Novel Non-Invasive Biomarkers For Irradiation, Chemical or Infectious Agent Induced Gastro-Intestinal Injury And Subsequent Recovery

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Overview

- Several studies pertaining to gastro-intestinal (GI) damage have been conducted in different species including non-human primates (NHPs) and mice, e.g., radiation-induced GI inflammation/injury, but so far no useful validated biomarkers are available for non-invasive detection of this condition. Hence, our aim was to establish for the first time new fecal biomarkers of GI inflammation/injury/recovery in NHPs and mouse animal models.
- **Calprotectin** is a calcium binding protein derived mainly from the cytosolic fraction of neutrophils and its elevated fecal levels are associated with increased neutrophil activity in GI.
- **Lactoferrin**, an iron-binding glycoprotein, is secreted by most mucosal membranes and is a major component of the secondary granules of neutrophils. During GI inflammation, granulocytes transmigrate through the intestinal wall and render these proteins detectable in the feces.
- In addition, several new markers were established including **intestinal fatty acid binding protein (I-FABP)**, an unbound cytosolic enterocyte protein and sensitive marker for enterocyte damage in the small intestine, along with **L-FABP** another marker of intestinal epithelial integrity. I-FABP and L-FABP are very small proteins which are released rapidly from GI enterocytes into the blood after cellular damage.
- Furthermore a unique ROS (Reactive Oxygen Species)-sensitive compound **NMAA-1** was established as a novel GI irritation/oxidative stress biomarker in irradiated mouse model.

Purpose

The **objective** of this study was to qualify feasibility of the human assays for monkeys and develop a new assay for establishing for the first time the novel fecal biomarker of GI inflammation/injury in different animal species.

VALIDATION OF CALPROTECTIN AS A BIOMARKER

- A human calprotectin ELISA kit was validated (10-600 µg/g) for the assay of NHP fecal samples. LLOQ was 10 µg/g and precision and accuracy were ≤ 20% and 80-120%, respectively.

- However, to obtain a more species specific and sensitive assay the Meso Scale Discovery (MSD) platform was utilized. Several monkey and mouse specific antibody pairs were tested and the combination with the best linearity was selected for validation. For monkeys, LLOQ was 0.825 µg/g (~ 12 times lower than ELISA) (LLOD= 0.528 µg/g).

- MSD's ultra sensitive electrochemiluminescence detection technology uses labels, which emit light upon electrochemical stimulation initiated at the electrode surface of MULTI-ARRAY microplates (Fig 1).

