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MEETING REPORT

Development of Biomarkers for Radiation Biodosimetry and Medical Countermeasures Research: Current Status, Utility, and Regulatory Pathways

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Biomarkers are important indicators of biological processes in health or disease. For this reason, they play a critical role in advanced development of radiation biodosimetry tools and medical countermeasures (MCMs). They can aid in the assessment of radiation exposure level, extent of radiation-induced injury, and/or efficacy of a MCM. This meeting report summarizes the presentations and discussions from the 2020 workshop titled, "Biomarkers in Radiation Biodosimetry and Medical Countermeasures" sponsored by the Radiation and Nuclear Countermeasures Program (RNCP) within the National Institute of Allergy and Infectious Diseases (NIAID). The main goals of this meeting were to: 1. Provide an overview on biomarkers and to focus on the state of science with regards to biomarkers specific to radiation biodosimetry and MCMs; 2. Understand developmental challenges unique to the role of biomarkers in the fields of radiation biodosimetry and MCM development; and 3. Identify existing gaps and needs for translational application.

INTRODUCTION

In 2004, the Radiation and Nuclear Countermeasures Program (RNCP) within the National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH) was tasked with supporting research and development of diagnostics and medical countermeasures (MCMs) for use during a radiological or nuclear mass casualty incident. To fulfill this mandate, the RNCP works closely with the Biomedical Advanced Research and Development Authority (BARDA), which supports many late-stage activities needed for product approval and is responsible for procurement of diagnostics and MCMs, and the U.S. Food and Drug Administration (FDA), that guides and facilitates regulatory aspects of radiation biodosimetry and MCM development. This ongoing and continuous interaction has resulted in approval of four MCMs to treat hematopoietic complications resulting from radiation exposure - filgrastim (Neupogen®, March 2015; Amgen),[2] pegfilgrastim (Neulasta®, November 2015; Amgen),[3] sargramostim (Leukine®, March 2018; Partner Therapeutics)[4] and romiplostim (Nplate®, January 2021, Amgen).[5] Although no device or biodosimetry test has been cleared by the U.S. FDA for use in a radiological/nuclear incident as of this writing, several approaches are in advanced development under BARDA's biodosimetry program.

Evaluation and understanding of biomarkers is critical to all stages of drug and device development (1), and issues surrounding their use represents an important part of understanding disease (2). The detection of biomarkers that are altered after radiation exposure, and accompanying device development for radiation triage, are likewise critical to the accurate and rapid assessment of exposure levels during a radiation public health emergency. This capability allows first responders and healthcare providers to separate the ''worried well'' or those who are concerned, but otherwise in good health, from individuals who would benefit from treatment because they have been exposed to large, absorbed radiation doses and/or have concomitant injuries. In the field of radiation biodosimetry, although cytogenetics (e.g., DNA damage markers) represent the

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[2] https://bit.ly/2ZJO9KH.
[3] https://bit.ly/2U8OwdE.
[4] https://bit.ly/2XYai6h.
[5] https://www.accessdata.fda.gov/drugsatfda_docs/label/2021/125268s167lbl.pdf.

''gold standard,'' there are a number of ''omics'' approaches that are also being explored, such as genomics, transcriptomics, proteomics, lipidomics and metabolomics. These assessments may be useful to both triage individuals into treatment categories, and later, track efficacy of a MCM that is administered as a treatment. From another perspective, these kinds of biomarkers can also be used purely as a process tool for researchers developing MCMs for therapy, since they can potentially be used as a bridge, to tie together perturbations seen across animal models to humans (e.g., a pharmacodynamic marker of effect). The use of biomarkers as surrogates for expected human responses to radiation and drug treatments is critical (3); however, since the efficacy of a particular MCM cannot be feasibly or ethically assessed in humans (21 Code of Federal Regulations (CFR) 314.600-650 for drugs; 21 CFR 601.90-95 for biologics), it must be studied in animal models under the FDA Animal Rule. From a regulatory perspective, the proposed use of biomarkers could span different FDA Centers, including the Center for Devices for Radiological Health (CDRH) and the Center for Drug Evaluation and Research (CDER). CDRH is responsible for reviewing pre-market submissions for products such as radiation biodosimetry devices that measure specific biomarkers to assess radiation exposure. CDER has oversight of biomarkers, particularly when alterations in their levels are used as pharmacodynamic markers of radiation injury and potential drug efficacy.

In summary, for both research areas, biodosimetry and MCM advancement, biomarkers are integral in the development pathway (1). For this reason, on June 1, 2020, in Rockville, MD, the NIAID/RNCP sponsored a workshop on ''Biomarkers in Radiation Biodosimetry and Medical Countermeasures.'' Speakers included academicians as well as U.S. Government (USG), industry and agency partners (Table 1). The objectives of this meeting were to: 1. Capture the role and utility of biomarkers and assess the state of the science on biomarkers specific to radiation countermeasures and biodosimetry; 2. Better understand developmental challenges unique to the role of biomarkers in the fields of radiation countermeasures and biodosimetry; 3. Identify existing gaps, and needs for translational application; and 4. Provide a platform for an open, informal dialogue among researchers, USG representatives and regulatory agencies with expertise in the development of MCMs toward FDA approval. Participating USG panelists at the NIAID-led discussion included RNCP and Office of Regulatory Affairs (ORA) staff from NIAID, BARDA personnel, and individuals from several FDA offices. Discussion topics centered on 1. biomarkers in biodosimetry platforms, 2. biomarkers in the context of radiation MCM development, and 3. translation of biomarkers in both areas from the bench to clinical settings. This report summarizes the talks, and the main points presented during these dialogs. Where unpublished data or personal communications are mentioned, presenter names are provided in parentheses.

Background

Effective biomarker use should span a continuum that ranges from early discovery and analytical validation through clinical validation and qualification. The biomarker can be at the beginning stages of development, with work involving standardization of the sample source and the detection method to be used. Investigators then need to develop performance metrics and determine the precision, accuracy, and sensitivity of the biomarker. For advanced, development, once clinical trials are underway, researchers then need to evaluate the utility of the biomarker for clinical practice and drug development, and if appropriate, submit for regulatory consideration.

Because of their widespread utility in product development and the clinical setting (1), the types of biomarkers were defined as well as their potential use. These classifications were based on a review authored by Califf, who proposed biomarker definitions, and how those definitions could be used to delineate their purpose (4). That review looked at biomarker definitions that were recently established by a cooperative effort between the FDA and the NIH, as part of a joint task force. A continuously-updated source document was generated by the FDA-NIH Biomarker Working Group called Biomarkers EndpointS and other Tools (BEST) (5), accessible in several locations online.[6] The working group, led by NIH Director Francis Collins and former FDA Commissioner Robert Califf, developed a glossary of harmonized terminology for biomarkers and endpoints. At its essence, a biomarker is defined as a characteristic measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention. Biomarkers that have been used in the development of radiation approaches can be binned into several different categories, including molecular (e.g., miRNAs), histologic (inflammation, fibrosis), radiographic [computed tomography (CT) quantitative findings], physiological (e.g., lung or kidney function) or other (e.g., metabolites). Because biomarker development has increased and they are being developed with different model systems and treatment settings, it is a challenge to keep up with their progress in product development, clinical use and policy development.

To streamline the types of biomarkers, they have been further categorized based on the timing and details of their use. These include the following:

- Diagnostic, to detect a disease or condition.
- Monitoring, to assess disease status.
- Pharmacodynamics, to define levels of response to a product.
- Predictive, to forecast an effect from administration of a product or agent.

[6] www.fda.gov/media/99221/download and https://www.ncbi.nlm.nih.gov/books/NBK326791/.

TABLE 1
Workshop Speakers and their Areas of Expertise

Speaker	Affiliation	Area of expertise
Andrea DiCarlo, PhD	NIAID, NIH, Rockville, MD	Radiation biology, advanced development, animal models
Sally Amundson, PhD	Columbia University, New York, NY	Radiation biodosimetry, gene expression, radiation measurements
Matthias Port, MD, PhD	Institut für Radiobiologie der Bundeswehr, Germany	Radiobiology, gene expression, EPR medicine
Naresh Menon. PhD	Chromologic LLC, Monrovia, CA	Transcriptomics, radiation biodosimetry, instrumentation
Francisca Reyes-Turcu, PhD	CDRH, FDA, White Oaks, MD	Device advanced development, biodosimetry guidance
Maureen Kane, PhD	University of Maryland, Baltimore, MD	Disease mechanism, radiation models, biomarkers
Meetha Medhora, PhD	Medical College of Wisconsin, Milwaukee, WI	Radiation countermeasures, models, novel imaging biomarkers
Marjan Boerma, PhD	NIAID, NIH, Rockville, MD	Toxicology, product development, MCMs, immunology
Sue-Jang Wang, PhD	CDER, FDA, White Oak, MD	Regulatory development of MCM, Animal Rule
Libero Marzella, MD, PhD	CDER, FDA, White Oak, MD	Regulatory development of MCM, Animal Rule
Lynne Wathen, PhD	BARDA, HHS, Washington DC	Radiation biodosimetry, device clearance, bridging studies
Radia Tamarat, PhD	Institut de Radioprotection et de Sûreté Nucléaire, France	Stem cell therapies, radiological accident management

- Prognostic, to predict likelihood of a biological event (e.g., disease).
- Safety, to evaluate potential toxicity of exposure to an agent.
- Susceptibility/risk, to implicate disease development before clinically apparent symptoms.

