MALDI and LCMS Identifications of Early Protein and LA-ICP-MS Identification of Early Metal Biomarkers of Ionizing Radiation

P. Pevsner[1], G. Li[2], N. Sobol[1], D.C Miller[1], D. Robertson[2,3]

[1]Department of Pathology and Anatomical Sciences
University of Missouri School of Medicine
[2] University of Missouri Department of Chemistry
[3]University of Missouri Research Reactor (MURR)
Columbia, MO, USA

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Abstract:
The goal of this project was identification of early protein biomarkers of total body ionizing radiation (TBI). Ionizing radiation (IR) experiments demonstrated radiation dose-related changes in exposed murine buccal mucosa and tongue tissue. Matrix assisted laser desorption ionization mass spectrometry (MALDI) imaging (IMS) co-registered with contiguous histopathologic sections demonstrated free tissue albumin and other proteins after 2 Gy TBI but not in control non-irradiated tissues. These findings were confirmed by MALDI and liquid chromatography mass spectrometry (LCMS) of protein extracts from irradiated tissue. Laser ablation ion coupled plasma mass spectrometry (LA-ICP-MS) measurements of Fe, Cu, and Zn demonstrated early changes as well, especially in the elevated levels of Zn in the first four days after 2 Gy, TBI, the threshold dose for radiation sickness. These results are proof of principle that there are protein, peptide, and elemental biomarkers of acute IR, and they could be used to identify victims of radiation exposure, specifically, the 2 Gy threshold dose for radiation sickness in a civilian or terrorist nuclear mass casualty.

Background and Significance:
A self administered lateral flow device to detect any proteins induced by radiation exposure at or beyond the 2Gy threshold could be used for rapid triage of victims. Anyone identified as exposed to less than 2 Gy would not require acute care and could return home. In previous experiments, Swiss mice were treated with 3 Gy to the head and neck. MALDI analysis of proteins and peptides extracted from the buccal mucosa demonstrated new proteins and peptides at 15 and 30 minutes post-radiation compared to the control samples. MALDI in reflectron mode identified peptide changes at 15 minutes post 3 Gy. Newly present peptides or proteins not seen in non-irradiated controls included msgprkaped, corresponding to gi|45593111| transaldolase [Mus musculus], an enzyme of the non-oxidative phase of the pentose phosphate pathway; and akdyygtgyf, gi|1333908|unnamed protein product [Mus musculus]. At 30 minutes post 3 Gy, an additional peptide, mrhvqaels, gi|168984609| novel protein (B230312A22Rik) [Mus musculus], became detectable The corresponding pro¬teins were identified from the NCBInr protein data base. These initial experiments in murine tongue demonstrated new protein and peptide biomarkers post 3 Gy local head and neck IR compared to normal controls. The tongue was chosen because it is a radiosensitive epithelial gastrointestinal tissue. The findings in this preliminary study were proof of principle that IR dose-related protein biomarkers could be identified in tissue with mass spectrometry. This report extends that study. The subject of this report is the determination of protein changes following acute TBI that could be the basis for a diagnostic lateral flow device (tongue test strip).

