Am J Physiol Gastrointest Liver Physiol 318: G439–G450, 2020.
First published January 21, 2020; doi:10.1152/ajpgi.00351.2019.

RESEARCH ARTICLE | *Nutrient Sensing, Nutrition, and Metabolism*

Methionine dietary supplementation potentiates ionizing radiation-induced gastrointestinal syndrome

Isabelle R. Miousse,[1,2] Laura E. Ewing,[1,3] Charles M. Skinner,[1,4] Rupak Pathak,[5] Sarita Garg,[5] Kristy R. Kutanzi,[1] Stepan Melnyk,[6] Martin Hauer-Jensen,[5] and Igor Koturbash[1,4]

[1]*Department of Environmental and Occupation Health, Fay W. Boozman College of Public Health, University of Arkansas for Medical Sciences, Little Rock, Arkansas;* [2]*Department of Biochemistry, College of Medicine, University of Arkansas for Medical Sciences, Little Rock, Arkansas;* [3]*Department of Pharmacology and Toxicology, College of Medicine, University of Arkansas for Medical Sciences, Little Rock, Arkansas;* [4]*Center for Dietary Supplements Research, Fay W. Boozman College of Public Health, University of Arkansas for Medical Sciences, Little Rock, Arkansas;* [5]*Division of Radiation Health, Department of Pharmaceutical Sciences, College of Pharmacy, University of Arkansas for Medical Sciences, Little Rock, Arkansas; and* [6]*Arkansas Children's Research Institute, Little Rock, Arknsas*

Submitted 20 November 2019; accepted in final form 9 January 2020

Miousse IR, Ewing LE, Skinner CM, Pathak R, Garg S, Kutanzi KR, Melnyk S, Hauer-Jensen M, Koturbash I. Methionine dietary supplementation potentiates ionizing radiation-induced gastrointestinal syndrome. *Am J Physiol Gastrointest Liver Physiol* 318: G439–G450, 2020. First published January 21, 2020; doi: 10.1152/ajpgi.00351.2019.—Methionine is an essential amino acid needed for a variety of processes in living organisms. Ionizing radiation depletes tissue methionine concentrations and leads to the loss of DNA methylation and decreased synthesis of glutathione. In this study, we aimed to investigate the effects of methionine dietary supplementation in CBA/CaJ mice after exposure to doses ranging from 3 to 8.5 Gy of ^{137}Cs of total body irradiation. We report that mice fed a methionine-supplemented diet (MSD; 19.5 vs. 6.5 mg/kg in a methionine-adequate diet, MAD) developed acute radiation toxicity at doses as low as 3 Gy. Partial body irradiation performed with hindlimb shielding resulted in a 50% mortality rate in MSD-fed mice exposed to 8.5 Gy, suggesting prevalence of radiation-induced gastrointestinal syndrome in the development of acute radiation toxicity. Analysis of the intestinal microbiome demonstrated shifts in the gut ecology, observed along with the development of leaky gut syndrome and bacterial translocation into the liver. Normal gut physiology impairment was facilitated by alterations in the one-carbon metabolism pathway and was exhibited as decreases in circulating citrulline levels mirrored by decreased intestinal mucosal surface area and the number of surviving crypts. In conclusion, we demonstrate that a relevant excess of methionine dietary intake exacerbates the detrimental effects of exposure to ionizing radiation in the small intestine.

NEW & NOTEWORTHY Methionine supplementation, instead of an anticipated health-promoting effect, sensitizes mice to gastrointestinal radiation syndrome. Mechanistically, excess of methionine negatively affects intestinal ecology, leading to a cascade of physiological, biochemical, and molecular alterations that impair normal gut response to a clinically relevant genotoxic stressor. These findings speak toward increasing the role of registered dietitians during cancer therapy and the necessity of a solid scientific background behind the sales of dietary supplements and claims regarding their benefits.

dietary supplementation; gastrointestinal toxicity; ionizing radiation; methionine; microbiome

INTRODUCTION

The role of diet and dietary supplements in health and disease is becoming increasingly recognized. Of particular interest are protein supplements, the global market for which is rapidly expanding and estimated to reach US $21.5 billion by 2025 (73). Amino acid supplements have been promoted for various applications, including muscle performance enhancement, and the reversal of muscle loss due to long-term inactivity after an illness or injury, particularly in the elderly. Amino acid supplementation is also an important aspect of the management strategy for cancer patients experiencing cachexia, a life-threatening form of muscle wasting found in 80% of patients with advanced cancer.

Methionine is an essential amino acid and, as such, must be acquired through the diet. It is needed for protein synthesis and is a key molecule in the one-carbon metabolism pathway, a cascade of biological transformations that is vital for the synthesis of nucleotides, glutathione, and proper maintenance of DNA methylation. On the other hand, toxicity associated with excess of methionine is well documented (4, 16, 27). Recent studies also indicate that dietary methionine overload negatively regulates intestinal physiology in the mouse model (52). Furthermore, dietary methionine restriction is reported to be effective in cancer therapy in several preclinical models, and a number of clinical trials utilizing dietary methionine restriction are ongoing (24, 54, 67).

Ionizing radiation (IR), a potent genotoxic stressor and an important diagnostic and treatment modality, negatively affects one-carbon metabolism and DNA methylation (a process dependent on one-carbon metabolism) (51, 53). Thus, methionine supplementation was proposed to improve the organismal response to radiation exposure (53). Indeed, several studies reported positive effects of methionine supplementation on the short-term response to total body irradiation (TBI). For instance, Batra and colleagues (6, 7) reported that dietary L-methionine supplementation mitigates radiation-induced global

Address for reprint requests and other correspondence: I. Koturbash, Dept. of Environmental and Occupational Health, Univ. of Arkansas for Medical Sciences, 4301 W. Markham St., Slot 8210-11, Little Rock, AR 72205 (e-mail: ikoturbash@uams.edu).

DNA hypomethylation shortly after exposure (24 and 48 h). However, the long-term effects of methionine supplementation and its potential for mitigation of radiation-induced effects remain unknown. Furthermore, as per recent epidemiological studies, dietary ingestion of supraphysiological amounts of methionine are common in Western populations (62, 66).

In this study, we aimed to investigate the effects of methionine dietary supplementation in CBA/CaJ mice after exposure to doses ranging from 3 to 8.5 Gy of ^{137}Cs TBI. We report that mice fed a methionine-supplemented diet (MSD) develop acute radiation toxicity at doses as low as 3 Gy. Partial body irradiation (PBI) performed with hindlimb shielding resulted in a 50% mortality rate in mice fed MSD and exposed to 8.5 Gy, suggesting prevalence of the radiation-induced gastrointestinal syndrome (RIGIS) in the development of acute radiation toxicity. To explore the mechanisms of MSD-induced potentiation of acute RIGIS, we analyzed the intestinal microbiome and investigated physiological, biochemical, and molecular end points. We demonstrate that a physiologically relevant excess of methionine potentiates the detrimental effects of exposure to IR in the small intestine.

MATERIALS AND METHODS

Animals, diets, and radiation exposures. Seven- to eight-week-old CBA/CaJ and C57Bl6/J male mice, purchased from Jackson Laboratory (Bar Harbor, ME) were used throughout the study. The animals were housed at the University of Arkansas for Medical Sciences (UAMS) animal facility under a 12:12-h light-dark cycle with free access to food and water. The Institutional Animal Care and Use Committee (IACUC) at UAMS reviewed and approved the experimental protocols. Animals were given a 1-wk acclimation period before initiation of experimental procedures. Using the Excel 2013 platform, we randomly divided the mice into the following groups (n = 10–24/group): sham or whole body irradiation to 3, 5.5, or 8.5 Gy of ^{137}Cs. During the entire experiment, sham-irradiated mice were not housed together with irradiated mice. Irradiation was performed with a J. L. Shepherd Mark I [model 25 ^{137}Cs irradiator (J. L. Shepherd & Associates, San Fernando, CA)]. For irradiation, unanesthetized mice were placed in cylindrical, well-ventilated Plexiglas chambers (J. L. Shepherd & Associates) divided into eight equally sized "pie slice" compartments by vertical dividers made of Plexiglas. Two chambers were stacked on top of each other and placed on a turntable rotating at 5 rpm in the position farthest away from the radiation source, allowing all mice that belonged to the same exposure regimen to be irradiated simultaneously. The average dose rate was 1.21 Gy/min.

For partial body irradiation (PBI), hindlimbs were protected by medical-grade lead to rescue 5–10% of the bone marrow to prevent the development of hematopoietic acute radiation syndrome.

Immediately after irradiation, animals were randomly assigned to receive the methionine adequate diet (MAD) or MSD (300% methionine compared with MAD). All diets were ordered from Teklad Diets/Harlan Laboratories (Madison, WI). The detailed composition of each diet is provided in Supplemental Table S1, and the experimental setup is depicted in Supplemental Fig. S1 (for supplemental materials, see https://doi.org/10.6084/m9.figshare.c.4805361.v1). Experiments involving TBI were repeated three times in all aspects, including ordering mice and diets from new lots, to confirm the nature of the effect.

On *days 3.5*, and *6*, animals were anesthetized with isoflurane (3% in oxygen), and blood samples were collected from the retroorbital plexus into EDTA-coated tubes. Blood was centrifuged at 10,000 *g* for 20 min at 4°C. Plasma was collected, flash-frozen in liquid nitrogen, and stored at −80°C for subsequent analyses. Anesthetized mice were then euthanized by cervical dislocation, and intestines were collected immediately and snap-frozen in liquid nitrogen or fixed in Carnoy's solution (60:30:10 methanol/chloroform/acetic acid) for metabolic, molecular, and histological analyses.

Analysis of the gut microbiome. DNA, extracted with the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA), was sent to Research and Testing Laboratories (RTL, Lubbock, TX). Sequencing of 16S ribosomal RNA and data analysis were done using the Illumina MiSeq System at RTL, as described previously (32, 61). Detailed description can be also found in supplemental materials. Sequence data for each individual sample has been uploaded to a publicly available database and can be accessed under "Project Data" at the following link: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA397387/.