Radiation biomarkers can fall into several categorizations as mentioned above. For instance, radiation-induced cytogenetic changes can serve as diagnostic biomarkers (6); radionuclide body burdens can be used to monitor the efficacy of decorporation agents (7); absolute neutrophil counts after radiation exposure and growth factor treatment have been used as pharmacodynamic markers (8); procalcitonin, a marker of total-body irradiation (TBI), can predict radiation lethality at 10 days postirradiation (9), and glutathione peroxidase can predict lethality from radiation-induced lung disease in mice (10); DNA damage in cells from Fanconi anemia patients is a prognostic marker for radiation sensitivity (11); cardiovascular biomarkers such as troponin T can be used as safety biomarker for patients undergoing left-sided irradiation for breast cancer (12); and biomarkers detected as part of retrospective radiation biodosimetry can indicate an increased susceptibility/risk of later cancer development (13). Because biomarkers represent surrogates of an outcome, one cannot assume that correlations are meaningful. In fact, most biomarkers do not end up being valid surrogates. As biomarkers and the methods used to evaluate them continue to develop, electronic measurements will continue to gain traction as a means of monitoring them in real time in both humans and animals and will prove critical to determine their use and robustness. In addition, it is always important to consider regulatory concerns in the development of these biological markers, so that they can be optimally used in the future.

Session I: Biomarkers in the Biodosimetry Framework

In the area of radiation biomarkers research, gene expression profiles can be developed for purposes of 1. triage, 2. dose reconstruction, and 3. prediction of outcome. Several biomarker studies were designed to reconstruct dose to determine actual radiation dose (Gy) exposure for medical triage and treatment purposes (S. Amundson). Using two independent blood sample data sets (Agilent microarray data), together with polynomial models, a proof-of-principle study was conducted to select radiation responsive genes to quantitatively reconstruct radiation dose up to 4.5 ± 0.35 Gy and up to 6 ± 1.74 Gy (14). This simple model showed that construction of a gene expression radiation dose response is possible, but translation between species is not as straightforward. While changes in the expression of some genes are similar in mice and humans, a significant number show opposing trends. For example, transcription factors such as p53 activate and inhibit genes based on species-specific phosphorylation sites that can be changed or missing between species; therefore, p53 may downregulate certain genes in mice and upregulate those same genes in humans (15). To minimize these differences and derive meaningful data, a nonobese diabetic/severe combined immunodeficiency (NOD SCID), humanized hematopoietic radiation mouse model was utilized, where the immune system was genetically abolished and then human hematopoietic stem cells were transplanted. Once the humanized immune system was established in the mouse, the mice were irradiated, and the resulting relative gene expression data between the human- and humanized mouse-derived blood samples were found to be similar.

This divergence in interspecies gene expression is not exclusive to mice, as nonhuman primates (NHPs) also exhibit species-specific radiation differences that are not

representative of the human radiation response. Efforts have been made to enrich biodosimetry signatures for ''interspecies'' radiation-responsive genes with strong correlation between NHPs and humans (16). While 52 genes were identified and correlated in both species by heatmap assessments, the relative signal strengths were different once assessed by RT-PCR. In order to lower the relative NHP signal strength so that it compares to the lower human gene expression level, a conversion must be done using the AdaBoost multi-gene conversion model.[7] In turn, NHP samples can then be used to create a radiation dose reconstruction curve predicative of human dose.

Inter-individual variation is yet another issue to be considered when using gene expression for dose reconstruction. Confounding factors such as underlying chronic inflammation, immunological problems, or DNA repair defects can alter the typical gene expression profile and may alter the response to radiation. To understand the potential impact of these differences on dose reconstruction, blood samples from mouse models with genetic defects in DNA repair, pro-inflammatory, and anti-inflammatory pathways were assessed (17, 18). The gene expression response to an $LD_{50/30}$ radiation dose is subdued in mouse strains with DNA repair defects, which makes it difficult to classify a sample as irradiated vs. non-irradiated if only wild-type mice are used for gene selection. Interestingly, if classifiers are built including samples from the DNA repair deficient mice, then samples from all genotypes, including the wild-type, can be classified with equal high accuracy. In general, the datasets with issues in the inflammatory pathways also exhibited significant differences from wild-type data, potentially impeding classification as irradiated vs. non-irradiated; however, the gene set selected using mixed wild-type and DNA repair deficient samples performed equally well when challenged with samples from the immune or inflammatory genotypes. In general, it appears that inclusion of samples from potential confounding conditions in the process of gene selection is more likely to produce a robust classification system.

Animal model studies certainly highlight some of the gaps and challenges that exist in using gene expression dose reconstruction in humans. Genetic differences, stochastic differences, pre-existing disease, medications, age and sex are a few of the inter-individual differences that can impact radiation sensitivity. External variations such as dose rate, radiation source (e.g., neutron, gamma), and presence of combined injuries can also have an impact on dose reconstruction. Some of these challenges are being addressed, and models are being combined to provide more robust classification systems, but much is still to be learned.

While gene expression can be used to establish actual dose received, it is also being used to predict the effective dose (i.e., individual biological effect or disease potential) after radiation exposure (19). A simple knowledge of the absorbed dose is not enough; predicting effective dose requires a triangulation of physical measurements, biological, and clinical parameters (M. Port) (Fig. 1). Dose is only a surrogate because radiation exposure is dependent on multiple factors, such as dose rate, homogenous vs. inhomogeneous irradiation, TBI vs. partial-body (PBI) irradiation, radiation quality (e.g., gamma, neutron), internal vs. external radionuclide contamination, and fractionated vs. high-dose, acute exposure (20). Using case studies from actual radiation accidents, the Medical TREatment ProtocOLs (METREPOL) hematopoietic (H)-acute radiation syndrome (ARS) severity scoring system of 0–4 was correlated with published dose estimates using dicentric chromosomes and physical dosimetry (21). An H-ARS score of 0 corresponds to $<$1.5 Gy, H-ARS scores of 1, 2, 3 correspond to 1–6 Gy, and an H-ARS score of 4 corresponds to $>$6 Gy (22). While a high degree of correlation was found between H-ARS scores and determination of absorbed radiation dose, some cases fell outside of the scoring range, and it was difficult to determine the true biological/delayed effects. In an effort to better predict delayed effects of H-ARS, peripheral blood samples from baboons were collected at days 1 and 2 after 2.5 Gy TBI or 5 Gy PBI (23). RNA was extracted from the samples and microarray technology was used to screen for 19,596 genes of interest. Using gene enrichment analysis, 89 mRNAs were selected for qRT-PCR validation, which led to the identification and validation of genes predictive of H1-3 (22 genes) and H2-3 (7 genes) H-ARS severity scores.

Third generation gene expression signatures have expanded to include the use of miRNAs to assess other endpoints, such as pre- and post-exposure pancytopenia (24, 25), persistent gene expression (26), and the ability to distinguish between PBI and TBI (27). A down selection of genes associated with radiation exposure resulted in the identification of six promising biomarkers (*WNT3, POU2AF1, CCR7, ARG2, CD177, WLS*) as well as confirmation of three genes (*FDXR, PCNA, DDB2*) previously identified using ex vivo blood samples (28). Validation of these genes was conducted in human in vivo blood samples from normal volunteers and radiotherapy patients using qRT-PCR. A variety of patient dose ranges were incorporated from: diagnostic CT scans (low dose range: 0.004–0.018 Sv), patients receiving local radiotherapy for prostate cancer (low dose range: 0.25–0.3 Sv), and TBI (high dose range: 3–4 Sv) scenarios. All gene expression levels were confirmed except for FDXR, which had an inverse relationship and was downregulated in baboons while upregulated in human samples. Similar results were obtained and confirmed using ex vivo blood samples. These studies show that cross-species validation is possible and necessary if a dose response is to be established. These gene expression markers were also examined for robustness to inter-individual variance using 200 healthy human donors (29). While significant sex- and

[7] https://towardsdatascience.com/understanding-adaboost-for-decision-tree-ff8f07d2851.