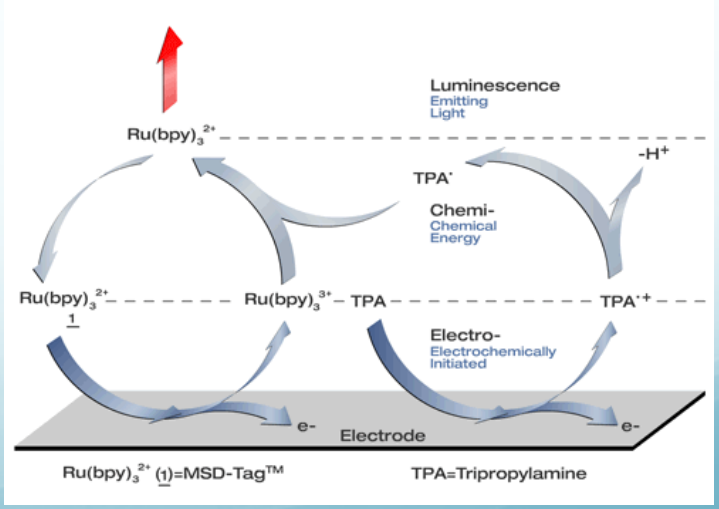


Fig. 1 Electrochemiluminescence chemistry

VALIDATION SUMMARY FOR CALPROTECTIN

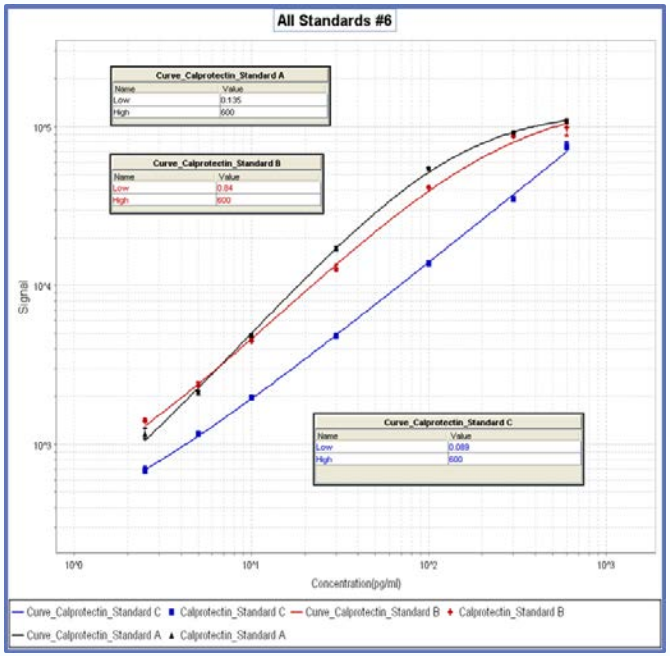


Fig.2: Selection of Antibodies and method optimization

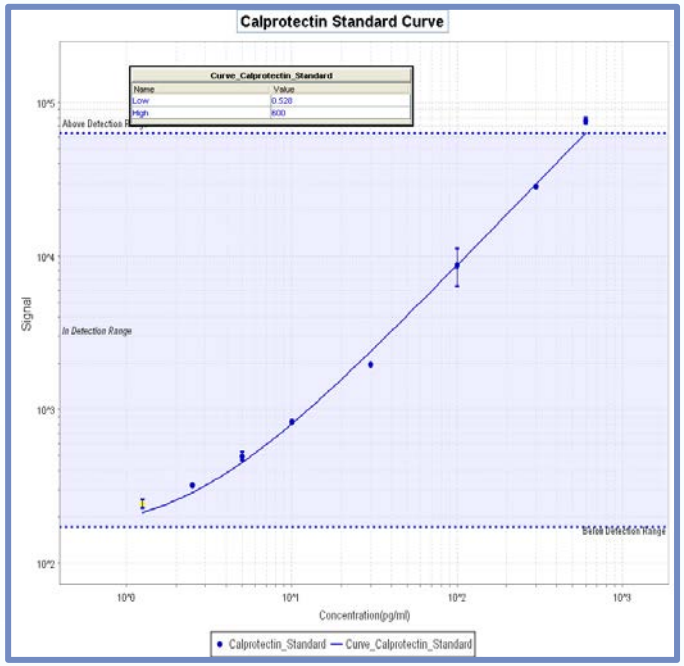


Fig. 3: The standard curves fit well over the range of this Calprotectin assay (0.8 to 600 ug /g).

SENSITIVITY	
LLOD (ug/g)	0.528
LLOQ (ug/g)	0.825
ULOD (ug/g)	600

LLOQ was 1.25ug/g at 2500x dilution and was 0.825 ug/g at 1650x dilution (LLOD=0.528 ug/g).

Intra and inter plate variability

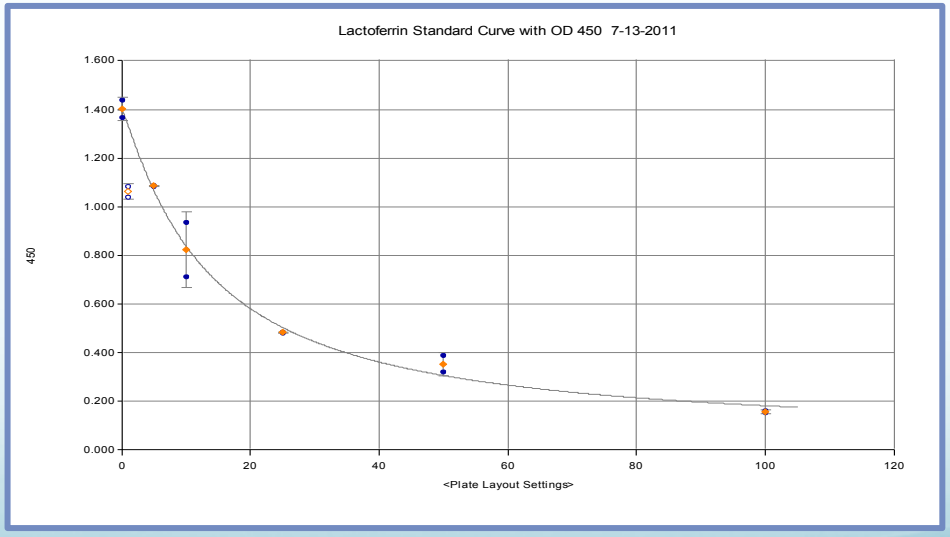
PRECISION			Intra-Plate			Inter-Plate
Control (ug/g)	Plates	Avg.Conc. (pg/mL)	Avg. %CV	Max %CV	Min %CV	%CV
5	2	5.10	1.53	2.29	0.74	13.03
2.5	2	2.52	6.32	9.48	4.56	0.93
1.25	1	1.55	1.65	4.39	1.17	