Gene expression analyses. RNA and DNA were extracted simultaneously from flash-frozen tissue by use of the AllPrep DNA/RNA extraction kit (QIAGEN). cDNA was synthesized using the SuperScript reverse-transcriptase kit (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed with TaqMan Universal Master Mix (Life Technologies). Primers were added at a final concentration of 5 μM (Supplemental Table S2). Expression of mRNA targets was normalized to the internal control genes *Hprt, Gapdh*, and *TBP*. Results are expressed as fold change from the average of the three housekeeping genes according to the $\Delta\Delta C_T$ method and then normalized to the average of the MAD 0-Gy group. Mouse tight-junctions PCR array (SA Biosciences, array no. PAMM143Z) was used to analyze the expression of genes involved in the regulation of tight-junction-related proteins according to the manufacturer's protocol.

Analysis of bacterial 16S rRNA in the liver. DNA was extracted from liver tissue under sterile conditions as described above. Amplification of the bacterial 16S DNA gene was performed from 5 ng of liver gDNA by using the primers referenced in Supplemental Table S2.

Intestinal crypt colony assay. Microcolony crypt cell survival was performed as previously described (8, 72). Fixed jejunum sections were paraffin embedded, sliced, and stained with hematoxylin and eosin (H&E) by the UAMS Histopathology Core. Surviving crypts, defined as crypts containing 10 or more adjacent chromophilic non-Paneth cells, were counted in transverse cross-sections. Four circumferences were scored per mouse, and microcolony survival was expressed as the average number of crypts per circumference, with the average from each mouse considered as a single value for statistical purposes.

Mucosal surface area analysis. Mucosal surface area was measured as described previously, using an adaptation of the methods of Baddeley and coworkers and Langberg and coworkers (2, 25, 42, 52). Briefly, an automated software was used to measure mucosal surface area in vertical H&E-stained jejunum sections, which utilized a computer-assisted image analysis program (Image-Pro Premier, Meyer Instruments, Inc., Houston, TX). All measurements were done with a ×10 objective lens, and a total of 3–5 areas were measured from each intestinal segment.

Tissue and plasma metabolite analyses. Components of the one-carbon metabolism were analyzed from jejunum and liver tissues by using an HPLC-EC method, as previously described (47, 48). Plasma levels of the amino acids, including citrulline, were determined using the high-throughput liquid chromatography-tandem mass spectrometry (LC-MS/MS) methodology, as previously described (25, 29).

Analysis of long interspersed element-1 DNA methylation. Recent advances in computational biology have led to classification of long interspersed element-1 (LINE-1) elements based on their evolutionary age and respective 5′-untranslated region (UTR) sequences (64). In this study, we assessed the DNA methylation status of six LINE-1 elements that belong to the evolutionary youngest A-type promoter. LINE-1 families' consensus sequences were obtained from the Genetic Information Research Institute (GIRI) Database (http://www.girinst.org/), and the 5′-UTRs of six LINE-1 elements were analyzed using NEBcutter (http://nc2.neb.com/NEBcutter2/) (5). The five most

frequent CpG sites that can be cleaved by the methylation-sensitive restriction enzymes (AciI, BstUI, HhaI, HpaII, and SmaI) were identified, and individual RT-PCR assays for each LINE-1 element were developed and validated. Analysis of the LINE-1 DNA methylation was performed as previously described (58). Briefly, 1 μg of genomic DNA was digested with 1 U of SmaI in 1× CutSmart buffer at 25°C for 2 h, HpaII, HhaI, and AciI at 37°C for 16 h, and then 0.5 U of BstUI for 4 h at 60°C (New England Biolabs, Ipswich, MA). Digested DNA was analyzed by qRT-PCR on a ViiA 7 Real-Time PCR System (Applied Biosystems, Forrest City, CA). DNA samples not digested with the restriction enzyme mix were used for normalization, along with primers for regions not containing restriction sites. Fold changes were calculated from C_T values using the $\Delta\Delta C_T$ method. Primer sequences for LINE-1 element DNA methylation are provided in Supplemental Table S2.

Statistical analyses. Data are presented as means (SD). Statistical differences between groups was assessed with a two-way ANOVA with a Bonferroni correction for multiple comparisons (when there was a significant radiation, diet, and/or interaction effect), a repeated-measures two-way ANOVA with a Bonferroni correction, a Student's *t* test with Welch's correction as appropriate, or a log rank Mantel-Cox test and Mantel-Haenszel hazards ratio to compare survival curves. GraphPad Prism (GraphPad Software, La Jolla, CA) was used for all statistical analyses.

RESULTS

Methionine dietary supplementation potentiates acute radiation toxicity. Exposure of mice to 8.5-Gy TBI (generally known to be ~$LD_{100/30}$ for this mouse strain) resulted in 100% mortality in both MAD- and MSD-fed animals. However, mortality associated with acute radiation syndrome (ARS) occurred earlier in irradiated mice fed MSD (Fig. 1*A*). Exposure to lower doses, such as 5.5-Gy and 3-Gy TBI resulted in 50 and 0% mortality, respectively, in mice fed MAD. At the same time, exposure to similar doses in mice fed MSD resulted in 100 and 35% mortality, respectively (Fig. 1*A*). The Mantel-Cox test revealed a statistically significant difference in all survival curves ($P < 0.0001$). There was a trend toward a difference between diets at 5.5 Gy but not at 3 or 8.5 Gy (at 3 Gy, $P = 0.2119$; at 5.5 Gy, $P = 0.0622$; and at 8.5 Gy, $P = 0.9198$). There was also an increase in hazard ratio at 3 and 5.5 Gy (4.232 and 4.173, respectively) but not at 8.5 Gy (0.9294).

The death of mice was observed mostly between *days 6* and *15*, suggesting mortality associated with either gastrointestinal (GI-) or hematopoietic (H-) ARS. To identify which syndrome was prevalent in mice, we used PBI (8.5 Gy), sparing 5–10% of the mouse bone marrow from irradiation, thus precluding the development of severe H-ARS. Among the animals fed MAD, only one mouse succumbed within the 30-day study; lethality occurred on *day 26*, which is typical for H-ARS (Fig. 1*B*). On the other hand, 50% of mice fed MSD were found dead by *day 9*, indicating a prevalence of GI-ARS. Even though the curves were not significantly different (Mantel-Cox test, $P = 0.0906$), the effect of diet was biologically significant, with a hazard ratio of 4.988 (MSD/MAD).

To further confirm the primary role of methionine dietary intake in the development of GI-ARS, mice were exposed to 8.5-Gy TBI and were provided diets with different amounts of methionine (from 0 to 300%). The onset and severity of ARS exhibited methionine dose dependence, with animals fed MSD succumbing first, and the animals receiving methionine-deficient diet (MDD, 0% methionine) succumbing last (Fig. 1*C*). There was a significant effect of diet (Mantel-Cox test, $P < 0.0001$), and significant difference between MSD and MRD curves ($P < 0.0001$) and between MSD and MDD curves ($P < 0.0001$), as well as between MAD and MRD ($P = 0.0013$), between MAD and MDD ($P = 0.0001$), and between MRD and MDD ($P = 0.0009$), indicating that decreased percentage

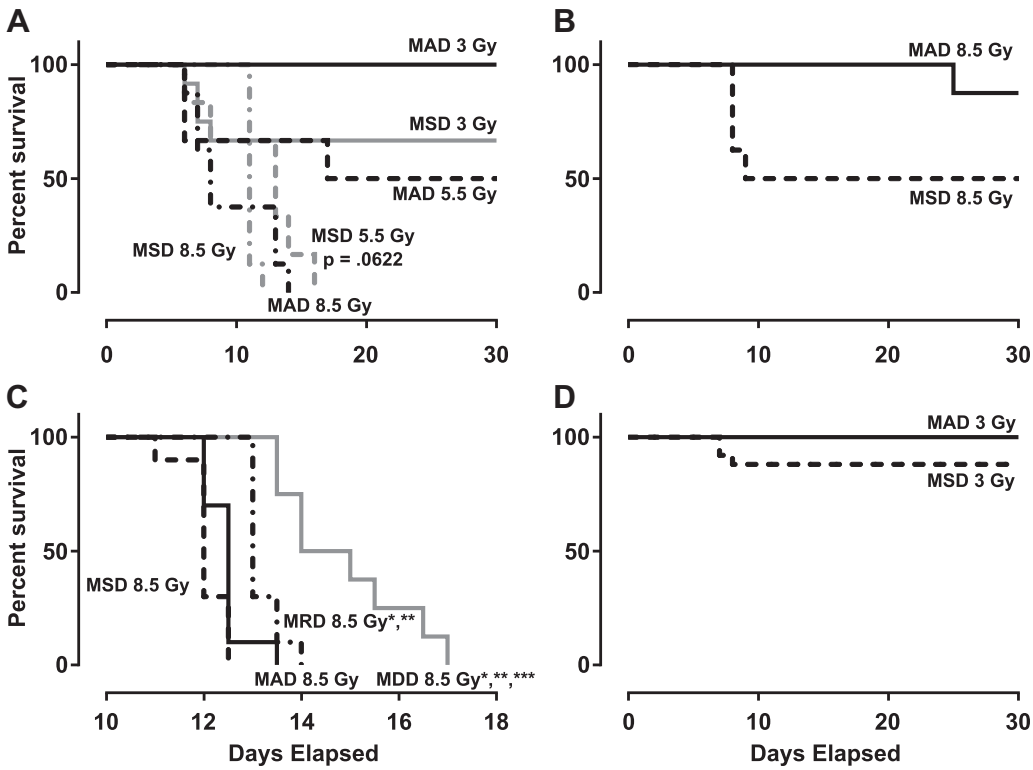

Fig. 1. Dietary methionine supplementation potentiates acute ionizing radiation (IR)-induced gastrointestinal toxicity. *A*: exacerbation of acute radiation toxicity by methionine dietary supplementation in CBA/CaJ mice after exposure to 3, 5.5, or 8.5 Gy of total body irradiation (TBI). *B*: partial body irradiation (PBI; hindlimb protection) to 8.5 Gy of IR indicates prevalence of gastrointestinal toxicity in CBA/CaJ mice. *C*: methionine restriction delays onset of acute radiation syndrome in CBA/CaJ mice. *Significantly different from methionine-supplemented diet (MSD) (300% of required intake); **significantly different from methionine-adequate diet (100% of required intake) (MAD); ***significantly different from methionine-restricted diet (25% of required intake) (MRD); Mantel-Cox test. *D*: exacerbation of acute radiation toxicity by methionine dietary supplementation in C57BL/6J mice after 3 Gy of TBI. For all groups, $n = 10–24$ mice. MDD, methionine-deficient diet (0% of required intake).

of methionine in diet is associated with increased survival after 8.5-Gy TBI. Mice on MSD also had a higher risk than the other three diets (hazard ratios: MSD/MAD 4.422, MSD/MRD 28.86, and MSD/MDD 22.49).