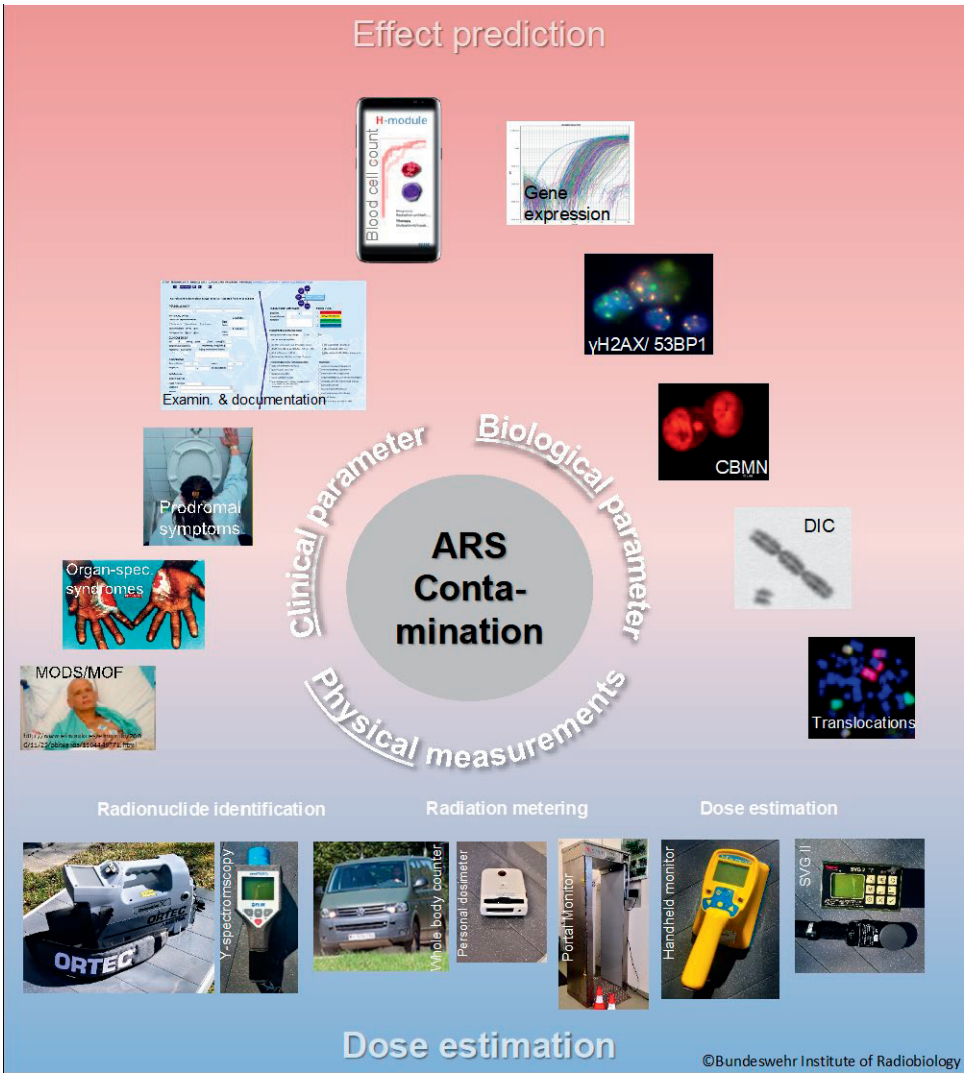

FIG. 1. Concept of the R/N Medical Task Force in the Bundeswehr Institute of Radiobiology (reprinted with permission from M. Port). CBMN: Cytokinesis-block micronucleus assay; DIC: Dicentric chromosome assay; MODS/MOFS: Multiorgan dysfunction syndrome, multiorgan failure, radiation metering: estimate radiation contamination.

age-dependent expression differences were noted, the variances were well within the twofold gene expression difference and contributed less than 20–30% of the inter-individual variance; therefore, it was considered to have minimal to no impact.

While these preliminary experiments are important, their diagnostic use is limited, and do not have the high throughput that would be needed in a mass-casualty radiation incident. Therefore, targeted next-generation sequencing (NGS) was used to evaluate gene expression changes of *FDXR, DDB2, POU2AF1* and *WNT3* and the results were validated by qRT-PCR (30). Blood was either sham or X-ray irradiated with 0.5 or 5.0 Gy, and NGS allowed for the classification of these samples into H-ARS severity scores of H0, H1 and H2–4 -with 90–97% agreement. This demonstrates the successful use of an automated methodology to discriminate 100× more samples in a third of the time it takes to assess samples using standard cytogenetic studies. Although the process was streamlined, it still required many pieces of laboratory equipment; therefore, the goal is to optimize the technology into a small microfluidic ‘‘lab-on-a-chip’’ point-of-care (POC) device.

A miRNA-based, tissue specific biomarker approach can consider the complexity of a radiation incident, including varied mechanisms (e.g., radiation type, dose rate, PBI vs. TBI), biology (e.g., confounding conditions, age, diet, lifestyle, ethnicity) and logistics (e.g., polytrauma, access, training, limited resources) (N. Menon). The final goal is to develop a ‘‘patient first’’ POC approach that will help the end user make a treatment decision based on an individual’s response to radiation exposure and/or predicted health outcome. One such consideration is the use of acute phase biomarkers to predict late onset of lung fibrosis/pneumonitis

or acute respiratory distress syndrome (ARDS), which ChomoLogic, LLC hopes to accomplish using circulating miRNAs. These molecules are plasma stable and robust tissue-specific biomarkers that can be used for diagnosis and prognosis of diseases. To identify a set of suitable miRNAs, whole blood samples were obtained from whole thorax lung irradiated (WTLI) and TBI studies conducted using different species, including irradiated mice (C3H and C57BL/6), NHPs (rhesus macaque), and stem cell transplant and lung cancer patients (31, 32). Although a miRNA-based biomarker diagnostic seems logical, major challenges exist, such as the sheer number of potential miRNAs to choose from, functional redundancy and complexity of miRNAs, and inefficient algorithms for the identification of robust miRNA panels. In addition, assay development requires miRNAs that are sufficiently abundant and have a significant change in expression ($P < 0.05$).

To address these issues, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were used to sort out the mechanistic roles of the miRNAs of interest. In addition, Kaplan-Meier curves were used to distinguish circulating miRNAs by survival outcome, and CT scans were used to track actual disease prognosis. As an example, an NHP WTLI study was presented in which samples were sorted and tracked by early death and survivor cohorts (31). Pathway analysis identified miRNAs in the early response (days 6–15) that significantly interacted with the p53 signaling pathway and activated pro-apoptotic pathways, leading to early death in irradiated animals. In the NHP survivors, miRNAs were significantly associated with TGF-b signaling and activation of pro-fibrotic pathways, perhaps leading to a delayed death in irradiated animals. Similarly, blood samples from WTLI mice were also subjected to miRNA-gene annotation studies as described above (32). While the species-specific miRNAs were different, common gene ontology networks were common to both species; for example, the TGF-β/SMAD signaling pro-fibrotic pathway was closely associated with lung disease outcomes. Major challenges exist in the development of miRNA-based biomarker diagnostics, even as miRNA correlation to injury pathways evolves and new miRNAs continue to be discovered. It is essential to understand how miRNAs relate to the biological pathways that underline the disease and how they can be predictive of an actual clinical outcome.

Clearly, the development of biomarker assays for use as biodosimetry tools is complex and validation is critical; therefore, it is important to consider any regulatory perspectives and guidance early in the process (F. Reyes Turcu). The types of biodosimetry needs in a mass casualty scenario that would help manage patients are: 1. POC devices with low false negative or positive rate to triage all victims, so that patients exposed to <2 Gy can be evacuated to safety; 2. High-throughput (HT) diagnostic assays as a second tier in sorting patients exposed to >2 Gy for further clinical follow-up; and 3. Clinical management assays with a low-false negative and positive rate that would help refine the needs of the patient. Radiation biodosimetry devices (33) described here are in vitro diagnostic devices (IVDs) that could estimate absorbed radiation dose in a field triage setting following a radiation mass casualty scenario, and are not meant to be used to measure doses delivered from radiation therapy. Radiation biodosimetry devices could be developed to be used for subsequent clinical evaluations in a radiation incident. These devices may provide a qualitative output (e.g., positive or negative) for triage or a quantitative output to provide actual absorbed dose, which may help with clinical management. Radiation biodosimetry devices may employ a variety of approaches including molecular (e.g., mRNA, miRNA), cytogenic (e.g., micronuclei, dicentric chromosomes), or protein biomarkers. These biomarkers are often paired with technologies such as enzyme-linked immunosorbent assay (ELISA), lateral flow, multiplex beads that provide a certain output upon detection of complex biomarker signatures. In all cases, biomarker changes would reflect biological responses to radiation, which could include DNA damage, inflammation, tissue damage repair.

To help navigate this complex process, the U.S. FDA has issued a *Radiation Biodosimetry Medical Countermeasure Devices* guidance document, to provide recommendations for the type of information typically requested for marketing authorization (33). In addition, sponsors may request feedback from FDA through the pre-submission process regarding regulatory recommendations for their proposed radiation biodosimetry devices (please refer to *Requests for Feedback and Meetings for Medical Device Submissions: The Q-Submission Program* guidance (34). Notwithstanding the unique concerns associated with MCMs, radiation biodosimetry devices are regulated just like every other medical device. The primary regulatory mechanisms that are available for the development and emergency use of medical device MCMs are: 1. Emergency Use Authorization (EUA), 2. Investigational Device Exemption (IDE), and 3. pre-market submission.

Devices used in a ''significant risk'' clinical investigation,[8] require an IDE to be submitted to FDA prior to initiation of the clinical study.[9,10] ''Nonsignificant risk'' (NSR) device clinical investigations must address labeling, institutional review board (IRB) approval, informed consent, monitoring, records, reports, and prohibition against promotion [21 CFR 812.2 (b)]. However, there is no need to make progress or final reports to FDA. NSR device studies do not have to have an IDE application approved by FDA.

Device classification (Class I, II, III) depends on the risk to patients.[11] This in turn guides the type of regulatory

[8] https://www.fda.gov/medical-devices/investigational-device-exemption-ide/ide-guidance.

[9] https://www.fda.gov/media/71075/download.

[10] https://www.fda.gov/media/75459/download.