MSD OUTCOME

- Ofers higher specificity due to use of simian antibody versus the use of human antibody in the ELISA assay.
- The linear range is wider with a much lower quantitation limit as compared to the ELISA procedure.
- Further optimization to decrease LLOQ is ongoing.
- Volume of sample needed is lower for the MSD method.

LACTOFERRIN AS A BIOMARKER

Assay Kit:	Simian plasma lactoferrin (LTF) ELISA kit
Standard curve dynamic range:	5-50 ng/mL
LLOQ :	5 ng/mL
Plate to plate variability:	≤ 10% at 10X dilution of monkey fecal samples



ASSAY PRINCIPLES

- This LTF enzyme linked immunosorbent assay applies a technique called a quantitative sandwich immunoassay.
- The microtiter plate in this kit is pre-coated with a monoclonal antibody specific for LTF.
- Standards or samples are added to the microtiter plate wells and LTF, if present, will bind to the antibody pre-coated wells.
- In order to quantitatively determine the amount of LTF present in the sample, a standardized preparation of horseradish peroxidase (HRP)-conjugated polyclonal antibody, specific for LTF is added to each well to "sandwich" the LTF immobilized on the plate.
- After washing, subsequent addition of substrate and incubation, the enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm.

REPRESENTATIVE DATA

Name	Concentration (ng/ml)	Mean	CV (%)	Final Concentration (ng/ml)
8090 G4D3 5x	40.643	44.05		**220.25**
	47.457		10.9	
8090 G4D3 10x	28.637	26.574	11	**265.74**
	24.511			

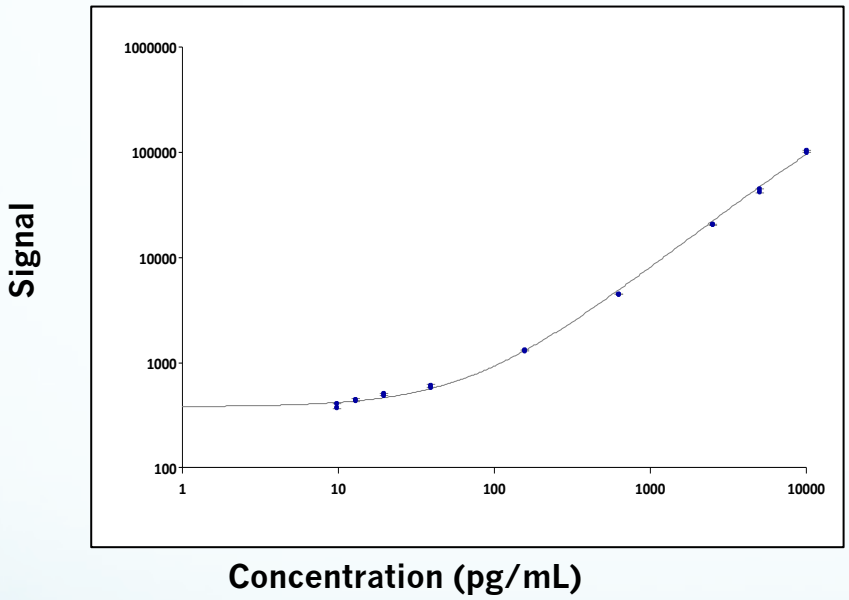
OUTCOME

A lactoferrin standard curve (5-50 ng/mL) was generated using a simian plasma lactoferrin ELISA kit and was optimized for analysis of monkey fecal extracts. LLOQ was established at 5 ng/mL and at 10X dilution the monkey fecal samples showed ≤ 10% plate-to-plate variability.