Finally, to investigate the strain-dependent nature of the effect, we exposed C57BL6/J mice (a strain generally known to be much more resistant to IR exposure than CBA/CaJ mice) to 3-Gy TBI, the dose that is not known to cause an acute ARS in mice. After irradiation, mice were assigned to either MSD or MAD. By *day 8*, 2 of 12 C57BL/6J MSD-fed mice had succumbed, indicating that, although different mortality rates were observed between the IR-sensitive and IR-resistant mouse strains, the effect itself was strain independent (Fig. 1*D*). Mice on MSD also had a 7.596-fold higher risk of death from radiation than mice on MAD (Mantel-Haenszel hazard ratio).

The surviving mice in the 3-Gy TBI experiments were further monitored for a period of up to 60 days. No mortality was observed during that period; however, the body weights of mice in the 3-Gy+MSD group remained significantly lower compared with MSD only, MAD only, or 3-Gy+MAD groups (Supplemental Fig. S2).

Methionine dietary supplementation alters the gut microbiome. Both MSD and IR can alter gut ecology, and the development of severe RIGIS is usually associated with compromised gut permeability and sepsis (17, 19, 20, 52). Therefore, we next sought to analyze the gut microbiome in mice fed MSD or MAD after exposures to either 8.5-Gy TBI (~$LD_{100/30}$ independently of the diet) or 3-Gy ($LD_{0/30}$ with MAD and $LD_{35/30}$ with MSD) 6 days after irradiation, 24 h before the clinical manifestation of RIGIS in our model. This choice of time point was made to delineate the mechanisms of the syndrome rather than its consequences.

Next generation sequencing analysis revealed that there was no difference in Chao1 richness ($P = 0.3040$) or in Shannon diversity ($P = 0.7874$), both measures of the diversity of operational taxonomic units, between the groups. However, there was a trend to a decreased degree of Chao1 richness with MSD and TBI compared with the overall richness of all samples (Supplemental Fig. S2). The most interesting finding was an increase in *Burkholderiales*, an order of pathogenic bacteria commonly found in hospital-borne infections, in the colon of MSD/sham-irradiated mice, while the levels of *Burkholderiales* in the colon of MAD/sham irradiated was mice was negligible (69). Furthermore, only minor increases in *Burkholderiales* were observed in the colon of MAD mice exposed to 3-Gy TBI, whereas more robust increases were observed in 8.5-Gy+MAD and 3- and 8.5-Gy+MSD mice, the treatment regimens strongly associated with severe RIGIS and mortality (Fig. 2*A*).

Radiation/methionine-induced shifts in gut ecology alter gut transcriptome. Exposure to high doses of IR is usually associated with the leaky gut syndrome, in which negative regulation of tight-junction-related proteins plays the driving role (26). Our previous study indicated that MSD-induced shifts in the gut microbiome are associated with altered expression of those key regulators of gut permeability (52).

Here, we identified that IR and MSD interact to affect the expression of a number of tight-junction-related proteins. At *day 6* after IR, the combination of IR with MSD resulted in the most pronounced effects, with a loss of mRNA abundance of the key proteins, such as *Cldn1*, *Cldn8*, *Cldn9*, and *Cldn10* greater than 10-fold compared with MAD/sham-irradiated mice (Fig. 2*B*). The expression of tight-junction-related proteins correlated with the shifts in the gut ecology and survival of mice where the extent of deregulation was much higher in 3-Gy+MSD mice compared with 3-Gy+MAD mice. Summaries of the two-way ANOVA results of the genes that were significantly affected by TBI, diet, or both are in Supplemental Table S6.

Proliferation of pathogenic bacteria together with impaired gut permeability may lead to the development of sepsis and death. Therefore, we measured the levels of bacterial 16S RNA in the livers of experimental mice as a sensitive indicator of sepsis. By *day 6*, significantly higher levels of bacterial DNA were detected in the mice fed MSD and exposed to 8.5-Gy TBI (Fig. 2*C* and Supplemental Table S6).

Combination of methionine dietary supplementation and irradiation negatively regulates one-carbon metabolism pathway and amino acid balance. Next, we sought to determine the extent to which IR and/or MSD affect the plasma and tissue (intestinal and hepatic) levels of methionine. Exposure to IR caused significant decreases in the plasma methionine concentrations on *day 6* at both 3 Gy and 8.5 Gy with MAD and at 8.5 Gy with MSD (Fig. 3*A*). When both factors were combined, the spike in plasma methionine induced by MSD was abrogated dose-dependently by IR (Fig. 3*A*).

In the proximal jejunum, MSD led to over twofold significantly lower methionine concentration observed at *day 6* (Fig. 3*B*). IR caused decreases in intestinal and hepatic one-carbon metabolites, with more pronounced effects in both diet groups at 8.5 Gy and greater differences between diets at 3 Gy. As methionine is central to the one-carbon metabolism pathway, the observed decreases in methionine concentrations in the intestinal tissue was associated with a decrease in downstream products of methionine biotransformation, namely SAM/SAH (*S*-adenosylmethionine-to-*S*-adenosylhomocysteine), cysteine/cystine, and GSH/GSSG ratios (Fig. 3, *B* and *C*, and Supplemental Table S3).

Contrary to the intestinal tissue, the liver is characterized by a strong expression of *Bhmt* and *Cbs* enzymes, which contribute alternative pathways within one-carbon metabolism (74). Despite this and the fact that liver is generally a mitotically inactive organ, the trend toward decreased hepatic methionine concentrations was observed in IR+MSD mice (Fig. 3*C*), with methionine levels being significantly decreased with MSD at 8.5 Gy. Changes in SAM/SAH and cysteine/cystine ratios observed after exposure to IR further underline the severity and multiorgan nature of the effect (Fig. 3*C*). Also, and consistent with oxidative stress induced by IR, MSD negatively affected synthesis of glutathione in the liver (Supplemental Table S4).

Finally, given the magnitude of the observed effects, we sought to determine whether alterations in one-carbon metabolism were associated with the large-scale perturbations in plasma amino acid concentrations (Supplemental Table S5). The IR effect was predominant at *day 3*.5, largely affecting the plasma levels of leucine, isoleucine, valine, phenylalanine, lysine, and arginine. At *day 6*, diet explained much of the variance for serine, asparagine, and proline. Interestingly, the strongest effect at *day 6* was the interaction between IR and MSD on the plasma concentration of both leucine and isoleucine, determining over 50% of the variance in both cases.