[11] https://www.fda.gov/regulatory-information/search-fda-guidance-documents/de-novo-classification-process-evaluation-automatic-class-iii-designation.

submission required for marketing of the device. For first-of-a kind device for which there is no legally marketed predicate device, the *De Novo* process provides a pathway to Class I or Class II classification if controls can be established to sufficiently mitigate the risks to health. When a new device has been classified through the *De Novo* process, it can serve as a predicate for subsequent devices, when appropriate. An EUA allows for a product to be used during a declared emergency, but authorization for such use must be obtained from FDA as per the *Emergency Use Authorization of Medical Products and Related Authorities* FDA guidance document (35).

These FDA guidance documents help provide a path to follow for approval, authorization or clearance of radiation biodosimetry devices, but ultimately their analytical and clinical validity needs to be sufficiently demonstrated. In addition, the benefit of use should outweigh the risks associated with use. Furthermore, laboratories and physicians will need clear instructions for use and interpretation of data and must be sufficiently informed of the limitations of the radiation biodosimetry. Overall, the information provided would provide clinical value and utility.

Major elements of radiation biodosimetry device submissions include intended use, device description (e.g., biomarkers measured, platform, software), specimen handling, analytical performance, clinical performance, instrumentation and software validation, and labeling. For premarket approval (PMA), the marketing submission will also describe the manufacturing, design controls, and quality system requirements (21 CFR 820) applicable to the device.

Regulatory submissions for a radiation biodosimetry device typically include descriptions of the biomarker(s) being measured, the type of specimen needed (e.g., whole blood, urine, saliva), the targeted population(s), field use setting (e.g., triage or clinic/professional use only), whether the output is quantitative or qualitative, and timeframes for use (e.g., hours to 7 days postirradiation). Disclaimers, explicit warnings, and limitations may also be described. If software is needed, software validation may be required, as described in the *Guidance for the Content of Premarket Submissions for Software Contained in Medical Devices* (36).

The development of radiation biodosimetry devices is highly dependent on biological samples for validation studies. Since clinical samples are difficult to obtain, samples may be contrived in a variety of ways including, but not limited to, ex vivo irradiation of the appropriate matrix to induce the biomarker response, spiking the biomarker of interest into an appropriate matrix, and/or use of animal derived specimens (e.g., specimens from irradiated animals as appropriate). Samples from non-irradiated subjects may be used to assess normal range (reference internal) or expected values for qualitative or semi-quantitative assays.

Because ethical considerations limit the availability of samples for radiation biodosimetry device development, laboratory animal models may be used for ARS biomarker development. To be useful and provide a bridge to humans, animal models would ideally be well-defined, and any animal biomarker chosen would need a degree of homology similar to the response observed in humans. Comparisons to show similar output in normal ranges, and fold changes, kinetics, and error in irradiated samples for both species are typically needed. If bridging is achieved, then animal studies may be useful to address device performance at conditions that cannot be addressed using human clinical studies, such as acute radiation doses, dose rate effects, radiation quality and exposure time, and other confounding conditions. Analytical validation needs to demonstrate reproducibility, stability, and specificity of the assay. Clinical validation is also needed, and while limitations exist, prospective and/or retrospective studies from patients undergoing TBI for radiotherapy purposes may be used. Normal samples are typically used to establish normal ranges of biomarker(s) expression, and irradiated samples are used to establish dose output of the assay. FDA supports the principles of the ''3Rs,'' to reduce, refine, and replace animal use in testing when feasible. They encourage sponsors to consult with the agency if they wish to use a non-animal testing method that they believe is suitable, adequate, validated, and feasible. The alternative method should be assessed for equivalency to an animal test method. Finally, interactions with the FDA are encouraged to happen early and often, to consider the best path forward to market.

Session II: Biomarkers in the Medical Countermeasure Setting

Biomarkers of Radiation-Induced Injury in Animal Models: Identification and Development

Although many natural history studies, including those focused on biomarkers, have been conducted on various sub-syndromes, the focus of this presentation was gastrointestinal (GI)-ARS-related biomarkers and how their histological and clinical endpoints correlate. Characterization of the natural history of radiation injury in animal models including related biomarkers is important for MCM development under the FDA Animal Rule. Additionally, if biomarkers are validated and correlate to histological endpoints, the number of animals in a study may be decreased, saving time and money. Biomarkers are characteristics that are objectively measured and evaluated, and may be indicators of normal biologic processes, pathologic processes, and/or biological responses to a therapeutic intervention. Defining the relationship between the biomarker of interest and a clinical endpoint is essential to give the biomarker interpretable meaning for drug development, triage, or any other use. Clinical endpoints

can include, but are not limited to, histological analyses, functional assays, imaging analyses, clinical observations, and survival (M. Kane).

Biomarker development encompasses many phases and must start with well-defined small or large laboratory animal models (e.g., mouse and NHP). Discovery of biomarkers often involves high-throughput evaluation of many ''omics,'' followed by identification of radiation injury-responsive candidate biomarkers from these broad initial studies. Candidate biomarkers must then be validated including assessing time- and dose-dependence. Ideal biomarkers should reflect the target sub-syndrome or organ-specific injury through a clear relationship between the biomarker and clinical endpoint. They should also be present in the circulation and be easily accessible through blood tests or other biofluids. Most importantly, biomarkers should have cross-species utility to allow for translation to humans.

Biomarkers have been assessed in plasma and jejunal tissues in both NHP (7.5–13 Gy up to 60 days) and mouse (6–15 Gy up to 6 days) TBI models, and NHP PBI models with 2.5% or 5% bone marrow sparing (10–12 Gy up to 180 days) (37). These models were developed to mimic intentional or accidental radiation exposure in humans, which is likely to involve some bone marrow sparing. They allow the concurrent analysis of short- and long-term damage to organ systems in a time- and dose-dependent manner, with established curves and time estimates for survival, body weight, stool consistency, dehydration, mucositis, fibrosis, and more (37–39). High-throughput metabolomics analyses of NHP tissue and plasma were conducted using liquid chromatography tandem mass spectrometry (LC-MS/MS). This detection scheme allows for flexible experimental design and is also used in clinical settings, making the data more easily translatable. Plasma and jejunum metabolites that were significantly different between irradiated animals and naïve animals were identified. Both specimens were studied to identify metabolites with high changes in response to radiation injury at both the tissue injury site and in circulation. Such a finding would further validate that the biomarker was tissue injury specific. A variety of parallel studies were conducted in both NHP and mouse models to identify metabolites that are repeatable in a wide variety of scenarios. Similar results in both models increases the likelihood of cross-species utility and ultimate translation to humans.

Many metabolites had similar results across the NHP and mouse models, and those with similar changes in plasma and jejunum tissue were prioritized. Citrulline was noted as an example of a strong-performing biomarker candidate. The principal source of circulating citrulline is the small intestine, and plasma citrulline concentration tracks directly with intestinal cell mass (40, 41). Citrulline levels have also been reported to be affected by radiation exposure (42), radiotherapy (43), chemotherapy (44), Crohn's disease (45), and HIV (46). Additionally, in humans, a 20 μM or lower level of citrulline in the plasma is a highly sensitive (92%) and specific (90%) threshold for permanent intestinal failure with positive (95%) and negative (85%) predictive values (42). The normal range for citrulline in healthy individuals is 27–80 μM (47). Citrulline levels were found to decrease after irradiation in both the jejunum tissue and plasma and correlated with the established GI histological endpoint, corrected crypt number (CCN), out to day 21 postirradiation (48). The correlation coefficient between jejunum citrulline and CCN was $R = 0.54$ ($P = 0.0051$) and $R = 0.67$ ($P = 0.0003$) between plasma and CCN (48). Other metabolites had high correlation with CCN, but few had significant correlation with CCN in both jejunum tissue and plasma, possibly due to being biomarkers of general GI damage, not specifically CCN. Citrulline levels in irradiated animals were found to be below the 20 μM threshold level set in humans on days 1–21 postirradiation. Plasma citrulline concentrations have been found to be similar in NHPs and humans (49, 50) further supporting it's translation ability. Over time, citrulline levels did recover to pre-irradiation levels, correlating with intestinal recovery seen via histology. Trends in citrulline levels after irradiation were consistent across mouse, NHP, and minipig TBI models (51–53). Further, time and dose dependence studies were conducted in the mouse model to determine biomarker correlation with GI histological endpoints – studies that would have been very difficult and expensive to do in an NHP (51, 52). These studies further supported citrulline's correlation to CCN. Ornithine aminotransferase, involved in the intestinal synthesis of citrulline and highly expressed in the GI villus epithelium, was also correlated with CCN in a proteomics study (54). Taken together, these findings highlight the importance of using animal models with well-defined natural histories and relevance to MCM development. Laboratory models need to show robust, reproducible findings across studies, and should be used to establish detailed and specific histological, functional, and imaging analyses, observational, and/or survival data that correlate to clinical endpoints.