I-FABP AND L-FABP AS BIOMARKERS

I-FABP was assayed on a MSD multi-spot plate coated with a specific antibody, whereas L-FABP was analyzed using ELISA. LLOD for I-FABP and L-FABP were 5.95 pg/mL and 102 pg/mL, respectively. Normal ranges in monkeys were established. These biomarkers were tested and proved helpful for assessment of drug therapeutic effect and potentially survival prognosis following irradiation GI damage. These markers correlated well with citrulline levels (measured by a LC-MS/MS method developed by us) and histopathology findings.

Detection of Monkey/Human Intestinal FABP (I-FABP) Using MSD Technology – a novel method is developed in our facilities



Calibrator (pg/mL)	Monkey / Human I-FABP		
	Average signal	StdDev	%CV
0	362	0.00	0.20
9.77	391	9.79	5.79
13.0	444	4.60	2.71
19.5	500	5.63	3.11
39.1	600	7.51	3.66
156	1,318	3.15	0.81
625	4,530	1.99	0.17
2,500	20,760	49.6	1.09
5,000	43,502	364	4.13
10,000	103,147	434	2.26

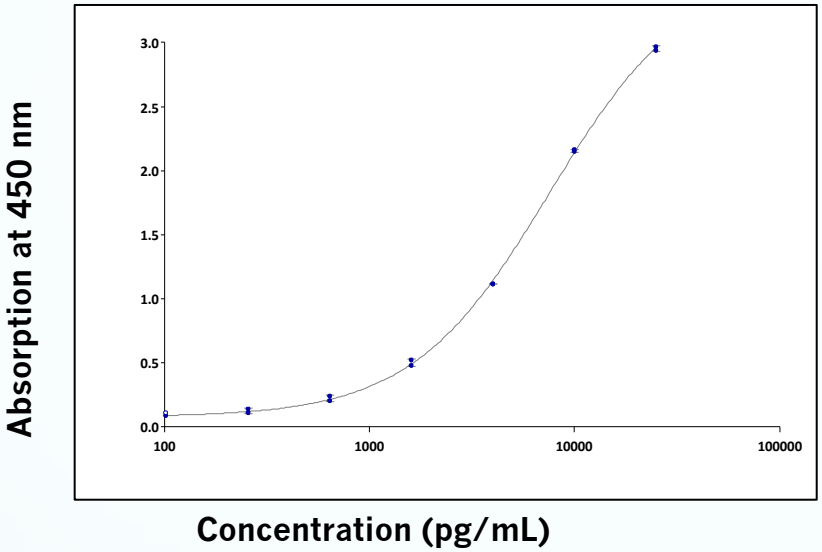
LLOD = 2.5 SD above background

I-FABP Detection Limit	5.95 pg/mL

Calibrators and serum samples were assayed on MSD MULTI-SPOT 96-Well plates coated with anti-I-FABP antibody (selected by us). I-FABP was detected with MSD SULFO-TAG labeled anti-I-FABP antibody. The average of duplicate wells from a representative experiment were plotted against concentration to generate a standard curve.

Normal monkey ranges were established at 34 – 83 pg/mL.