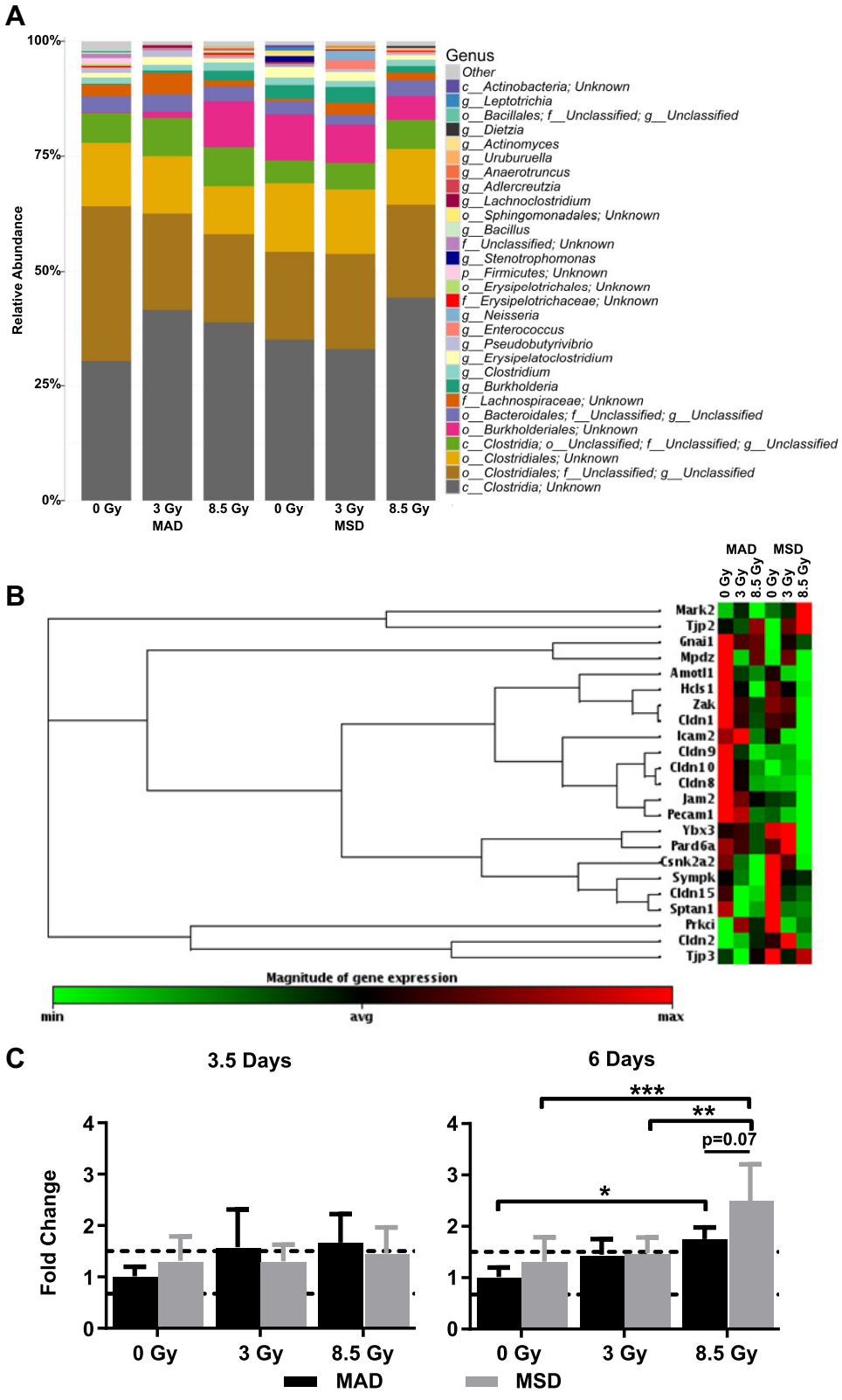

Fig. 2. Methionine dietary supplementation leads to alterations in gut ecology, resulting in development of "leaky gut" syndrome and bacterial translocation (n = 3–6 mice). MAD, methionine-adequate diet (100% of required intake); MSD, methionine-supplemented diet (300% of required intake). *A*: microbiome profile of top 30 genera present in colon tissue samples at 6 days after total body irradiation (TBI) of 3 or 8.5 Gy. *B*: expression of tight-junction-related genes in jejunum of mice at 6 days post-TBI. Data are presented as heat map of z-scores, with green indicating lowest expression and red indicating highest expression. *C*: 16S ribosomal DNA in livers of mice at 3.5 or 6 days post-TBI. Dashed lines indicate 1.5-fold change level that were arbitrarily selected for biological significance. Data are presented as means ± SD and analyzed with two-way ANOVA with Bonferroni correction. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Impairment of normal gut physiology as a result of the combination of methionine dietary supplementation and irradiation. Citrulline is a nonessential amino acid produced by enterocytes. Therefore, its plasma concentration is generally considered reflective of enterocyte mass and is extensively used as a biomarker of postexposure enterocyte regeneration (9, 20, 33, 34, 44, 55). At *day 3.5*, a time point characteristic of nearly 100% enterocyte renewal, a significant decrease in citrulline concentrations was observed in 8.5-Gy+MSD mice compared with sham and 3-Gy (Fig. 4*A*). At the 6-day time-point,

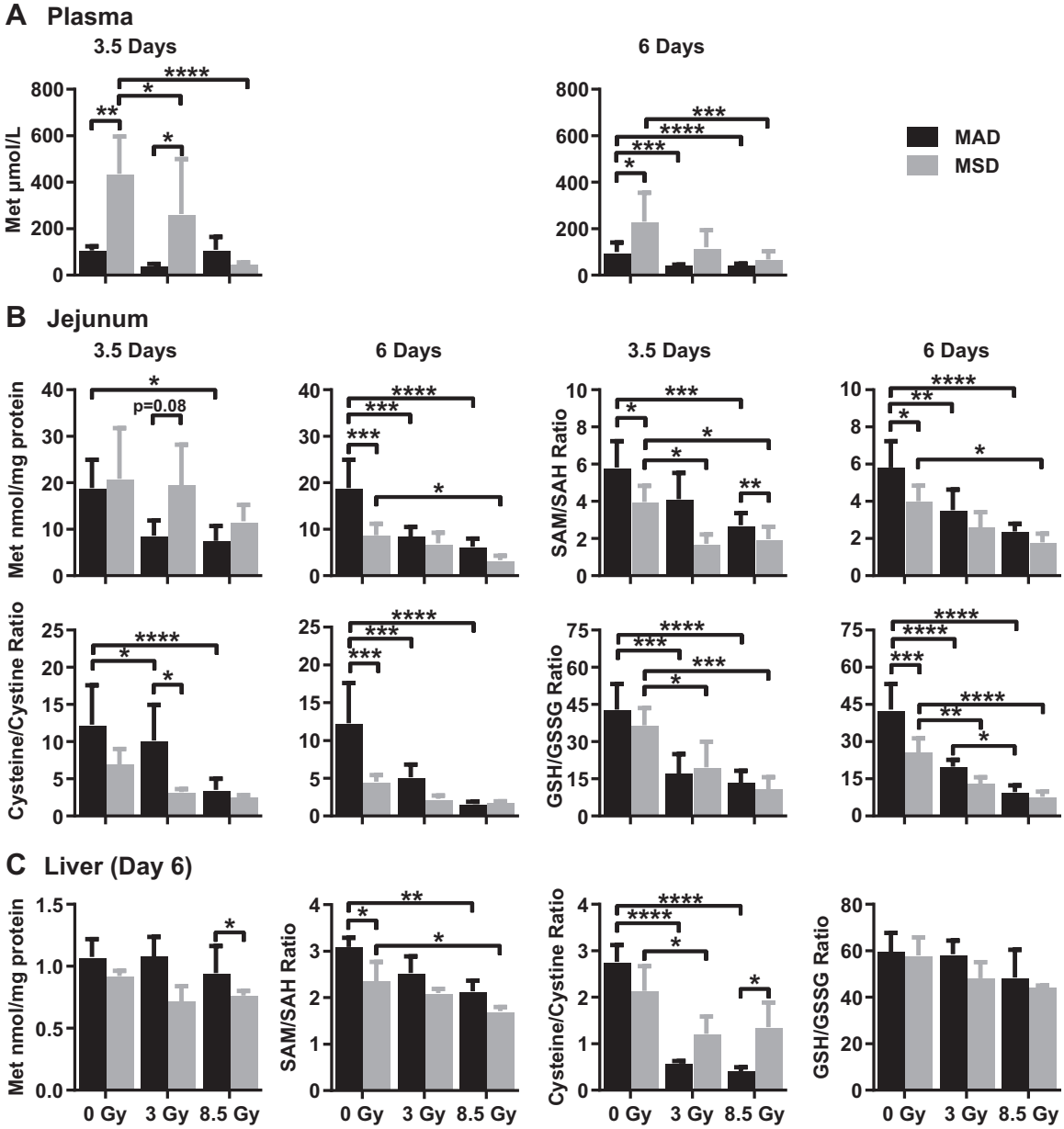

Fig. 3. Combination of methionine dietary supplementation and irradiation negatively regulates one-carbon metabolism pathway and amino acid balance (n = 3–6 mice). *A*: plasma methionine concentrations. *B*: methionine content and SAM/SAH, cysteine/cystine, and GSSG/GSH ratios in proximal jejunum at 3.5 and 6 days post-TBI. *C*: methionine content and SAM/SAH, cysteine/cystine, and GSSG/GSH ratios in liver at *day 6* post-TBI. MAD, methionine-adequate diet (100% of required intake); MSD, methionine-supplemented diet (300% of required intake); Met, methionine; SAM, *S*-adenosyl methionine; SAH, *S*-adenosyl homocysteine; GSSG, oxidized glutathione; GSH, total glutathione. Data are presented as means ± SD and analyzed with two-way ANOVA with Bonferroni correction. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

citrulline levels spiked in 3-Gy+MAD mice, indicative of a compensatory postexposure enterocyte regeneration burst. At the same time, this effect was absent in 3-Gy+MSD as well as in both 8.5-Gy groups.

Both IR and MSD negatively affected the histology of the gastrointestinal tract, as measured by the number of surviving crypts (those with ≥10 adjacent, chromophilic, non-Paneth cells) in the jejunum. Specifically, IR and MSD both led to a decrease in crypt colonies at 8.5 Gy at both 3.5 and 6 days post-IR (Fig. 4*B*). Assessment of the mucosal surface area further confirmed the findings, indicative of decreased absorption ability and altered gut physiology, with 8.5-Gy+MSD mice having significantly lower mucosal surface area than 8.5-Gy+MAD on *day 6* (Fig. 4*C* and Supplemental Table S6).

Methionine dietary supplementation and irradiation lead to epigenetic reprogramming in the mouse proximal jejunum. Methionine is a precursor of *S*-adenosylmethionine, the methyl donor for DNA methylation. Given the gross alterations in the one-carbon metabolism pathway observed in the gut tissue, we sought to delineate the potential alterations in the gut epigenome with a focus on DNA methylation.

LINE-1 is the most abundant repetitive element in mammals and occupies ~20% of the mouse genome. The methylation

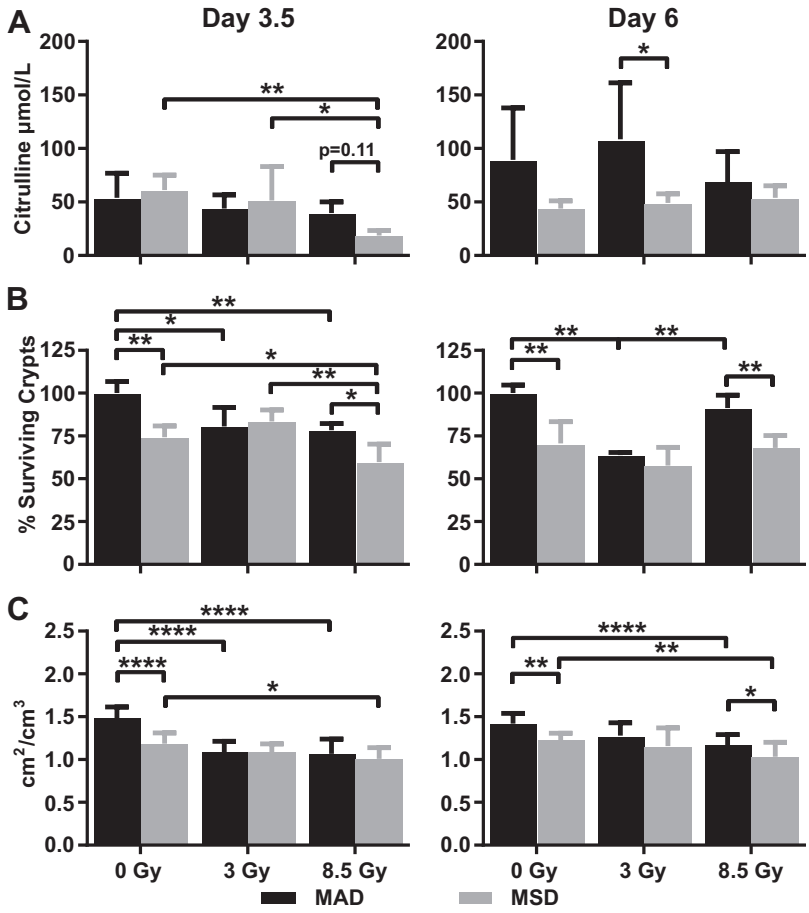

Fig. 4. Impairment of normal gut physiology as a result of combination of methionine dietary supplementation and irradiation (n = 3–7 mice). MAD, methionine-adequate diet (100% of required intake); MSD, methionine-supplemented diet (300% of required intake). *A*: plasma citrulline concentrations. *B*: percent surviving crypts in proximal jejunum. *C*: ratio of internal mucosal surface area of proximal jejunum to total volume of tissue. Data are presented as means ± SD and analyzed with two-way ANOVA with Bonferroni correction. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$.

status of the LINE-1 element is generally considered a surrogate biomarker for global DNA methylation (49). Furthermore, numerous studies have demonstrated that IR-induced DNA hypomethylation primarily stems from LINE-1 elements (39, 49–51, 58). Because of age-related differences in the methylation of LINE-1 elements, we evaluated the methylation status of the 5′-UTRs in six LINE-1 elements that substantially differ in their evolutionary age (from 0.21 to 6.43 million years).