Materials and Methods:
The initial experimental protocol was approved by the NYU Investigational Animal Use and Care Committee (IACUC), and a subsequent protocol by the University of Missouri IACUC. Forty Swiss Webster (CFW®) Mice (10 controls and 30 experimental) were used. The animals received total body ionizing radiation (TBI) to total doses of 1 Gy (10 mice), 2 Gy (10 mice) and 3 Gy (10 mice), in groups of five. Under terminal anesthesia, the tongue and heart were removed following perfusion and placed in 3.7% paraformaldehyde for one hour (10:1 tissue: solution, V/V) at 4°C. The tissue was then immersed in 30% sucrose (10:1 tissue: solution, V/V) at 4°C until equilibration. The tissue was rapidly frozen by immersion into liquid nitrogen-cooled isopentane to minimize any freezing artifact in the tissue 4 . One micrometer (µm) serial sections were obtained for histology, MALDI imaging (IMS), and liquid chromatography mass spectrometry (LCMS), respectively. The sections for histology were with hematoxylin and eosin (H&E) (Sigma Aldrich, St. Louis). Low and high power (oil immersion) light microscopic images were obtained for correlation with the MALDI images. The sections for MALDI were applied to conductive stainless steel plates (76 x 26 x 1.1mm). The sections for LCMS were immersed in 100mM ammonium bicarbonate in pressure cycling pulse tubes, and proteins extracted with 35,000 psi alternating pressure in a Barocycler (Pressure BioSciences, South Easton, MA). 4,7 The extracted samples were trypsinized. The trypsin digest was analyzed with a Hitachi NanoFrontier LCMS, (Hitachi High Technologies America, Dallas, TX). IMS and LCMS studies of control, 1, 2 and 3 Gy post TBI tongue tissue, and control and 2 Gy post TBI heart tissue were obtained. MALDI images were obtained on a Shimadzu Axima, TOF2 mass spectrometer (Shimadzu, Kyoto, Japan) operated in linear mode. Each image is composed of spectra obtained in a rectangular serpentine raster of each tissue section, at 70µm spot intervals, with a cumulative average of 50 profiles (spectra) per spot. The calibrated mass accuracy in linear mode was ± 0.3 Da, or 50 ppm.
The LA-ICP-MS measurements were performed with a 5 micron laser spot size on contiguous tissue sections. Measurements were repeated in sets of four on all tongue tissue at the spicule cellular and muscle zones (Figure 8). Additional measurements were obtaine in multiple zones in murine brain, heart, liver, kidney, and colon.

Results:
IMS of normal tongue (Figure 1) and post 1 Gy TBI (Figure 2) demonstrated corresponding progressive increases in tissue albumin with increasing IR dose (red in images). At 2 Gy (Figure 3), there was an increase in peripheral albumin in the IMS. At 3 Gy (Figure 4), the IMS showed that the albumin was now virtually absent in the periphery due to the extensive peripheral epithelial destruction. LCMS studies were performed on protein extracts of control, post 1, 2 and 3 Gy IR tongue tissue, and control and post 2 Gy IR heart tissue. Protein identifications were made with MASCOT software query of MSDB and Swissprot databases. The proteins from the control, 1, 2, and 3 Gy tongue tissue extracts identified by LCMS are summarized in Table 1. Albumin was demonstrated by LCMS in the mouse tongue 1 hour post 1 Gy, 2 Gy and 3 Gy. LCMS confirmed the finding of Hemoglobin Subunit α in the IMS post 1 Gy, Figure 5; Fatty Acid-Binding Protein Adipocyte in the IMS post 2 Gy, Figure 6. ICP-MS measurement of elemental iron in the spicule layer, cellular layer, and muscular layer of the tongue demonstrated progressive loss of Fe in the most radiosensitive zone, the actively dividing cellular layer, as the radiation dose increases from 2Gy total body radiation (TBI) threshold dose for radiation sickness, to 7 Gy, the LD 50/30 (Figure 9). LA-ICP-MS measurement of elemental Cu and Zn in the spicule layer, cellular layer, and muscular layer of the tongue. There was an initial ,dramatic rise in Zn in the most radiosensitive zone, the actively dividing cellular layer, from day 1 through day four post 2 Gy, the (TBI) threshold dose for radiation sickness (Figure 10). Acute changes were also identified in murine brain, heart, liver, kidney, and colon.

Discussion:
With this project we hoped to confirm and expand upon our pilot data concerning early protein biomarkers following TBI that would distinguish dose levels. These biomarkers could then be used as potential biomarkers of IR dose, specifically, for the determination of 2 Gy threshold for radiation sickness. These biomarkers could then be used to triage victims that received greater than the 2 Gy threshold for radiation sickness who would need treatment from those that would not need treatment.