Detection of Monkey/Human Liver FABP (L-FABP) Using ELISA

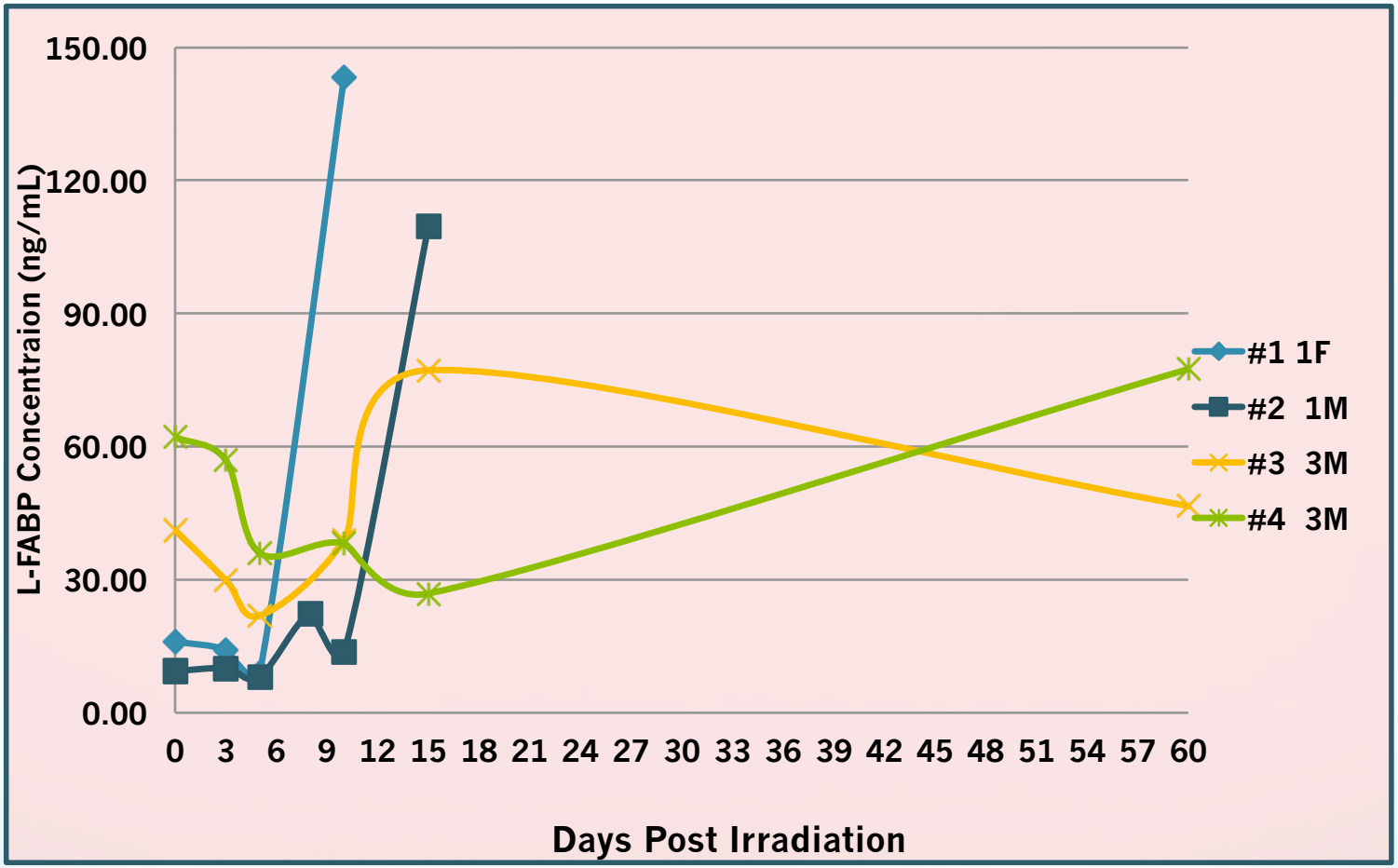


Calibrator (pg/mL)	Monkey / Human L-FABP		
	Average signal	StdDev	%CV
0	0.05	0.00	4.37
102	0.08	0.00	0.00
256	0.14	0.00	1.96
640	0.31	0.02	5.25
1,600	0.80	0.01	1.25
4,000	1.72	0.00	0.04
10,000	2.75	0.26	9.50
25,000	3.19	0.40	13.86