Exposure to either 3- or 8.5-Gy TBI resulted in global LINE-1 DNA hypermethylation patterns in MAD mice compared with sham-irradiated mice (Fig. 5*A*). In agreement with the decreased levels of intraintestinal methionine, DNA hypomethylation was observed in 8.5-Gy+MSD in evolutionarily young LINE-1 elements (Fig. 5*A*). This effect was largely independent from DNA methyltransferase expression, with little change in *Dnmt1* (DNA methyltransferase-1) and *Dnmt3a*. However, a significant increase of the de novo DNA methyltransferase *Dnmt3b*, potentially of a compensatory nature, was observed at the 3.5-day time point (Fig. 5*B*). Increased expression of *Mecp2* (methyl-CpG-binding protein-2), which is a protein that binds to methylated DNA to inhibit transcription, was found with TBI at 3 Gy at both 3.5 and 6 days and at 8.5 Gy only at 6 days with MAD; however, with MSD, expression increased only at 3 Gy on *day 3.5* and at 8.5 Gy on *day 6*.

DISCUSSION

With the currently high levels of radiological and nuclear threats, there is considerable risk of exposure to IR due to terroristic attacks or catastrophic events (13). Furthermore, IR is a primary treatment modality for more than half of cancer therapies. H-ARS and GI-ARS are the major concerns of exposure to IR. Whereas a number of strategies exist to mitigate H-ARS, there are no FDA-approved strategies for mitigation or prevention of GI-ARS. In clinical settings, damage to the GI tract is a dose-limiting factor during radiation therapy to the abdominal and pelvic area. It is estimated that of the 10 million cancer survivors in the United States, 1.5 to 2 million suffer from postradiation intestinal dysfunction (30).

There are several known factors that increase the likelihood of experiencing side effects following radiotherapy that can be classified as IR related or host related. IR-related factors include the dose, distance to the source, length of exposure (in the case of accidental exposure), volume of normal gut irradiated, and treatment plan specifics. Host-related factors include previous surgeries to the GI tract (14, 15, 23, 35, 43, 56), inflammatory bowel disease (71), diabetes (31), vascular disease (12, 15, 57), and smoking (3, 18).

Our previous study demonstrated that diet may be another determinant of the severity of RIGIS, as the short-term administration of MSD led to the development of the bacterial overgrowth of small intestine (BOSI) syndrome (52). In the latter, shifts in the gut microbiome manifested as an increase in pathogenic and conditionally pathogenic bacteria at the expense of commensal bacteria, as well as in the development of a "leaky gut," leading to an altered gut physiology (52). We hypothesized that this condition may further exacerbate RIGIS,

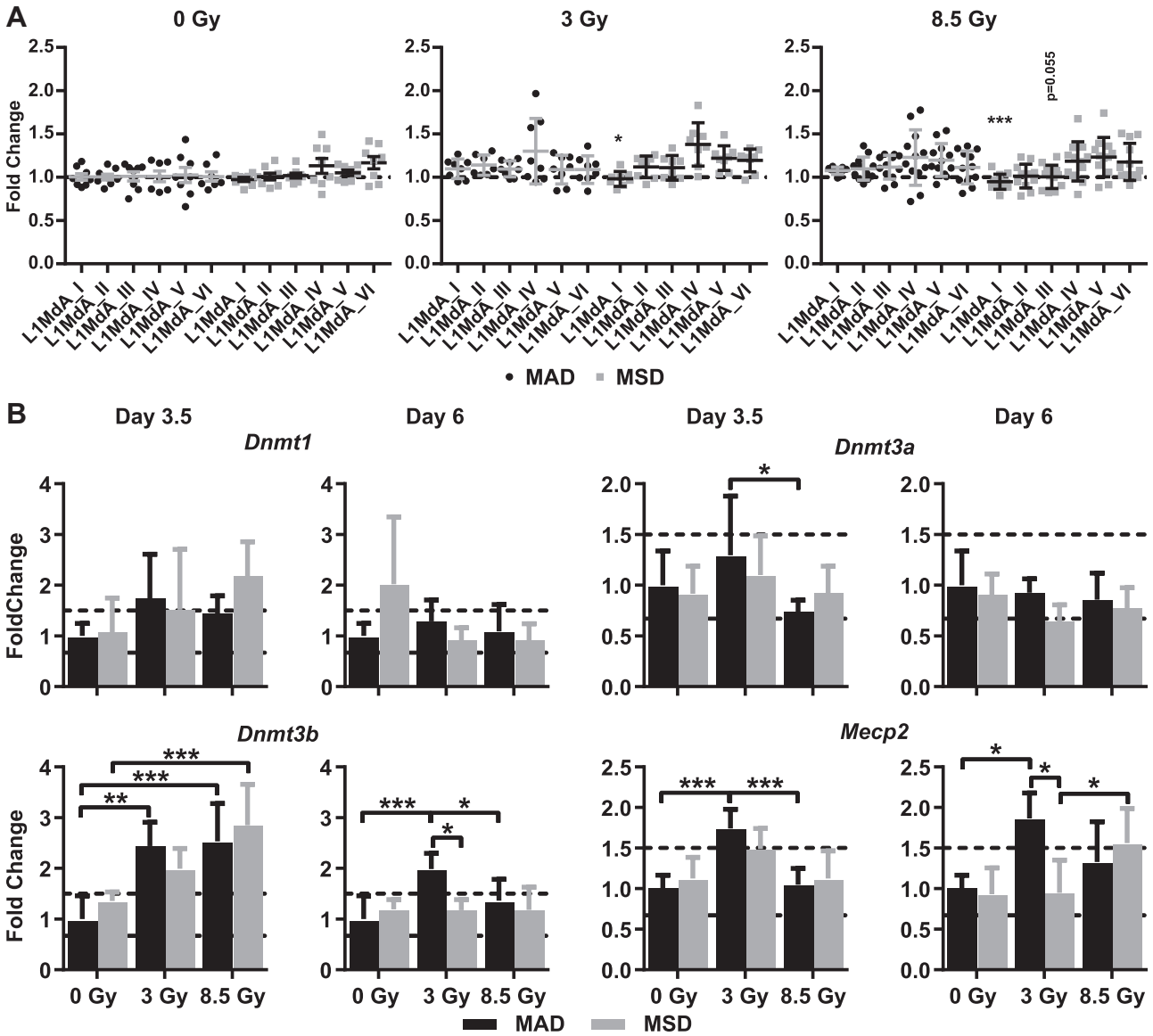

Fig. 5. Methionine dietary supplementation and irradiation lead to epigenetic reprogramming in mouse proximal jejunum (n = 8–12 mice). MAD, methionine-adequate diet (100% of required intake); MSD, methionine-supplemented diet (300% of required intake). *A*: altered DNA methylation of long interspersed element 1 (LINE-1) L1MdA_I–VI elements (in order of evolutionary age) in proximal jejunum at 6 days following 0, 3, or 8.5 Gy of total body irradiation (TBI). LINE-1 methylation status is a proxy measure of global DNA methylation. Data in other groups are normalized to total methylation of MAD at 0 Gy and analyzed with a *t* test between diets for each LINE-1 element at each dose. *B*: gene expression profiles of DNA methyltransferases (*Dnmt1, Dnmt3a*, and *Dnmt3b*) and methylation-binding protein (*Mecp2*) in proximal jejunum at 3.5 and 6 days post-TBI. Dashed lines indicate 1.5-fold change level that was chosen for biological significance. Circles represent MAD; squares represent MSD. Data are presented as means ± SD and analyzed with two-way ANOVA with Bonferroni correction. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

as exposure to IR causes similar effects in the gut (11, 28). Here, we demonstrate that supplementation of diet with supraphysiological concentrations of methionine (supplementation comparable to methionine dietary intake in a typical Western diet or ingestion of amino acid-based athletic dietary supplements), sensitizes the mouse gut to IR, leading to the development of severe RIGIS and even death.

Specifically, we observed that administration of MSD resulted in reduced tolerance of mice to high doses of IR and led to death even at doses that were not previously reported as lethal or sublethal in this species. Although we cannot discern the role of H-ARS in this syndrome, the 50% mortality observed while protecting hindlimb bone marrow from irradiation clearly indicated the prevalence of GI-ARS.

Exposure to IR, both TBI and as radiotherapy, for abdominal and pelvic cancers, induces changes in gut ecology that are becoming increasingly recognized as the driving forces of GI-ARS (10, 22, 40, 41, 59). While due to interindividual differences in the gut microbiome it is often difficult to define commensal microbiota from pathogenic or conditionally pathogenic sources, some bacterial orders and species are becoming more frequently associated with various pathological states. In this regard, of particular interest is an upsurge in the order of *Burkholderiales* that was observed in the intestines of mice

receiving MSD (both control and irradiated) as well as in MAD mice after exposure to 8.5 Gy. First described in the late 1970s, *Burkholderiales* represent an order of versatile bacteria commonly present in the environment, mainly in soil and water. Many species of this Gram-negative bacteria are characterized by natural resistance to antibiotics (60) and are commonly identified in various health care units across the globe (36, 65, 68). Of particular concern are *Burkholderia pseudomallei* and *cepacia*, associated with cystic fibrosis and melioidosis, an emerging infectious disease often characterized by life-threatening sepsis with systemic inflammatory responses and organ dysfunction (70). Recent years are characterized by increased rates of intrahospital bacteremia outbreaks associated with *Burkholderia* (1, 37, 63). Given that immunosuppressed patients are particularly vulnerable to *Burkholderia*, patients receiving anticancer therapy represent a potentially susceptible population.

