101.2	14.88	101.2	14.88		1.05	0.1071
Mg	1.9	0.16	1.9	0.16	1.9	0.16		1.01	0.6094
Ph	4.3	0.58	4.3	0.77	4.3	0.77		1.01	0.6008

^aStatistical test used: Wilcoxon matched-pairs signed rank test.

^bSignifies $p < 0.05$.

Table 2

Fold Changes from Global Profiling

Identity or m/z_{rt}	Combined males and females				Male/Female			
	6h/Pre	Relative to Pre	24h/Pre	Relative to Pre	24h/6h	Pre	6h	Trend (6h)
								24h
								Trend (24h)
Oleic acid	1.31	↑	1.91	↑	1.45	1.36	1.15	↑
279.2319_8.17	1.37	↑	1.72	↑	1.26	1.18	0.92	↓
174.0388_0.34	0.95	-	1.58	↑	1.66	0.38	1.00	↓
152.0571_0.34	0.83	↓	1.41	↑	1.70	0.36	0.88	↓
149.0815_7.61	1.38	↑	1.32	↑	0.96	0.63	1.36	↑
L-alpha-Glycerophosphocholine	0.75	↓	0.50	↓	0.67	0.44	0.35	↓
25.0875_8.39	0.57	↓	0.66	↓	1.15	0.46	2.32	↑
alpha-Linolenic acid (ALA)	1.29	↑	1.43	↑	1.11	0.99	1.00	↓
Ubiquinone Q2	1.08	-	1.32	↑	1.20	1.29	1.09	↑
Phenylalanine+Na	0.90	-	0.84	↓	0.94	0.85	0.88	-
MG(18:1)	2.03	↑	2.59	↑	1.28	1.34	1.00	↓
MG(18:2)	2.04	↑	2.26	↑	1.11	1.29	1.13	↓
PC(38:8)	1.21	↑	0.36	↓	0.30	0.65	0.67	↓
PC(40:10)	0.98	-	0.39	↓	0.39	2.06	1.21	↑
PG(34:0)	0.98	-	1.11	-	1.14	0.98	1.02	-
PG(32:0)	1.01	-	0.70	↓	0.69	0.68	0.83	-
TG(40:1)	0.28	↓	0.01	↓	0.05	0.76	0.60	↓
TG(42:1)	0.60	↓	0.05	↓	0.08	1.28	0.58	↑
TG(42:2)	0.55	↓	0.02	↓	0.03	0.93	0.72	↓
TG(42:4)	0.61	↓	0.02	↓	0.04	0.54	0.90	↑
TG(44:2)	0.75	↓	0.08	↓	0.11	0.95	0.67	↑
TG(44:3)	0.62	↓	0.04	↓	0.07	0.72	0.75	↓
TG(46:3)	0.87	-	0.22	↓	0.25	0.93	0.78	↑
TG(48:0)	0.88	-	2.25	↑	2.57	1.26	0.90	-
TG(48:1)	0.35	↓	0.04	↓	0.11	1.76	0.72	↑
TG(48:2)	0.23	↓	0.01	↓	0.03	1.55	0.28	↓

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Identity or <i>m/z</i> _rt	Combined males and females				Male/Female					
	6h/Pre	Relative to Pre	24h/Pre	Relative to Pre	24h/6h	Pre	6h	Trend (6h)	24h	Trend (24h)
TG(48:5)	0.69	↓	0.08	↓	0.12	0.63	1.00	-	22.29	↗
TG(50:3)	0.78	↓	2.27	↗	2.93	1.14	1.00	-	0.96	-
TG(50:6)	1.01	-	0.19	↓	0.19	1.00	0.98	-	3.15	↗

Table 3

Fold Changes from GC-MS Profiling

Identity	Combined males and females				Male/Female					
	6h/Pre	Relative to Pre	24h/Pre	Relative to Pre	24h/6h	Pre	6h	Trend (6h)	24h	Trend (24h)
Linoleic acid	1.2	↑	1.4	↑	1.2	0.9	1.0	-	0.9	-
Oxalic acid	1.0	-	1.4	↑	1.4	0.9	1.2	-	0.7	↓
Palmitoleic acid	1.1	-	1.7	↑	1.5	1.2	1.1	-	0.8	↓
Palmitic acid	1.1	-	1.3	↑	1.2	1.1	1.1	-	1.0	-
Oleic acid	1.2	↑	1.5	↑	1.2	1.2	1.4	↑	1.1	-