Biomarkers To Trace Radiation-Induced Lung Injury: Progress and Challenges

Studies to identify biomarkers predictive of lung injury were also presented (M. Medhora). The aim was to identify candidate biomarkers that appear before symptoms manifest, and before mitigators are effective. The biomarkers should correlate with pathophysiological endpoints, such as vascular regression and reactivity or bone marrow depletion. The biomarkers were evaluated in a WAG/RijCmcr rat WTLI model (55) with no supportive care (e.g., antibiotics, hydration, interventions) or anesthesia, using opposed lateral fields of radiation at a dose rate of 1.43 Gy/min. In all rat models of irradiation, including the WTLI model, there is some bone marrow exposure that can result in lowered blood cell counts, and that has been taken into

consideration for analyses. The experimental design involved a training cohort of 9–10-week-old rats that received 15 Gy X ray WTLI resulting in no survival at day 120 postirradiation, 2 trial rat cohorts that received 13 Gy or 10 Gy WTLI and had ~30% and 100% survival, respectively, and a negative, naïve control group. A significant challenge that arose was validating whether the biomarker was specific to radiation injury alone vs. other lung injuries, such as those caused by lipopolysaccharide (LPS). Thus, a positive control set that was exposed to LPS to cause lung injury was also included. These researchers had previously found that when treating the WTLI rats with enalapril beginning 35 days postirradiation, survival significantly increased from 18% to 75% (56). Therefore, they sought biomarkers that would appear in weeks 1–4 postirradiation, allowing for time to identify individuals at high risk for lung injury prior to the 35-day timepoint.

A variety of assays were utilized to determine radiation-induced lung injury. These included qRT-PCR for changes in circulating miRNAs previously identified via discovery NGS, blood cell counts, cardiovascular imaging for regression of blood vessels in the lung, lung imaging for apoptotic cells, enzyme-linked immunosorbent assay (ELISA) for circulating cardiovascular protein markers, and invasive assays such as lung vascular resistance and permeability (Kf). Weight loss and breathing rates were also assessed after irradiation. Single-photon emission computed tomography/computed tomography (SPECT/CT) imaging (used clinically) was employed to measure lung perfusion by visualizing ^{99m}Tc-Macro-aggregated albumin (^{99m}Tc-MAA) lodged in the pulmonary arterioles (57). Mean ^{99m}Tc-MAA count dropped in the 15 Gy irradiated animals during weeks 1–4 postirradiation; however, at 2 weeks postirradiation the 10 Gy trial set had no difference and the 13 Gy trial set had only a slight drop compared to unirradiated control rats. At 2 weeks postirradiation (15 Gy) the irradiated animals had significantly higher vascular resistance and lung vascular permeability compared to unirradiated controls (58). In addition, white blood cell, neutrophil, lymphocyte, and monocyte counts were decreased in the irradiated animals in the training and trial sets compared to unirradiated controls. Interestingly, the neutrophil/lymphocyte ratio was not significantly different between the irradiated sets and controls but was significantly higher in the LPS injury set. The circulating miRNA markers were not as drastically changed by irradiation as anticipated; only miR-150-5p, which is related to circulating white blood cells, significantly decreased at 2 weeks in all irradiated groups. Each assay was assessed and three were selected for the predictive methodology: 1. ^{99m}Tc-MAA SPECT/CT analysis, 2. circulating lymphocytes, and 3. circulating monocytes. Statistical modeling was conducted using the following criteria:1. % lymphocytes $\geq$79.4%, 2. normalized MAA/CT volume $\geq$89.6%, and 3. % monocytes $<$2.0% – yielded 60-day survival prediction with an accuracy of 88.5% (95% CI: 77.8–95.3%, $P = 0.0000015$, McNemar's $P = 0.4497$) (data not yet published).

In summary, all assays were selected for ease of translation to the clinic, and a three-criteria prediction model allowed for prediction of mortality in a rat WTLI model with 88.5% accuracy at 2 weeks postirradiation. The group is exploring indocyanine green perfusion kinetics as another early lung injury detection method (59). More collaborations are underway to explore metabolomic and lipidomic biomarkers of lung injury to eventually translate these findings to humans.

Metabolomics Based Biomarkers in Delayed Radiation Injuries

Studies of biomarkers of delayed effects of acute radiation exposure (DEARE) are currently being conducted to examine progressive and irreversible symptoms that lead to organ damage of the kidney, lungs, cardiovascular system, central nervous system, and gastrointestinal system. The delayed effects can manifest in survivors of ARS months to years following radiation exposure (M. Boerma). The aim is to identify biomarkers that could predict DEARE in ARS survivors before symptoms occur, such that individuals at risk may be identified, monitored, and treated early to minimize organ damage and symptoms. The complex pathophysiology and multi-organ involvement of DEARE is a challenge, and it is hypothesized that each organ system has its own metabolic profile upon injury.

Current metabolomic studies are being conducted to identify DEARE predictive biomarkers for individual organ systems, with the ultimate goal of developing personalized monitoring tests and early treatment plans. A partial body, hind-leg shielded C57BL/6N mouse model was irradiated with 9.5 Gy γ rays to promote long-term ARS survival that would then lead to DEARE. Urine and plasma samples were collected at multiple time points out to six months from both female and male mice, and untargeted metabolomics and lipidomics analyses were conducted. At six months, Y-maze and Morris water maze neurocognitive function tests, echocardiography cardiac function tests, cardiac collagen deposition, and capillary density were assessed. To construct a prediction model, the outcomes of these tests were compared with early metabolite/lipid changes to determine if any correlations existed in a training data set ($n = 25$ mice). The model then was assessed in a separate testing data set ($n = 25$ mice) for validation.

The results of the brain and cardiac tests showed a mild difference between irradiated mice and unirradiated controls, with some differences between males and females noted as well. Cardiac collagen deposition was significantly increased in irradiated vs. unirradiated male mice ($P = 0.006$), though the difference was not large, and no significant difference was seen between irradiated and unirradiated female mice. Cardiac capillary density was decreased in both irradiated male and female mice

compared to unirradiated controls, but the decrease observed in female mice was much larger than in males. After radiation exposure, several urine metabolites were also very different in male compared to female mice. At six months in the training and testing sets, hundreds of metabolites were found to change longitudinally showing a variety of time kinetics, and many urine metabolites that were observed to change at 1-week and 1-month postirradiation correlated with cardiac collagen deposition and left ventricular ejection fraction (data not yet published). Moving forward, males and females will be analyzed separately, and long-term metabolite changes will be prioritized to expand the time window for assessing predictive biomarkers.

In summary, many urine and plasma metabolites were changed by exposure, with profiles differing dramatically between males and females, and across postirradiation time points. These metabolite profiles, identified early after irradiation, correlated with later cardiac functional changes. Ongoing studies in a PBI rat model are being conducted to identify biomarkers of DEARE. Since rats are larger than mice, more biofluids can be collected for more expansive studies. Male and female WAG/RijCmcr and Sprague-Dawley rats will be irradiated with a range of doses of X rays and neutrons. Urine and plasma samples will be collected at time points, again out to six months, with untargeted metabolomics analyses conducted. Kidney, heart and brain function tests will also be conducted, and metabolite profiles will be correlated with the functional test outcomes. Similar studies are proposed using banked plasma samples from previously irradiated (3.5–8.5 Gy γ rays) male and female NHPs. Markers discovered and validated in the rat model will be further validated in the NHP samples to identify biomarkers with cross-species utility. These comparisons will increase the likelihood that these biomarkers will be translatable to humans, which has been a consistent gap and challenge across institutions. Other considerations include onset of other pathologies that may interfere with biomarker assessment, methodological changes impacting metabolite stability, and development of algorithms for biomarker scoring, and identifying biomarkers that are specific to individual organ systems and to radiation injury.

Biomarker Qualification Considerations

Because of the importance of biomarkers in drug and device development, the U.S. FDA has developed a qualification process that provides sponsors with information intended to reduce uncertainties and accelerate regulatory decisions during drug development.[12] This process is voluntary, but once qualified, a biomarker is published and then can be used by anyone approaching the FDA. The process begins with a letter of intent, in which the sponsor proposes a context of use for a proposed biomarker in drug development. For a radiation MCM, the context of use could include a biomarker as a trigger for intervention (e.g., elevated levels of IL-6), or for the selection of a dose or regimen in humans. Another potential MCM context of use could be to support the mechanism of action of the product, as is required by the FDA Animal Rule (60). Sponsors are encouraged to submit a biomarker qualification plan that provides data for the proposed context of use. The 2018 *FDA Draft Guidance for Industry and FDA Staff on Biomarker Qualification Evidentiary Framework* provides details on the process (61). Additionally, sponsors seeking a biomarker qualification should provide information to address unmet drug development needs, and outline the benefits (i.e., added value to drug development) and risks (i.e., consequences if the biomarker is not suitable for its planned used) of its qualification. Included in the package submitted to the FDA should be the biological rationale, analytical information, and data that supports how the biomarker is relevant to the clinical endpoint. Qualified biomarkers span both nonclinical and clinical settings that originate from biological samples taken from urine, serum, plasma, and bronchoalveolar lavage, as well as biomarkers derived from radiographic imaging. Cardiac troponins T and I are examples of qualified nonclinical safety biomarkers developed for specific contexts of use in rats and dogs.[13]

In addition to biomarkers, the FDA also offers a mechanism for sponsors to qualify animal models.[14] A radiation animal model qualification implies that for an animal species, irradiated in a specific way with a specific radiation quality, the FDA accepts that it produces a condition that corresponds to what would be seen in humans. Whether or not an animal model is qualified depends on the availability of adequate data for human radiation exposures, as well as natural history data in the irradiated animal model. To date, there is not a qualified animal model for use in testing efficacy of radiation MCMs. A qualified model would greatly support the development of MCMs because fewer animals and fewer studies would be needed (S-J. Wang). There is a large animal model of H-ARS that was used for MCM efficacy testing of the four currently approved drugs for H-ARS. Those approvals were granted based on the efficacy of the growth factors in a rhesus macaque model exposed to TBI. This NHP model appears to have been well-established and understood, making it a candidate for the animal model qualification process. For other animal models of radiation exposure, the regulatory pathway is less clear. Many challenges remain in advancing products to address the different stages of radiation-induced lung injuries, including the development of sterile inflammation (pneumonitis) and late fibrosis. The

[12] www.fda.gov/drugs/cder-biomarker-qualification-program/about-biomarkers-and-qualification#what-is.