Although the gut integrity, although compromised, was mostly preserved in MSD-fed control mice, the cytotoxic effects of irradiation to the intestinal epithelium combined with MSD resulted in increased intestinal permeability. The latter was facilitated by the disrupted function of the tight-junction-related proteins that ensure the appropriate functioning of the intestinal epithelia as a barrier between the host's external and internal environments. Among the tight-junction proteins, integral membrane proteins (i.e., *Cldn 8*, *9*, and *10*) responsible for the formation of paracellular pores within the tight junctions rather than the junctional complex proteins were most affected. This has led to the development of bacteremia and sepsis, as translocation of bacterial 16S RNA from the gut to the liver was observed at least 24 h before manifestation of GI-ARS and was increased in a synergistic manner by the 8.5-Gy+MSD combination.

It is important to note that the observed IR-induced cytotoxicity was clearly exacerbated by MSD. While it is hard to dissect this role of MSD among the mice exposed to 8.5 Gy, this is clearly evident in mice from the 3-Gy exposure group. For instance, circulating citrulline levels provided a clear-cut differentiation between the surviving mice with GI-ARS (3-Gy+MAD vs. 3-Gy+MSD, 8.5-Gy+MAD, and 8.5-Gy+MSD). The biosynthesis of citrulline occurs predominantly in the small intestine enterocytes. Therefore, it is considered a well-established biomarker of the small intestine postirradiation crypt survival, including the murine TBI model (34, 45, 46). Shortly after exposure to IR, the circulating levels of citrulline decrease, showing a high degree of correlation with the significant decreases in enterocyte mass (9, 33, 34, 45, 46). However, within days after irradiation, circulating citrulline levels increase, often even exceeding the preirradiation levels, mirroring the robust regeneration process in the small intestine. In our study, by *day 6* after irradiation, we observed a significant difference in circulating citrulline levels between the 3-Gy+MAD mice (those that did not develop GI-ARS), and the 3-Gy+MSD mice (those that developed GI-ARS with lethality at 35% by *day 12*). This finding suggests that the early intestinal epithelium regeneration observed in 3-Gy+MAD group allowed for a fast restoration of intestinal integrity and prevented the development of leaky gut syndrome and significant bacterial translocation. On the other hand, mice in the 3-Gy+MSD group, as well as mice in both 8.5-Gy groups, did not restore the normal epithelium surface, which resulted in the development of GI-ARS and its associated effects. These findings also suggest that circulating levels of citrulline on or around *day 6* after exposure can be utilized as predictors of survival in experimental models.

The impaired ability for normal gut regeneration in MSD-fed mice was mediated, at least in part, by a significantly lower intraintestinal methionine concentrations observed in MSD-fed mice. The enterocyte usually utilizes apical rather than basolateral membrane transport. Therefore, while the circulating methionine levels were higher in MSD mice, apical transport of methionine could be impaired by rapid passage of methionine from the gut lumen into the blood stream via paracellular transport (facilitated by the nonfunctional tight junctions). Studies also indicate the importance of methionine for bacterial homeostasis, the role of which becomes especially critical for survival under the conditions of overwhelming oxidative stress, which exposure to IR is capable of causing (21, 75). Therefore, the remaining methionine in the lumen could be rapidly consumed by the abundant gut microbiome. Furthermore, IR is capable of depleting internal methionine concentrations (51). The role of methionine for a regenerating organ is vital, as it is needed for protein synthesis, providing building blocks for new enterocytes, and the lack of intraintestinal methionine significantly impairs regeneration after IR. This lack of methionine was further exacerbated by alterations in the one-carbon metabolism pathway, including decreases in cysteine and glutathione that are needed for normal tissue response under oxidative stress conditions.

The effects caused by IR + MSD were global, as they were observed in other organs (such as liver), resulted in altered plasma concentrations of numerous other amino acids (i.e., leucine, isoleucine, valine, phenylalanine, lysine, and arginine), and even led to epigenetic alterations in the small intestine exhibited as DNA hypomethylation of evolutionary young (and, thus, hypermethylated) LINE-1 elements and aberrant expression of DNA methyltransferases and methylated DNA-binding proteins. Elevated levels of *Mecp2* may protect methylated DNA and regulate gene expression in response to exposure to IR (38).

In conclusion, this study demonstrates that dietary methionine supplementation, instead of an anticipated health-promoting effect, sensitizes mice to GI-ARS. Since increased sensitivity to IR was observed in more than one mouse strain, these findings seem not to be limited to one particular genotype. These findings also speak toward increasing the role of registered dietitians during cancer therapy and the necessity of a solid scientific background behind the sales of dietary supplements and claims regarding their beneficial effects. Future studies are needed to delineate the applicability of the observed phenomena to clinically relevant models of radiotherapy, such as stereotactic body radiotherapy, to demonstrate the translational nature of methionine supplementation on the exacerbation of RIGIS. These studies are currently underway at our laboratories.

ACKNOWLEDGMENTS

We thank Cellular and Molecular Analytic, and Irradiation and Animal Core Facilities within the COBRE under National Institute of General Medical Sciences Grant 1P20 GM-109005. We thank Robin Mulkey and Bianca Schutte for providing excellent animal care at the UAMS Animal Facility.

GRANTS

Research reported in this publication was supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences (NIGMS) of the National Institutes of Health Grant 1P20 GM-109005; the NIGMS Grant T32 GM-106999; Clinical and Translational Science Awards UL1 TR-000039 and KL2 TR-000063; and the Arkansas Biosciences Institute. I. Koturbash acknowledges the guidance provided during preparation of this manuscript made possible via R25 CA-203650 (Yale University, PI: M. Irwin).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

I.K. conceived and designed research; I.R.M., L.E.E., C.M.S., R.P., S.G., K.R.K., and I.K. performed experiments; I.R.M., L.E.E., C.M.S., R.P., S.G., K.R.K., S.M., M.H.-J., and I.K. analyzed data; I.R.M., L.E.E., S.M., M.H.-J., and I.K. interpreted results of experiments; I.R.M. and L.E.E. prepared figures; I.K. drafted manuscript; I.R.M., L.E.E., K.R.K., and I.K. edited and revised manuscript; I.R.M., L.E.E., C.M.S., R.P., S.G., K.R.K., S.M., M.H.-J., and I.K. approved final version of manuscript.