Initial post IR MALDI and LCMS experiments in mice demonstrated radiation dose-related albumin and induced-proteins in radiosensitive buccal mucosa and tongue tissue 8. In this study, the initial experiments were expanded to include evaluation of induced protein changes in the tongue and heart of mice within 1 hour of exposure to 1-3 Gy TBI. This was well correlated with serial IMS images that demonstrated progressive increases in albumin and other proteins. Post 1 Gy TBI, minimal peripheral albumin was identified on IMS and LCMS. However, post 2 Gy IR, there was a dramatic increase in albumin identified in the peripheral epithelium of the tongue on IMS. This was confirmed by LCMS. The progressive detection of albumin in the IMS images post 2 and 3 Gy is probably a reflection of progressive vascular damage and increasing extravasation of plasma into the extravascular space; histologic analysis is in progress. Plasma leakage and extravasation of albumin into the extravascular space is a well known physiologic phenomenon following vascular injury and has been demonstrated on IMS in stroke 9.
There was other evidence of a dose-response to radiation in the tongue: Post 1 Gy, Hemoglobin Subunit α was identified in IMS and confirmed by LCMS. Post 2 Gy, Fatty Acid-Binding Protein Adipocyte was identified in IMS and confirmed by LCMS. Post 3 Gy Hemoglobin α Chains was identified in IMS and confirmed by LCMS.
The methods of IMS and LCMS, and pressure cycling technology (PCT) applied in our previous report were used successfully in this study. PCT was an important element in high yield tissue extraction of protein, 4. LA-ICP-MS added a new dimension by allowing us to measure early elemental changes.

Conclusion:
Induced early IR dose-related proteins and increasing extravascular space albumin were identified by IMS and confirmed by LCMS. These findings confirm our initial observations that early protein biomarkers of acute TBI can be identified by IMS, and LCMS. Furthermore, these biomarkers are dose-related proteins. IMS demonstrated concordant dose-related changes in protein biomarkers. The extravascular extravasation of plasma and albumin increased with increasing IR dose. At 1 Gy, there was minimal albumin at the periphery on IMS. At 2 Gy, there was high IMS signal of albumin at the periphery. At 3 Gy, the peripheral albumin was no longer present on IMS, but there was a central increase in IMS albumin at 3 Gy. At 3 Gy, the threshold radiation dose exceeded the higher intrinsic radioresistance of the central muscle tissue of the tongue. The proteins identified on IMS and confirmed by LCMS including post IR tissue albumin and induced proteins such as Hemoglobin Subunit α at 1 Gy; Fatty Acid-Binding Protein Adipocyte at 2 Gy; and Hemoglobin α Chains at 3 Gy; are all IR dose-related biomarkers. IMS and LMCS demonstrated both the presence and loci of these IR-induced protein biomarkers. The initial ,dose related, changes in Fe, Cu, and Zinc in the tongue in the most radiosensitive zone, the actively dividing cellular layer, from day 1 through day four post 2 Gy, the (TBI) threshold dose for radiation sickness, and the high correlation between the radiation dose and induced proteins and peptides suggests that these are dose related biomarkers. Similar elemental, protein and peptide changes were observed in brain, heart, liver, kidney, and colon.
These elemental, protein and peptide biomarkers could become the basis for a diagnostic lateral flow device (LFD or test strip) for triage of nuclear casualty victims. Preliminary work on an LFD for albumin and other induced proteins is in progress and the subject of another report.

References
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Table I

Normal Mouse Tongue	Mouse Tongue 1hr post 1 GY	Mouse Tongue 1hr post 2GY	Mouse Tongue 1hr post 3GY
		Q3TV03 - Mus: albumin 1 - full insert sequence.	**Q3TV03** - Mus: albumin 1 - full insert sequence.
Q9CXH5 – Mus :hemoglobin, beta adult major chain	**Q9CXH5** – Mus :hemoglobin, beta adult major chain		
PO1942 – Mus: hemoglobin subunit alpha Hemoglobin alpha chain Hba-a1 - HAMS	**PO1942** – Mus: hemoglobin subunit alpha Hemoglobin alpha chain Hba-a1 - HAMS	**PO1942** – Mus: hemoglobin subunit alpha Hemoglobin alpha chain Hba-a1 - HAMS	**PO1942** – Mus: hemoglobin subunit alpha Hemoglobin alpha chain Hba-a1 - HAMS
Q9JJ20 – Mus : 14-3-3 protein sigma; Sfn MGI 1891831 Synonyms: Mme1 EMBL AAF36093.1			
Q64475 Mus: histone H2B type 1-B (h2B-143) H2B1B			
Q9Z1R9 Mus: - trypsinogen 16	**Q9Z1R9** - Mus: - trypsinogen 16		
BAC33789 –Mus: Sgol1 shugoshin-like 1 [(S. pombe) (AK049517.1)]			
		Q9CY54 - Mus: hemoglobin, beta adult major chain Symbol: Hbb-b1	
		Q9CY06 - MUS:hemoglobin, beta adult major chain	
		P02088 – Mus: HBMS Hemoglobin subunit beta	
		Q3UJR8 –Mus: glutamate oxaloacetate transaminase 1 EC=2.6.1.1	
		P11404 Mus: fatty acid-binding protein, heart FABPH	
		P04117 – Mus: FABPA Fatty acid-binding protein, adipocyte (AFABP) also (Adipocyte lipid-binding protein) (ALBP)	
		S02654; **S00195** Mus: malate dehydrogenase DEMSMC - (EC 1.1.1.37), cytosolic – mouse, L-lactate dehydrogenase	
		Q3UBW7 – Mus: transferrin	
			Q9CY12 – Mus: hemoglobin, beta adult major chain
			Q3USS4 – Mus: glial fibrillary acidic protein
			Q3TWV0 – Mus: vimentin
			JC4030 – Mus: DnaJ-like protein MTJ1
			Q9QZ05 (E2AK4) Mus: eukaryotic translation initiation factor 2-alpha kinase 4 EC=2.7.11.1