[13] www.fda.gov/media/83152/download.

[14] https://www.fda.gov/drugs/drug-development-tool-ddt-qualification-programs/biomarker-qualification-program.

lack of clarity is because the mechanisms by which radiation exerts these different outcomes is not completely understood, and several irradiation paradigms, TBI with subsequent bone marrow transplant to allow for survival from hematopoietic H-ARS (62), PBI with a percentage of the bone marrow (e.g., 2.5, 5% or higher) shielded (39), or WTLI (63, 64), have all been used to demonstrate product efficacy.

Session III: Biomarkers in Transition: From Modeling to a Clinical Setting

H-ARS Biomarkers Used in Studies According to the FDA Animal Rule

Biomarkers play key roles in development of therapeutic medical countermeasures (L. Marzella). The most important are: 1. as secondary and safety endpoints, and guides to supportive care, 2. to establish mechanism of action of investigational product, and 3. as a means of bridging effective animal dose to human dose. A wide range of biomarkers have been characterized after radiation exposure, and the U.S. FDA is more confident in accepting biomarkers closer to the clinical outcome for the purposes of marketing approval. For instance, the FDA has accepted the use of neutrophil and platelet changes to demonstrate the myelosuppressive effects of irradiation in models as well as to demonstrate improvement after treatment with an MCM. In addition, markers such as respiratory rates, and oxygen saturation, which demonstrate functional outcomes and are considered more downstream are also acceptable. However, other indirect biomarkers such as cytokines (e.g., G-CSF, IFNγ, TGFβ), genetic markers (e.g., miRNA, mtDNA), metabolites (e.g., citrulline, retinoic acid), cell injury/death markers, indices of cell mass, proliferation and organ function markers have been less accepted given the inability to demonstrate a clear connection with outcome. Markers such as respiratory rates, and oxygen saturation demonstrate functional outcomes and are considered more downstream.

As described in earlier sections, biomarker utilities can be broadly classified into biomarkers for absorbed radiation dose estimation (e.g., triage, prognosis, clinical management), acute or delayed biologic effects of radiation, prediction of risk, or assessment of treatment response. Here, biomarkers in the context of drug development tools are described, with a focus on the pharmacologic target of an investigational drug in proof-of-concept studies, dose selection studies, and efficacy outcomes. For H-ARS, it is critical to use myelosuppressive biomarkers like serial peripheral blood counts and bone marrow histology, and outcomes that reflect clinical benefit, such as systemic effects of cytopenia (e.g., blood and tissue microbial cultures, gross and microscopic evidence of hemorrhage).

Biologics License Application (BLA) 103362 for Sargramostim (GM-CSF)

On March 29, 2018, the U.S. FDA approved a new H-ARS indication for Leukine (sargramostim)[15] ''to increase survival of adult and pediatric patients acutely exposed to myelosuppressive doses of radiation'' as could occur after a radiological or nuclear incident. Sargramostim was the third of four FDA-approved medical countermeasures indicated to increase survival in patients exposed to myelosuppressive doses of radiation. Sargramostim, a yeast-derived, molecularly cloned, hematopoietic growth factor and pleiotropic cytokine, supports proliferation, differentiation, maturation, and survival of cells of several myeloid lineages. Efficacy of sargramostim (7 μg/kg/day; sc) was evaluated in irradiated NHPs exposed to TBI ($LD_{50/60}$). The primary endpoint was day 60 survival, and the study was blinded. Sargramostim significantly increased day 60 survival to 78% vs. 42% in vehicle-treated controls ($P = 0.0018$). Neutrophil, platelet and lymphocyte recovery rates were accelerated and infection rates decreased (65). As a biomarker for outcome, the kinetics of neutrophil nadir and recovery demonstrated the clinical consequence. For instance, levels of neutropenia, along with the time to troughs and nadirs, are associated with increased risk of infections. In addition, a quantitative and correlative relationship was demonstrated between the development and resolution of cytopenia after radiation and sargramostim-treated groups, respectively. Since the risk of infection depends on the depth and duration of the nadir, the blunted neutrophil nadir and earlier recovery at therapeutic doses of sargramostim, supports the mechanism of action of the MCM. In part, given the strength of these observations, the U.S. FDA approved sargramostim for H-ARS. Another attribution of clinical benefit to the MCM was in the treatment of febrile neutropenia, as observed in patients undergoing myelosuppressive doses of chemotherapy (66). There is a hierarchy of endpoints, from neutropenia to febrile neutropenia, serious infections, and ultimately lethality, which were significantly reduced by administration of the MCM, adding to the confidence in this biomarker.

Another role of radiation-modified biomarkers is in drug dose conversion to calculate effective dose from an NHP model to human use.[16] In bridging between an NHP and human effective dose, the U.S. FDA recommends that the effective dose in humans exceed the effective dose in the animal models, with the caveat that there is sufficient safety data in humans to warrant administration at these higher dose levels. In illustrating the importance of biomarkers for dose conversion, the pharmacokinetics (PK) of neutrophil changes in NHP (7 μg/kg) was used as a baseline to model

[15] https://www.fda.gov/media/112441/download#:~:text=On%20March%2029%2C%202018%2C%20the,%2C%20or%20H%2DARS).

[16] https://www.accessdata.fda.gov/drugsatfda_docs/nda/2018/103362Orig1s5240.pdf.

neutrophil counts in adults and pediatric populations of various ages, 7 μg/kg in adult and pediatric patients weighing greater than 40 kg, 10 μg/kg in pediatric patients weighing 15 kg to 40 kg, and 12 μg/kg in pediatric patients weighing less than 15 kg, to show the comparable effective dose using area under the curve (AUC) and peak serum concentration (Cmax). Based on these data, BLA reviewers stated: ''Treatment of NHPs with sargramostim starting at day 2 postirradiation to 655 or 713 cGy TBI resulted in a significant increase in survival at 60 days compared to vehicle controls. In general, the clinical signs and the hematologic and microbiologic laboratory data appeared to be more favorable in the sargramostim treated groups compared to vehicle controls; overall these secondary assessments are judged to be supportive of the treatment effect of sargramostim.''

Similarly, neutrophil biomarkers were used to extrapolate human dose from animal efficacy studies for H-ARS for Neulasta as well (BLA 125031; pegfilgrastim).[17,18,19] BLA reviewer assessments stated that 'The main driver of the predicted survival benefit of pegfilgrastim is assumed to be the whole time-course of absolute neutrophil count (ANC) profile. The pharmacodynamic endpoint of absolute neutrophil count was chosen as the target for dose selection.'' More recently, platelets were used as biomarkers for efficacy outcomes and mechanism of action studies of thrombopoietin receptor agonists (67, 68), where Nplate improved platelet counts preventing severe radiation-induced thrombocytopenia, and the observed outcomes (platelet increase and survival) were correlated with Nplate administration.

Based on these observations and to address gaps for MCMs against GI-ARS and DEARE, it is advised that investigators focus on clinically relevant radiation exposures; ''natural history'' animal model studies that characterize pathophysiology, time-course, manifestations of pathophysiology relevant to clinical condition; and select survival or other quantifiable major morbidity endpoints for efficacy outcomes. Potential biomarkers for GI-ARS can be intestinal function measures, such as nutrient absorption and mucosal barrier functions, or enterocyte mass measures (e.g., villus atrophy, crypt apoptosis, enterocyte precursor proliferation or metabolites like citrulline) (69, 70). For cutaneous radiation injury (CRI), measures of area and depth of injury (e.g., ulceration, repair, regeneration) by planigraphy and histology are some of the biomarkers that can be used for potential primary efficacy outcome (71), while for pulmonary function testing, radiological and histological assessment of pneumonitis and fibrosis would be important for lung-DEARE (72). In summary, there are several established roles of biomarkers in animal efficacy studies for radiation MCMs, such as proof of concept, dose ranging, secondary efficacy pharmacology, and mechanism of action extrapolation of effective animal dose to humans.

Advancing Radiation Biodosimetry Biomarkers beyond the Bench

BARDA, which is part of the office of the Assistant Secretary for Preparedness and Response (ASPR) has at a part of its mission to save lives from 21st century health threats. BARDA develops and facilitates MCM availability for the public by forming unique public-private partnership with industry partners (L. Wathen). Biomarkers in biodosimetry can be a single or a panel of biomarkers that has the sensitivity, specificity, and accuracy for its intended use. Once an early verification phase is characterized, a validation plan is submitted to the U.S. FDA seeking input for the course of action to demonstrate validity and generate data for the pre-EUA and/or 510K filings. A validation phase may require 25 to 30 different studies. Once FDA authorization to market is granted, laboratories need to be established that can run the test, and Good Manufacturing Process (GMP) kit manufacturing and stockpiling activities need to begin in earnest, with the intent that these tests become accessible rapidly in response to a mass casualty incident. If applicable, use of these tests is appropriate in small accidents, to further establish clinical biomarker utility and validity. The pre-EUA submission is another important element in the regulatory pathway to advance biodosimetry biomarkers. Once the biomarker validation package is established, it is highly valuable for test developers to submit portions of the data to the FDA for review, so that in the event of a radiological or nuclear emergency, the USG triage and treatment response will be more streamlined.