REFERENCES

1. **Abdelfattah R, Al-Jumaah S, Al-Qahtani A, Al-Thawadi S, Barron I, Al-Mofada S.** Outbreak of Burkholderia cepacia bacteraemia in a tertiary care centre due to contaminated ultrasound probe gel. *J Hosp Infect* 98: 289–294, 2018. doi:10.1016/j.jhin.2017.09.010.
2. **Baddeley AJ, Gundersen HJ, Cruz-Orive LM.** Estimation of surface area from vertical sections. *J Microsc* 142: 259–276, 1986. doi:10.1111/j.1365-2818.1986.tb04282.x.
3. **Bagalà P, Ingrosso G, Falco MD, Petrichella S, D'Andrea M, Rago M, Lancia A, Bruni C, Ponti E, Santoni R.** Predicting genitourinary toxicity in three-dimensional conformal radiotherapy for localized prostate cancer: a dose-volume parameters analysis of the bladder. *J Cancer Res Ther* 12: 1018–1024, 2016. doi:10.4103/0973-1482.165871.
4. **Baker DH.** Comparative species utilization and toxicity of sulfur amino acids. *J Nutr* 136, *Suppl*: 1670S–1675S, 2006. doi:10.1093/jn/136.6.1670S.
5. **Bao W, Kojima KK, Kohany O.** Repbase ujpdate: a database of repetitive elements in eukaryotic genomes. *Mob DNA* 6: 11, 2015. doi:10.1186/s13100-015-0041-9.
6. **Batra V, Sridhar S, Devasagayam TP.** Enhanced one-carbon flux towards DNA methylation: effect of dietary methyl supplements against gamma-radiation-induced epigenetic modifications. *Chem Biol Interact* 183: 425–433, 2010. doi:10.1016/j.cbi.2009.11.010.
7. **Batra V, Verma P.** Dietary L-methionine supplementation mitigates gamma-radiation induced global DNA hypomethylation: enhanced metabolic flux towards S-adenosyl-L-methionine (SAM) biosynthesis increases genomic methylation potential. *Food Chem Toxicol* 69: 46–54, 2014. doi:10.1016/j.fct.2014.03.040.
8. **Berbée M, Fu Q, Boerma M, Wang J, Kumar KS, Hauer-Jensen M.** gamma-Tocotrienol ameliorates intestinal radiation injury and reduces vascular oxidative stress after total-body irradiation by an HMG-CoA reductase-dependent mechanism. *Radiat Res* 171: 596–605, 2009. doi:10.1667/RR1632.1.
9. **Bujold K, Hauer-Jensen M, Donini O, Rumage A, Hartman D, Hendrickson HP, Stamatopoulos J, Naraghi H, Pouliot M, Ascah A, Sebastian M, Pugsley MK, Wong K, Authier S.** Citrulline as a biomarker for gastrointestinal-acute radiation syndrome: species differences and experimental condition effects. *Radiat Res* 186: 71–78, 2016. doi:10.1667/RR14305.1.
10. **Carbonero F, Mayta A, Bolea M, Yu JZ, Lindeblad M, Lyubimov A, Neri F, Szilagyi E, Smith B, Halliday L, Bartholomew A.** Specific members of the gut microbiota are reliable biomarkers of irradiation intensity and lethality in large animal models of human health. *Radiat Res* 191: 107–121, 2018. doi:10.1667/RR14975.1.
11. **Casero D, Gill K, Sridharan V, Koturbash I, Nelson G, Hauer-Jensen M, Boerma M, Braun J, Cheema AK.** Space-type radiation induces multimodal responses in the mouse gut microbiome and metabolome. *Microbiome* 5: 105, 2017. doi:10.1186/s40168-017-0325-z.
12. **Cicchetti A, Rancati T, Ebert M, Fiorino C, Palorini F, Kennedy A, Joseph DJ, Denham JW, Vavassori V, Fellin G, Avuzzi B, Stucchi C, Valdagni R.** Modelling late stool frequency and rectal pain after radical radiotherapy in prostate cancer patients: results from a large pooled population. *Phys Med* 32: 1690–1697, 2016. doi:10.1016/j.ejmp.2016.09.018.
13. **Coleman CN.** The radiation stress response: of the people, by the people and for the people. *Radiat Res* 187: 129–146, 2017. doi:10.1667/RR0CNC.1.
14. **Cosset JM, Henry-Amar M, Burgers JM, Noordijk EM, Van der Werf-Messing B, Meerwaldt JH, van der Schueren E.** Late radiation injuries of the gastrointestinal tract in the H2 and H5 EORTC Hodgkin's disease trials: emphasis on the role of exploratory laparotomy and fractionation. *Radiother Oncol* 13: 61–68, 1988. doi:10.1016/0167-8140(88)90299-X.
15. **Defraene G, Van den Bergh L, Al-Mamgani A, Haustermans K, Heemsbergen W, Van den Heuvel F, Lebesque JV.** The benefits of including clinical factors in rectal normal tissue complication probability modeling after radiotherapy for prostate cancer. *Int J Radiat Oncol Biol Phys* 82: 1233–1242, 2012. doi:10.1016/j.ijrobp.2011.03.056.
16. **Dever JT, Elfarra AA.** The biochemical and toxicological significance of hypermethionemia: new insights and clinical relevance. *Expert Opin Drug Metab Toxicol* 6: 1333–1346, 2010. doi:10.1517/17425255.2010.522177.
17. **Duran-Struuck R, Hartigan A, Clouthier SG, Dyson MC, Lowler K, Gatza E, Tawara I, Toubai T, Weisiger E, Hugunin K, Reddy P, Wilkinson JE.** Differential susceptibility of C57BL/6NCr and B6.Cg-Ptprca mice to commensal bacteria after whole body irradiation in translational bone marrow transplant studies. *J Transl Med* 6: 10, 2008. doi:10.1186/1479-5876-6-10.
18. **Eifel PJ, Jhingran A, Bodurka DC, Levenback C, Thames H.** Correlation of smoking history and other patient characteristics with major complications of pelvic radiation therapy for cervical cancer. *J Clin Oncol* 20: 3651–3657, 2002. doi:10.1200/JCO.2002.10.128.
19. **Elliott TB, Bolduc DL, Ledney GD, Kiang JG, Fatanmi OO, Wise SY, Romaine PL, Newman VL, Singh VK.** Combined immunomodulator and antimicrobial therapy eliminates polymicrobial sepsis and modulates cytokine production in combined injured mice. *Int J Radiat Biol* 91: 690–702, 2015. doi:10.3109/09553002.2015.1054526.
20. **Elliott TB, Deutz NE, Gulani J, Koch A, Olsen CH, Christensen C, Chappell M, Whitnall MH, Moroni M.** Gastrointestinal acute radiation syndrome in Göttingen minipigs (Sus scrofa domestica). *Comp Med* 64: 456–463, 2014.
21. **Ferla MP, Patrick WM.** Bacterial methionine biosynthesis. *Microbiology* 160: 1571–1584, 2014. doi:10.1099/mic.0.077826-0.
22. **Ferreira MR, Muls A, Dearnaley DP, Andreyev HJ.** Microbiota and radiation-induced bowel toxicity: lessons from inflammatory bowel disease for the radiation oncologist. *Lancet Oncol* 15: e139–e147, 2014. doi:10.1016/S1470-2045(13)70504-7.
23. **Fiorino C, Fellin G, Rancati T, Vavassori V, Bianchi C, Borca VC, Girelli G, Mapelli M, Menegotti L, Nava S, Valdagni R.** Clinical and dosimetric predictors of late rectal syndrome after 3D-CRT for localized prostate cancer: preliminary results of a multicenter prospective study. *Int J Radiat Oncol Biol Phys* 70: 1130–1137, 2008. doi:10.1016/j.ijrobp.2007.07.2354.
24. **Gao X, Sanderson SM, Dai Z, Reid MA, Cooper DE, Lu M, Richie JP, Ciccarella A, Calcagnotto A, Mikhael PG, Mentch SJ, Liu J, Ables G, Kirsch DG, Hsu DS, Nichenametla SN, Locasale JW.** Dietary methionine influences therapy in mouse cancer models and alters human metabolism. *Nature* 572: 397–401, 2019. doi:10.1038/s41586-019-1437-3.
25. **Garg S, Wang W, Prabath BG, Boerma M, Wang J, Zhou D, Hauer-Jensen M.** Bone marrow transplantation helps restore the intestinal mucosal barrier after total body irradiation in mice. *Radiat Res* 181: 229–239, 2014. doi:10.1667/RR13548.1.
26. **Garg S, Zheng J, Wang J, Authier S, Pouliot M, Hauer-Jensen M.** Segmental differences in radiation-induced alterations of tight junction-related proteins in non-human primate jejunum, ileum and colon. *Radiat Res* 185: 50–59, 2016. doi:10.1667/RR14157.1.
27. **Garlick PJ.** Toxicity of methionine in humans. *J Nutr* 136, *Suppl*: 1722S–1725S, 2006. doi:10.1093/jn/136.6.1722S.
28. **Gerassy-Vainberg S, Blatt A, Danin-Poleg Y, Gershovich K, Sabo E, Nevelsky A, Daniel S, Dahan A, Ziv O, Dheer R, Abreu MT, Koren O, Kashi Y, Chowers Y.** Radiation induces proinflammatory dysbiosis: transmission of inflammatory susceptibility by host cytokine induction. *Gut* 67: 97–107, 2018. doi:10.1136/gutjnl-2017-313789.
29. **Gupta PK, Brown J, Biju PG, Thaden J, Deutz NE, Kumar S, Hauer-Jensen M, Hendrickson HP.** Development of high-throughput