Table 1. Summary of Proteins identified. Note that Albumin is only found post 2 and 3 Gy TBI.

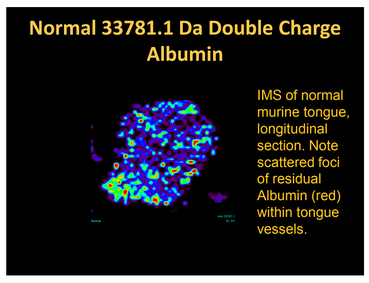

Figure 1. IMS of normal tongue. Minimal residual intra-vascular albumin (Red)

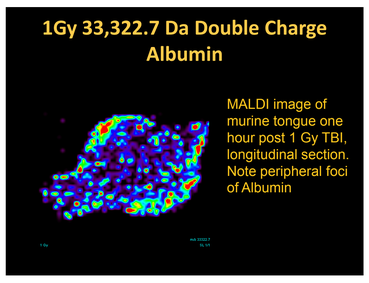

Figure 2. IMS of tongue post 1 Gy TBI demonstrated corresponding progressive increases in tissue albumin with increasing IR dose (red in image).

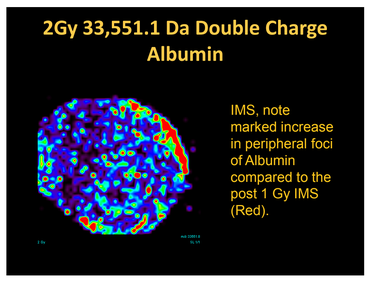

Figure 3. IMS of tongue post 2 Gy. There is an increase in peripheral albumin (red in the image).

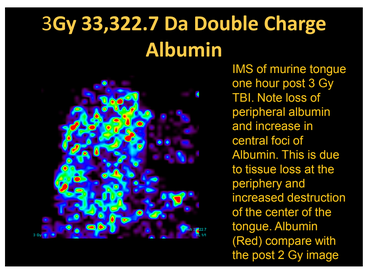

Figure 4. IMS post 3Gy. Albumin is virtually absent in the periphery due to the extensive peripheral epithelial destruction.

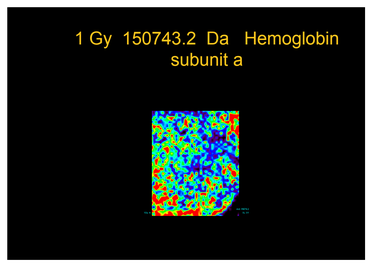

Figure 5. IMS of tongue post 1 Gy. Hemoglobin Subunit α (red in image).

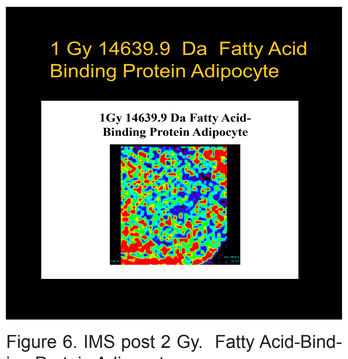

Figure 6. IMS post 2 Gy. Fatty Acid-Binding Protein Adipocyte (red in image).