There are several confounding factors that can impact translation of biomarkers from a research setting to clinical use. These can encompass person-to-person variability, such as individualized radiosensitivity, special populations, preexisting conditions, and medications; complexity and logistics of the test and turnaround time; interpretation of the signals, accuracy of the test, and lack of availability of the appropriate clinical population to tether research data. There are other conditions that assay developers must demonstrate do not cause biomarkers to change significantly, such as immune status (e.g., infection, autoimmune disorder, etc.), other injuries (e.g., burn, trauma, wound, etc.), health status (e.g., diabetes, elevated cholesterol, etc.) or pregnancy. Assay developers must also provide evidence that the special population reference range for a biomarker is within the normal population range (Table 2). Furthermore, developers must show that common medications and endogenous substances do not interfere with the chemistry or biology of the biomarker test (Table 2). For example, a particular biomarker that was proposed for triage use

[17] https://www.accessdata.fda.gov/drugsatfda_docs/label/2015/125031s180lbl.pdf.

[18] https://www.accessdata.fda.gov/drugsatfda_docs/appletter/2015/125031Orig1s180ltr.pdf.

[19] https://www.accessdata.fda.gov/drugsatfda_docs/nda/2015/125031Orig1s180.pdf.

TABLE 2

Special populations to be considered for biodosimetry biomarker development

Burn	Influenza	
Trauma	Immunocompromised	
Healthy geriatrics (>60 years)	Autoimmune disorders	
Healthy adolescent (13–21 years)	Diabetes (Type 1 and Type 2)	
Healthy pediatric (2–12 years)	Pregnancy	
Therapeutics in common use that could possibly alter biomarker outcomes		
Pioglitazone	Biotin	Heparin
Metformin	Albumin	Human IgG
Loperamide	Conjugated bilirubin	Intralipid® 20%
Bismuth subsalicylate	Unconjugated bilirubin	Amoxicillin + clavulanic acid
Acetaminophen	Naproxen	Ciprofloxacin
Acetylsalicylic acid	Hemoglobin	Clindamycin
Ibuprofen	Ondansetron	Ganciclovir
NOXAFIL® Posaconazole	Granisetron	Caspofungin

resulted in a false positive due to administration of G-CSF, and administration of a radioprotectant also confounded the results (L. Wathen). In the event of a radiological incident, it is likely that populations that received G-CSF or protectants may be excluded from receiving that test.

Theoretically, human therapeutic radiation biomarkers can be validated in human samples, if the radiation treatment regimen received was a single radiation dose; however, patients undergoing radiation therapy are not often administered a single dose. Instead, NHPs that receive a single radiation dose are used as laboratory models to mimic the human condition. Given the limited source of human samples, NHP samples from single and fractionated dose irradiation were compared followed by NHP and human fractionated dose comparisons. The time-course and fold-change correlation between the two species was examined. To do this, BARDA-funded the Arizona State University biodosimetry platform which used 14 biomarkers to demonstrate that the pattern of NHP to human fractionated irradiation response was very similar at radiation fractions and time-course, post-exposure ($r = 0.99$), although both human and NHP biomarkers could not distinguish between fractionated 3.6 and 7.2 Gy. Similarly, in a bridging study comparing three model systems, the BARDA contractor DxTerity demonstrated comparable patterns between human TBI fractionated dose and NHP TBI fractionated dose, and the test could distinguish between radiation doses (3.6, 7.2 and 10.8 Gy). However, caution must be exercised since baseline values in some TBI patients are confounded due to years of chemotherapy or a recent transplant. Furthermore, the NHP fractionated dose pattern is much lower than NHP single dose biomarker expression. Sometimes, the time course of biomarker expression is different in NHP and humans. For example, AMY1, a protein biomarker, increased in both NHPs and humans on day 1 postirradiation, but approached baseline in NHPs, while the signal was sustained in humans on days 2 and 3 postirradiation. Another biomarker, Flt3 ligand, increased on days 1–3 postirradiation in humans, but the time-course was much slower in NHPs, with elevation seen only on day 2 postirradiation. Another biomarker, AACT was expressed significantly in irradiated NHPs, but did not show a radiation-specific response in humans and was therefore cut from the panel.

Finally, in addressing the challenges of incorporating new biomarkers into a nuclear incident response, these areas must be considered:

- Establishment of a network of labs or instruments prepared to respond immediately with adequate training and proficiency demonstrated on regular basis.
- Supply chain certainty of kits, venipuncture items, and shipment of samples that efficiently maximize biomarker test utility.
- First responder and emergency medical system knowledge and training of the biomarker test.
- The biomarker test should be easy to interpret, and the results must be considered ''trustworthy.''

There are also gaps in terms of including radiation biomarkers in clinical practice, since the regulatory pathway for therapeutic radiation biomarkers is distinctly different from that of a single dose diagnostic. While most therapeutic radiation is fractionated to spare normal tissue, the acute, high dose exposure resulting from a radiation incident has a strong impact on normal tissues. Therefore, strong datasets correlating biomarkers to clinical outcomes would be needed to gain biomarker acceptance. Both laboratory technicians and clinicians will need to be educated about radiation biomarkers, so they can be best informed on how to apply the results. Scientifically strong literature, easily digestible training materials, and targeted workshops will facilitate acceptance of the use of radiation biomarkers.

Extracellular Vesicles as Biomarkers of Radiation Exposure: The Clinical Experience

Biomarker utility is not limited to the Animal Rule and radiation biodosimetry; it has clinical relevance with adverse effects in patients undergoing radiotherapy. Extracellular vesicles (EV), which are small, biological containers that can store or transport materials, and are enclosed by a lipid bilayer, are one such biomarker (R. Tamarat). EVs are classified based on origin, size, composition, and markers (73). Exosomes are intraluminal vesicles extruded into the cell/circulation, of 30–100 nm size, while microvesicles (MV) range from 0.1–1 μm and are released from the cell membrane, and apoptotic bodies are small cell fragments (>1 μm) released during apoptosis. These vesicles can contain mRNA, miRNA, proteins, DNA, and nuclear fragments that is dependent on the cell of origin and

the stimulus that causes release of EVs. Further, EVs are considered vectors of biological information and intercellular communication, as they can travel far from their site of origin to deliver their content.

Emerging evidence substantiates involvement of these EVs in different diseases and as emergent biomarkers in thrombotic states (74), endothelial dysfunction (75), and cardiovascular disease (76). Since MVs express a selection of molecules from the parental cells on their membrane, they play a pivotal role in key biological processes (77). The utility of circulating MVs as predictor biomarkers of severe complications of radiotherapy has been described in the retrospective analysis of the Epinal, France radiotherapy accident (78). Between 1987 and 2006; 5,000 patients undergoing radiotherapy for prostate adenocarcinoma at the Public General Hospital were overexposed to radiation (8–20%) due to errors in treatment processes, where patients who received higher doses presented with great anatomic and physiologic dilapidations, and also has the highest severity grade on the Common Terminology Criteria for Adverse Events (CTCAE) scale.[20] Patients were divided into 3 cohorts based on the chronology of exposures, and blood was collected from patients with 1–4 grading on the CTCAE scale. EVs were isolated from the blood, enumerated, and subjected to functional and proteomic analyses of the vesicle content. Both exosomes and MVs were increased, and increases in MVs, but not exosomes, had a positive correlation to severity of grade of injury. Using cell surface markers, it was shown that levels of platelet-derived MVs were significantly higher than MVs derived from other organs/cells, and there was a reduction in endothelial and monocyte cell-derived MVs. After performing logistical regression analyses on the ratio of platelet-derived MVs to endothelial cell and monocyte-derived MVs, risk prediction could be attributed to an increase in ratio, correlating to increased risk of higher toxicity. Again, this trend was observed in the MVs and not the exosomes. Next, functional analysis of the content of the MVs and exosomes was conducted by unsupervised hierarchical clustering, and proteomic signatures could distinguish between grade 2 and higher severity, which allows for identification of patients with higher severity grade, especially in radiation-induced late effects. Of importance is the relationship between the radiation dose, volume of irradiated tissue and the EV. There was a positive correlation between the platelet-derived MVs and the radiation dose to the bladder and rectum, while monocyte-derived MVs correlated to irradiation of the anterior prostate. Here, it is emphasized that the volume of the organ exposed has a direct correlation to quantity of the MVs secreted. If a small volume of an organ is irradiated with a high dose, the volume of MVs secreted is lower; if a larger volume of an organ is irradiated with lower radiation dose, the quantity of secreted MVs is higher. The main limitation to this study is the fact that it was conducted retrospectively (4–5 years postirradiation). Future prospective studies are planned for early prediction and detection of cardiotoxicity (79), and neurotoxicity (80) in patients undergoing radiotherapy. A development strategy also includes participation in a multicenter, multiethnic study with different partners from Europe, Japan, and elsewhere, to allow validation of these EVs in patients.[21] Parallel studies in laboratory animals are also planned.