HILIC-MS/MS methodology for plasma citrulline determination in multiple species. *Anal Methods* 3: 1759–1768, 2011. doi:10.1039/c1ay05213f.
30. **Hauer-Jensen M, Wang J, Boerma M, Fu Q, Denham JW.** Radiation damage to the gastrointestinal tract: mechanisms, diagnosis, and management. *Curr Opin Support Palliat Care* 1: 23–29, 2007. doi:10.1097/SPC.0b013e3281108014.
31. **Herold DM, Hanlon AL, Hanks GE.** Diabetes mellitus: a predictor for late radiation morbidity. *Int J Radiat Oncol Biol Phys* 43: 475–479, 1999. doi:10.1016/S0360-3016(98)00460-X.
32. **Javan GT, Finley SJ, Can I, Wilkinson JE, Hanson JD, Tarone AM.** Human thanatomicrobiome succession and time since death. *Sci Rep* 6: 29598, 2016. doi:10.1038/srep29598.
33. **Jones JW, Bennett A, Carter CL, Tudor G, Hankey KG, Farese AM, Booth C, MacVittie TJ, Kane MA.** Citrulline as a biomarker in the non-human primate total- and partial-body irradiation models: correlation of circulating citrulline to acute and prolonged gastrointestinal injury. *Health Phys* 109: 440–451, 2015. doi:10.1097/HP.0000000000000347.
34. **Jones JW, Tudor G, Li F, Tong Y, Katz B, Farese AM, MacVittie TJ, Booth C, Kane MA.** Citrulline as a biomarker in the murine total-body irradiation model: correlation of circulating and tissue citrulline to small intestine epithelial histopathology. *Health Phys* 109: 452–465, 2015. doi:10.1097/HP.0000000000000346.
35. **Kasibhatla M, Clough RW, Montana GS, Oleson JR, Light K, Steffey BA, Jones EL.** Predictors of severe gastrointestinal toxicity after external beam radiotherapy and interstitial brachytherapy for advanced or recurrent gynecologic malignancies. *Int J Radiat Oncol Biol Phys* 65: 398–403, 2006. doi:10.1016/j.ijrobp.2005.12.008.
36. **Kenna DTD, Lilley D, Coward A, Martin K, Perry C, Pike R, Hill R, Turton JF.** Prevalence of Burkholderia species, including members of *Burkholderia cepacia* complex, among UK cystic and non-cystic fibrosis patients. *J Med Microbiol* 66: 490–501, 2017. doi:10.1099/jmm.0.000458.
37. **Ko S, An HS, Bang JH, Park SW.** An outbreak of *Burkholderia cepacia* complex pseudobacteremia associated with intrinsically contaminated commercial 0.5% chlorhexidine solution. *Am J Infect Control* 43: 266–268, 2015. doi:10.1016/j.ajic.2014.11.010.
38. **Koturbash I, Jadavji NM, Kutanzi K, Rodriguez-Juarez R, Kogosov D, Metz GAS, Kovalchuk O.** Fractionated low-dose exposure to ionizing radiation leads to DNA damage, epigenetic dysregulation, and behavioral impairment. *Environ Epigenet* 2: dvw025, 2017.
39. **Koturbash I, Miousse IR, Sridharan V, Nzabarushimana E, Skinner CM, Melnyk SB, Pavliv O, Hauer-Jensen M, Nelson GA, Boerma M.** Radiation-induced changes in DNA methylation of repetitive elements in the mouse heart. *Mutat Res* 787: 43–53, 2016. doi:10.1016/j.mrfmmm.2016.02.009.
40. **Kumagai T, Rahman F, Smith AM.** The microbiome and radiation induced-bowel injury: evidence for potential mechanistic role in disease pathogenesis. *Nutrients* 10: 1405, 2018. doi:10.3390/nu10101405.
41. **Lam V, Moulder JE, Salzman NH, Dubinsky EA, Andersen GL, Baker JE.** Intestinal microbiota as novel biomarkers of prior radiation exposure. *Radiat Res* 177: 573–583, 2012. doi:10.1667/RR2691.1.
42. **Langberg CW, Sauer T, Reitan JB, Hauer-Jensen M.** Relationship between intestinal fibrosis and histopathologic and morphometric changes in consequential and late radiation enteropathy. *Acta Oncol* 35: 81–87, 1996. doi:10.3109/02841869609098484.
43. **LoIudice T, Baxter D, Balint J.** Effects of abdominal surgery on the development of radiation enteropathy. *Gastroenterology* 73: 1093–1097, 1977. doi:10.1016/S0016-5085(19)31864-5.
44. **Luiking YC, Poeze M, Ramsay G, Deutz NE.** Reduced citrulline production in sepsis is related to diminished de novo arginine and nitric oxide production. *Am J Clin Nutr* 89: 142–152, 2009. doi:10.3945/ajcn.2007.25765.
45. **Lutgens L, Lambin P.** Biomarkers for radiation-induced small bowel epithelial damage: an emerging role for plasma citrulline. *World J Gastroenterol* 13: 3033–3042, 2007. doi:10.3748/wjg.v13.i22.3033.
46. **Lutgens LC, Deutz NE, Gueulette J, Cleutjens JP, Berger MP, Wouters BG, von Meyenfeldt MF, Lambin P.** Citrulline: a physiologic marker enabling quantitation and monitoring of epithelial radiation-induced small bowel damage. *Int J Radiat Oncol Biol Phys* 57: 1067–1074, 2003. doi:10.1016/S0360-3016(03)00781-8.
47. **Melnyk S, Pogribna M, Pogribny I, Hine RJ, James SJ.** A new HPLC method for the simultaneous determination of oxidized and reduced plasma aminothiols using coulometric electrochemical detection. *J Nutr Biochem* 10: 490–497, 1999. doi:10.1016/S0955-2863(99)00033-9.
48. **Melnyk S, Pogribna M, Pogribny IP, Yi P, James SJ.** Measurement of plasma and intracellular S-adenosylmethionine and S-adenosylhomocysteine utilizing coulometric electrochemical detection: alterations with plasma homocysteine and pyridoxal 5′-phosphate concentrations. *Clin Chem* 46: 265–272, 2000. doi:10.1093/clinchem/46.2.265.
49. **Miousse IR, Chalbot MC, Lumen A, Ferguson A, Kavouras IG, Koturbash I.** Response of transposable elements to environmental stressors. *Mutat Res Rev Mutat Res* 765: 19–39, 2015. doi:10.1016/j.mrrev.2015.05.003.
50. **Miousse IR, Koturbash I.** The fine LINE: methylation drawing the cancer landscape. *BioMed Res Int* 2015: 1, 2015. doi:10.1155/2015/131547.
51. **Miousse IR, Kutanzi KR, Koturbash I.** Effects of ionizing radiation on DNA methylation: from experimental biology to clinical applications. *Int J Radiat Biol* 93: 457–469, 2017. doi:10.1080/09553002.2017.1287454.
52. **Miousse IR, Pathak R, Garg S, Skinner CM, Melnyk S, Pavliv O, Hendrickson H, Landes RD, Lumen A, Tackett AJ, Deutz NEP, Hauer-Jensen M, Koturbash I.** Short-term dietary methionine supplementation affects one-carbon metabolism and DNA methylation in the mouse gut and leads to altered microbiome profiles, barrier function, gene expression and histomorphology. *Genes Nutr* 12: 22, 2017. doi:10.1186/s12263-017-0576-0.
53. **Miousse IR, Tobacyk J, Melnyk S, James SJ, Cheema AK, Boerma M, Hauer-Jensen M, Koturbash I.** One-carbon metabolism and ionizing radiation: a multifaceted interaction. *Biomol Concepts* 8: 83–92, 2017. doi:10.1515/bmc-2017-0003.
54. **Miousse IR, Tobacyk J, Quick CM, Jamshidi-Parsian A, Skinner CM, Kore R, Melnyk SB, Kutanzi KR, Xia F, Griffin RJ, Koturbash I.** Modulation of dietary methionine intake elicits potent, yet distinct, anticancer effects on primary versus metastatic tumors. *Carcinogenesis* 39: 1117–1126, 2018. doi:10.1093/carcin/bgy085.
55. **Pawar SA, Shao L, Chang J, Wang W, Pathak R, Zhu X, Wang J, Hendrickson H, Boerma M, Sterneck E, Zhou D, Hauer-Jensen M.** C/EBPδ deficiency sensitizes mice to ionizing radiation-induced hematopoietic and intestinal injury. *PLoS One* 9: e94967, 2014. doi:10.1371/journal.pone.0094967.
56. **Peeters ST, Hoogeman MS, Heemsbergen WD, Hart AA, Koper PC, Lebesque JV.** Rectal bleeding, fecal incontinence, and high stool frequency after conformal radiotherapy for prostate cancer: normal tissue complication probability modeling. *Int J Radiat Oncol Biol Phys* 66: 11–19, 2006. doi:10.1016/j.ijrobp.2006.03.034.
57. **Potish RA, Twiggs LB, Adcock LL, Prem KA.** Logistic models for prediction of enteric morbidity in the treatment of ovarian and cervical cancers. *Am J Obstet Gynecol* 147: 65–72, 1983. doi:10.1016/0002-9378(83)90086-8.
58. **Prior S, Miousse IR, Nzabarushimana E, Pathak R, Skinner C, Kutanzi KR, Allen AR, Raber J, Tackett AJ, Hauer-Jensen M, Nelson GA, Koturbash I.** Densely ionizing radiation affects DNA methylation of selective LINE-1 elements. *Environ Res* 150: 470–481, 2016. doi:10.1016/j.envres.2016.06.043.
59. **Reis Ferreira M, Andreyev HJN, Mohammed K, Truelove L, Gowan SM, Li J, Gulliford SL, Marchesi JR, Dearnaley DP.** Microbiota- and radiotherapy-induced gastrointestinal side-effects (MARS) study: a large pilot study of the microbiome in acute and late-radiation enteropathy. *Clin Cancer Res* 25: 6487–6500, 2019. doi:10.1158/1078-0432.CCR-19-0960.
60. **Schweizer HP.** Mechanisms of antibiotic resistance *in Burkholderia pseudomallei*: implications for treatment of melioidosis. *Future Microbiol* 7: 1389–1399, 2012. doi:10.2217/fmb.12.116.
61. **Shah R, Cope JL, Nagy-Szakal D, Dowd S, Versalovic J, Hollister EB, Kellermayer R.** Composition and function of the pediatric colonic mucosal microbiome in untreated patients with ulcerative colitis. *Gut Microbes* 7: 384–396, 2016. doi:10.1080/19490976.2016.1190073.
62. **Shorter KR, Felder MR, Vrana PB.** Consequences of dietary methyl donor supplements: is more always better? *Prog Biophys Mol Biol* 118: 14–20, 2015. doi:10.1016/j.pbiomolbio.2015.03.007.
63. **Song JE, Kwak YG, Um TH, Cho CR, Kim S, Park IS, Hwang JH, Kim N, Oh GB.** Outbreak of *Burkholderia cepacia* pseudobacteraemia caused by intrinsically contaminated commercial 0.5% chlorhexidine solution in neonatal intensive care units. *J Hosp Infect* 98: 295–299, 2018. doi:10.1016/j.jhin.2017.09.012.
64. **Sookdeo A, Hepp CM, McClure MA, Boissinot S.** Revisiting the evolution of mouse LINE-1 in the genomic era. *Mob DNA* 4: 3, 2013. doi:10.1186/1759-8753-4-3.

65. **Southeast Asia Infectious Disease Clinical Research Network.** Causes and outcomes of sepsis in southeast Asia: a multinational multicentre cross-sectional study. *Lancet Glob Health* 5: e157–e167, 2017. doi:10.1016/S2214-109X(17)30007-4.
66. **Stipanuk MH.** Sulfur amino acid metabolism: pathways for production and removal of homocysteine and cysteine. *Annu Rev Nutr* 24: 539–577, 2004. doi:10.1146/annurev.nutr.24.012003.132418.
67. **Strekalova E, Malin D, Good DM, Cryns VL.** Methionine deprivation induces a targetable vulnerability in triple-negative breast cancer cells by enhancing TRAIL receptor-2 expression. *Clin Cancer Res* 21: 2780–2791, 2015. doi:10.1158/1078-0432.CCR-14-2792.
68. **Voronina OL, Kunda MS, Ryzhova NN, Aksenova EI, Semenov AN, Lasareva AV, Amelina EL, Chuchalin AG, Lunin VG, Gintsburg AL.** The variability of the order *Burkholderiales* representatives in the healthcare units. *BioMed Res Int* 2015: 1, 2015. doi:10.1155/2015/680210.
69. **Voronina OL, Kunda MS, Ryzhova NN, Aksenova EI, Sharapova NE, Semenov AN, Amelina EL, Chuchalin AG, Gintsburg AL.** On *Burkholderiales* order microorganisms and cystic fibrosis in Russia. *BMC Genomics* 19, *Suppl* 3: 74, 2018. doi:10.1186/s12864-018-4472-9.
70. **Wiersinga WJ, Virk HS, Torres AG, Currie BJ, Peacock SJ, Dance DAB, Limmathurotsakul D.** Melioidosis. *Nat Rev Dis Primers* 4: 17107, 2018. doi:10.1038/nrdp.2017.107.
71. **Willett CG, Ooi CJ, Zietman AL, Menon V, Goldberg S, Sands BE, Podolsky DK.** Acute and late toxicity of patients with inflammatory bowel disease undergoing irradiation for abdominal and pelvic neoplasms. *Int J Radiat Oncol Biol Phys* 46: 995–998, 2000. doi:10.1016/S0360-3016(99)00374-0.
72. **Withers HR, Elkind MM.** Microcolony survival assay for cells of mouse intestinal mucosa exposed to radiation. *Int J Radiat Biol Relat Stud Phys Chem Med* 17: 261–267, 1970. doi:10.1080/09553007014550291.
73. **Wood L.** *Protein Supplements: Global Market Size, Share & Trends Analysis, 2019 to 2025* Dublin, Ireland, April 18, 2019.
74. **Zeisel S.** Choline, other methyl-donors and epigenetics. *Nutrients* 9: 445, 2017. doi:10.3390/nu9050445.
75. **Zhao M, Wijayasinghe YS, Bhansali P, Viola RE, Blumenthal RM.** A surprising range of modified-methionyl S-adenosylmethionine analogues support bacterial growth. *Microbiology* 161: 674–682, 2015. doi:10.1099/mic.0.000034.