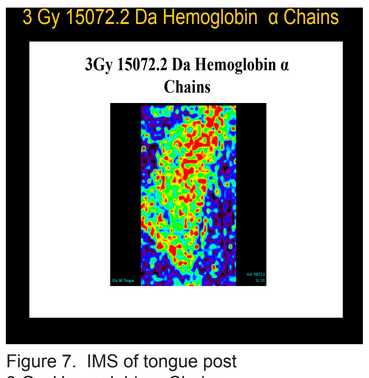

Figure 7. IMS of tongue post 3 Gy. Hemoglobin α Chains.

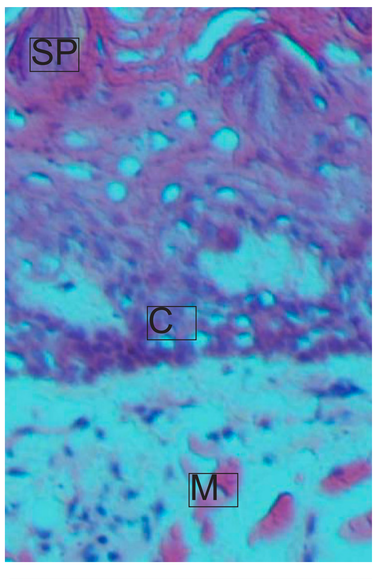

Figure 8. Histologic section of normal tongue. The upper surface of the tongue-spicules, sp. The cellular epithelium base cellular layer, C. The deeper muscular layer, M.

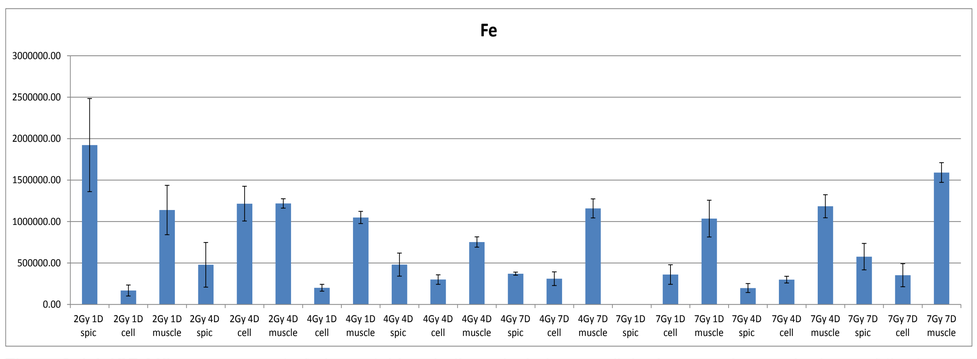

Figure 9. LA-ICP-MS measurement of elemental iron in the spicule layer, cellular layer, and muscular layer of the tongue. Note the progressive loss of Fe in the most radiosensitive zone, the actively dividing cellular layer, as the radiation dose increases from 2Gy total body radiation (TBI) threshold dose for radiation sickness, to 7 Gy, the LD 50/30.

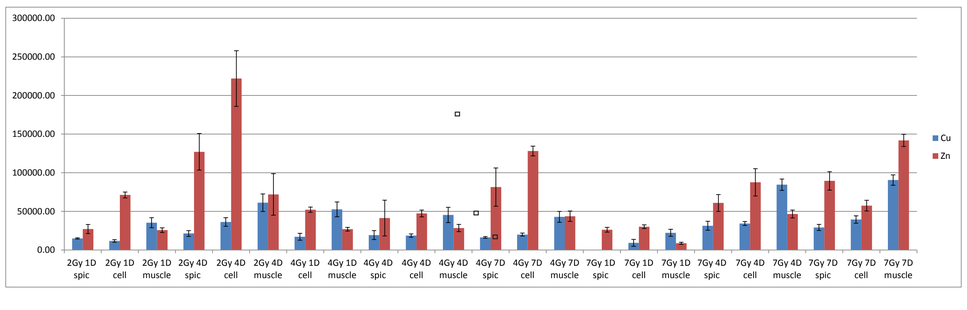

Figure 10. LA-ICP-MS measurement of elemental Cu and Zn in the spicule layer, cellular layer, and muscular layer of the tongue. Note the initial ,dramatic rise in Zn in the most radiosensitive zone, the actively dividing cellular layer, from day 1 through day four post 2 Gy, the (TBI) threshold dose for radiation sickness. **A simple self administered lateral flow device (tongue test strip) for elemental Zn may be able to identify and triage victims of 2 Gy TBI exposure.**