[20] https://ctep.cancer.gov/protocoldevelopment/electronic_applications/docs/ctcaev3.pdf.

[21] https://cordis.europa.eu/project/id/755523.

DISCUSSION

The workshop discussion was based on questions developed by the meeting planners to highlight key points under the topic areas presented during the scientific sessions, with the purpose of obtaining input from subject matter experts, panelists, and the audience. Discussions from each session are summarized here.

Radiation Biodosimetry Biomarkers

Because biodosimetry tests or radiation signatures vary due to homogeneity of the exposure, and since mass casualty exposures in humans will never be homogeneous, investigators described strategies to extrapolate their findings in TBI animal models to inhomogeneous radiation exposures, as would be experienced by cancer patients receiving pelvic irradiation. While PBI animal model studies (mice and NHPs) are useful, validation of the biodosimetry signature in TBI cancer patients, or hemi-body irradiated patients represent a valuable resource, and would be of considerable interest to the radiation biodosimetry community. In fact, a French PBI study in baboons found that dose reconstruction using this model was extremely difficult, implying that PBI samples may not be suited for this task (25). Although the biodosimetry markers measured over different time windows could accurately assess the H-ARS severity levels, they could not be used to distinguish between TBI and PBI. Therefore, to inform patient treatment, a multiparametric strategy is needed for filling this critical gap.

Another point raised was centered on different radiation sensitivities of cell types. For example, it was demonstrated that peripheral blood cells, or stem and progenitor cells in either human or murine models, had different radiation sensitivities (81). Also, after a nuclear detonation, energy deposition will vary based on distance from the source, and the individual may also be exposed to blast trauma in addition to radiant energy. An individual's response is further complicated by inter-individual variations such as size. For example, in a person with a large body habitus, the depth penetration of dose may be higher at the surface, with the marrow and deep tissues may not receive a high dose. In

fact, an individual would not receive a homogenous dose, with the skin receiving the highest dose and the bone marrow and GI system receiving almost none. Orientation to the radiation source could also affect the radiation dose received. Ultimately, the models used in research and development may not really reflect what happens in reality.

One way to approach this dilemma is to look at outcomes, irrespective of what the exposures might be. For instance, in the clinical setting, oncology patients can range from those receiving only chemotherapy, to a combination of chemotherapy and irradiation, to only radiation therapy. Using circulating miRNA, the focus can be on the health outcomes presenting early, latent, or delayed, ranging from one week to one month to 2 years postirradiation or chemotherapy. Different samples can provide a wealth of information and, if the samples are well maintained and phenotyped using TBI, PBI or WTLI, and even using different species, researchers can use multi-parameter data to examine underlying effects and outcome.

Another support for the PBI model in radiation biodosimetry is the availability of clinical hemi-body irradiation samples. Most clinical protocols use PBI, as even very ill patients can better tolerate this approach. Furthermore, regulatory considerations require information of how the biodosimetry test or signature performs in TBI as well as PBI models (i.e., bone marrow sparing models). While most studies in the radiation biodosimetry field focus on acute exposures, there is need to fill in the gaps for biomarkers of more complex irradiation conditions, such as protracted irradiation, which are relevant to a fallout scenario, or using different quality of radiation exposures (i.e., mixed fields with some percentage of neutrons) (82).

It is also important to understand the biokinetics of a select signature/biomarker in relation to time postirradiation Most of the data presented at the workshop focused on 24 h post-TBI, or 1–7 days postirradiation, and certainly biomarkers will change in relation to time. Gene expression is especially dynamic after irradiation (83) with some changes persisting 30 days postirradiation.

There is no one perfect biomarker; therefore, for successful translation of in vitro diagnostic devices, the biomarker's intended use (e.g., triage, definitive dose or predictive biodosimetry), analytical and clinical validity, accuracy and reliability must be demonstrated.

Radiation MCM Biomarkers

Rodent models are helpful in the identification of radiation biomarkers, be it for animal model qualification, or to estimate the efficacy of an MCM. Rodents are more homogeneous and give results with tight standard deviation/coefficient of variations (SD/CV), but when the same is estimated in humans or NHPs, the SD/CV deviates significantly. The SD/CV of the biomarker is affected by many variables, including the homogenous nature of an inbred strain, the nature of the marker, fasting and feeding, or dietary conditions. It is important to consider these variables during the discovery phase of biomarkers, so that the biomarkers that are not down selected have the highest potential to inform when they are translated to other species. Traditionally, following the discovery phase of biomarkers in rodents or lower mammals, advancement of a biomarker requires a similar trend in both NHPs and humans in response to an insult or disease. For instance, the mechanism of citrulline is well understood in humans and appears to be consistent across species (53). However, it is important to consider if the regulatory pathway will differ if biomarker panels are discovered by algorithm, rather than association to those with known mechanisms and utility.

Given that four MCMs have been approved by the FDA for mitigation of radiation-induced hematopoietic injury, details of the use of biomarkers in those licensures has been described in earlier sections. However, the role of biomarkers in other radiation sub-syndromes and delayed effects of acute radiation exposure (DEARE) is yet unclear. For radiation-induced, non-heme syndromes, the role of biomarkers is not as well-described, since non-heme mitigators have yet to be FDA approved. However, researchers continue to gather data to establish robust biomarkers. For instance, breathing rates to assess radiation-induced lung injury are being explored. There is a radiation dose-dependent relationship with increases in breathing rate in irradiated rats, which can be correlated to pneumonitis (58). Specifically, breathing rate can be used as a biomarker to indicate the efficacy of the administered MCM; however, if the end-use is to predict pneumonitis, measuring breathing rate is not necessarily as useful, due to the latency and no change in breathing rates before the onset of pneumonitis. There is other organ specific subsyndromes, with specific pathophysiology, latency, and biomarkers that need to be addressed in the continuum of radiation injury.

Clinical Applications

Successful translation of a biodosimetry signature or MCM to clinical use requires an understanding of the regulatory processes that are available for approval/clearance. MCM advanced development is well-described in the Animal Rule Guidance; however, for non-traditional biodosimetry approaches, FDA guidance does not describe a concrete path forward, and there is little commercial or federal incentive to specifically develop companion biomarkers as a standalone IVD. To resolve this, it is helpful to consider that a diagnostic classifies as a device. A device is something that can be used for diagnosis and for the management of patients, so the product can be legally marketed. Therefore, researchers need to move from the biomarker space to a development area in which a diagnostic device can be marketed, as outlined in the

FDA guidance for companion diagnostics.[22] There are therapeutic products in cancer that require demonstration that the specific target the agent is addressing is present. The regulatory standard is that the IVD device has to be essential for the safe and effective use of the therapeutic. That requirement is key and implies that the therapeutic be marketed with the diagnostic.[23] Using that same paradigm for therapeutics for radiation-induced syndromes is challenging. Possible linkages can be made by focusing on outcomes from radiation injury and building a biomarker panel to predict those outcomes. Therefore, even in the absence of specific, predictive outcome-based biodosimeters, communicating with the regulatory agency can help form a development strategy.

Lessons learned in MCM development are not restricted to U.S. researchers alone. For instance, France has a new rule that requires hospital-associated laboratories to work in conjunction with expert/research-level facilities in response to mass casualty events, and to work together in performing these radiation exposure analyses. This approach allows for sharing of expertise and improves robustness in approaches and analyses. Further, individual laboratories are obtaining certification for evaluations and preparation of EVs, with the specific goal of implementation of these practices in the clinics. The approach has been successful for the cardiovascular field, and French scientists are implementing similar strategies for radiological and nuclear incidents.

CONCLUSIONS

Many of the biomarker approaches mentioned here have varying strengths and weaknesses, especially concerning the timing of when assessments are made after radiation exposure. There is also consensus that radiation type, and dose rate could cause significant and unpredictable changes in the readouts for most biomarkers, and there is a belief that TBI is unlikely to be a realistic model for the kinds of exposures that would occur because of detonation of an improvised nuclear device or other mass casualty incident. Therefore, much more work still needs to be done with PBI models to understand whether the data currently established with TBI is representative of exposure responses in PBI. This is an important concern in the current U.S. concept of operations, since there is no FDA-cleared, rapid and accurate POC method for assessing PBI exposures. Current biomarker methods do not appear to be capable of accurate identification of PBI exposures, making PBI dose reconstruction problematic. A recurring theme is the variability in responses among the laboratory animals used in these studies, and the challenges of finding markers that can reliably bridge responses between species. These issues must be addressed to translate these model findings to what might be expected in humans. There was a robust discussion of the regulatory requirements that may need to be met to have a radiation exposure biomarker qualified by the FDA and how the radiation field, with respect to mass casualty incidents, presents a high bar and many challenges to qualification. The work on MVs and EVs may be closer to translation under current French regulations than it would be under U.S. requirements. It is, therefore, important for funding agencies, regulatory partners, and researchers (national and international) to support and communicate freely and frequently on the use of biomarkers for biodosimetry and MCM development, to establish long term solutions to resolving radiation injury.

[22] https://www.fda.gov/medical-devices/in-vitro-diagnostics/companion-diagnostics.

[23] https://www.fda.gov/regulatory-information/search-fda-guidance-documents/developing-and-labeling-vitro-companion-diagnostic-devices-specific-group-oncology-therapeutic.

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