L-FABP Detection Limit	102 pg/mL

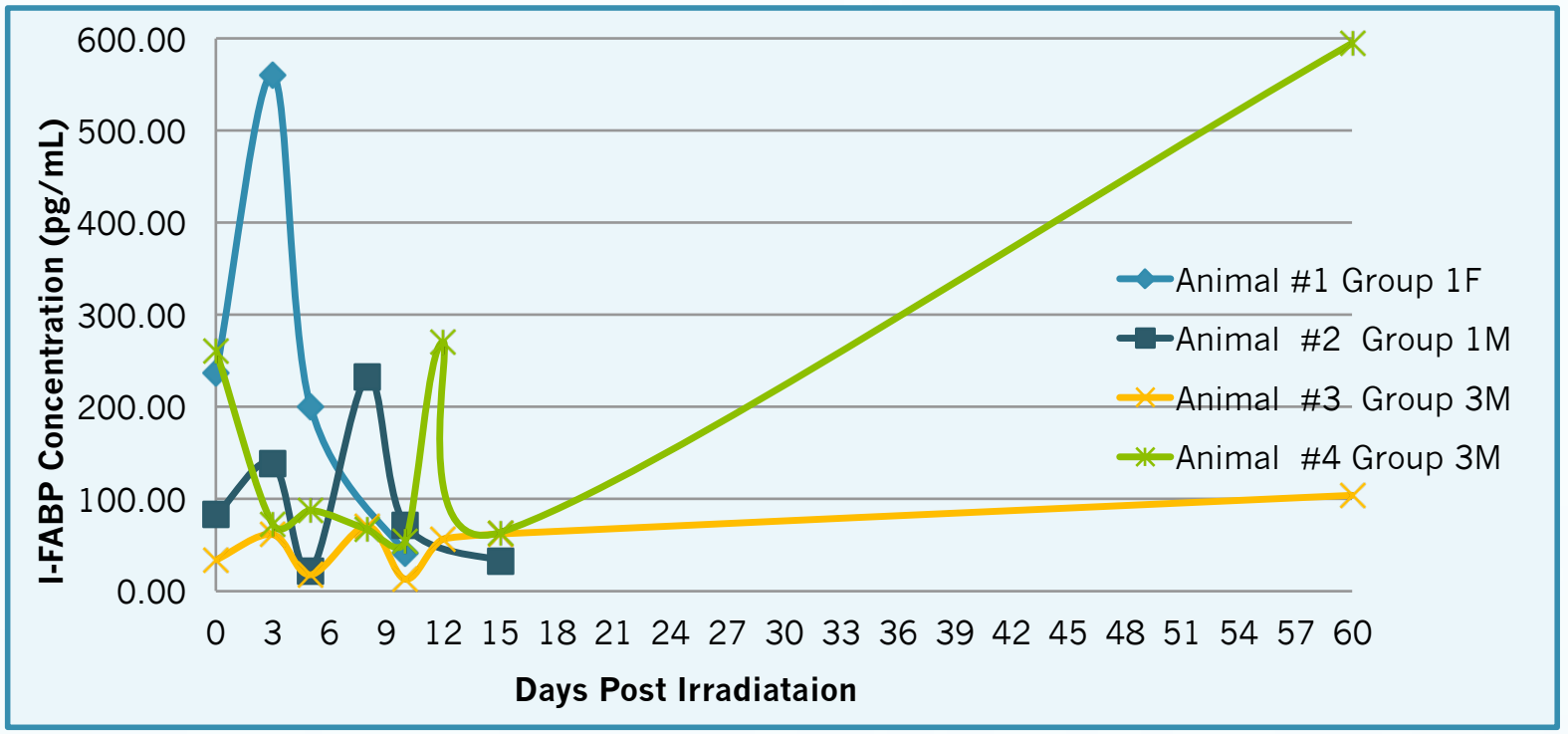
Calibrators and serum samples were assayed on 96-well plates coated with anti-L-FABP antibody. L-FABP was detected with a second (detection) anti-L-FABP antibody. Mean absorptions at 450 nm are the average of the duplicate wells from a representative experiment to generate a standard curve. Normal average monkey ranges 9,000 – 62,000 pg/mL. This assay uses 20X dilution.

L-FABP ELISA ASSAY in serum of irradiated monkeys



- Group 1 male and female (vehicle non-treated control- Animal Nos. 1 and 2) both died by day 15 and demonstrated high levels of L-FABP.
- In contrast, Group 3 (drug treated) males (Animal Nos. 3 and 4) lived for 60 days and L-FABP levels never exceeded 100 ng/mL.
- L-FABP elevation by day 60 in Animal No. 4 correlated with histopathology findings and increase in I-FABP.

I-FABP MSD ASSAY in serum of irradiated monkeys



- **Both animals in group 1 (non-treated controls) had elevated peak levels seen before day 10 and died by day 15.**
- **The animal with much higher and earlier (day 3) peaked I-FABP levels (Animal No. 1) died first (day 10).**
- **Both animals in group 3 (drug-treated) survived to day 60.**
- **However, Animal No. 4 had elevated levels on day 12 and much higher I-FABP levels on day 60 which perfectly correlated with histopathology findings in this animal while the second surviving animal (Animal No. 3) had low levels of I-FABP and histopathologically normal GI by day 60.**

ESTABLISHING A NOVEL FREE RADICAL BASED BIOMARKER

The compound NMAA-1 is a small molecule which upon reaction with H_2O_2 produces cleaved NMAA-1. We proposed to use NMAA-1 as a biomarker for gastrointestinal inflammation associated with the generation of reactive oxygen species.

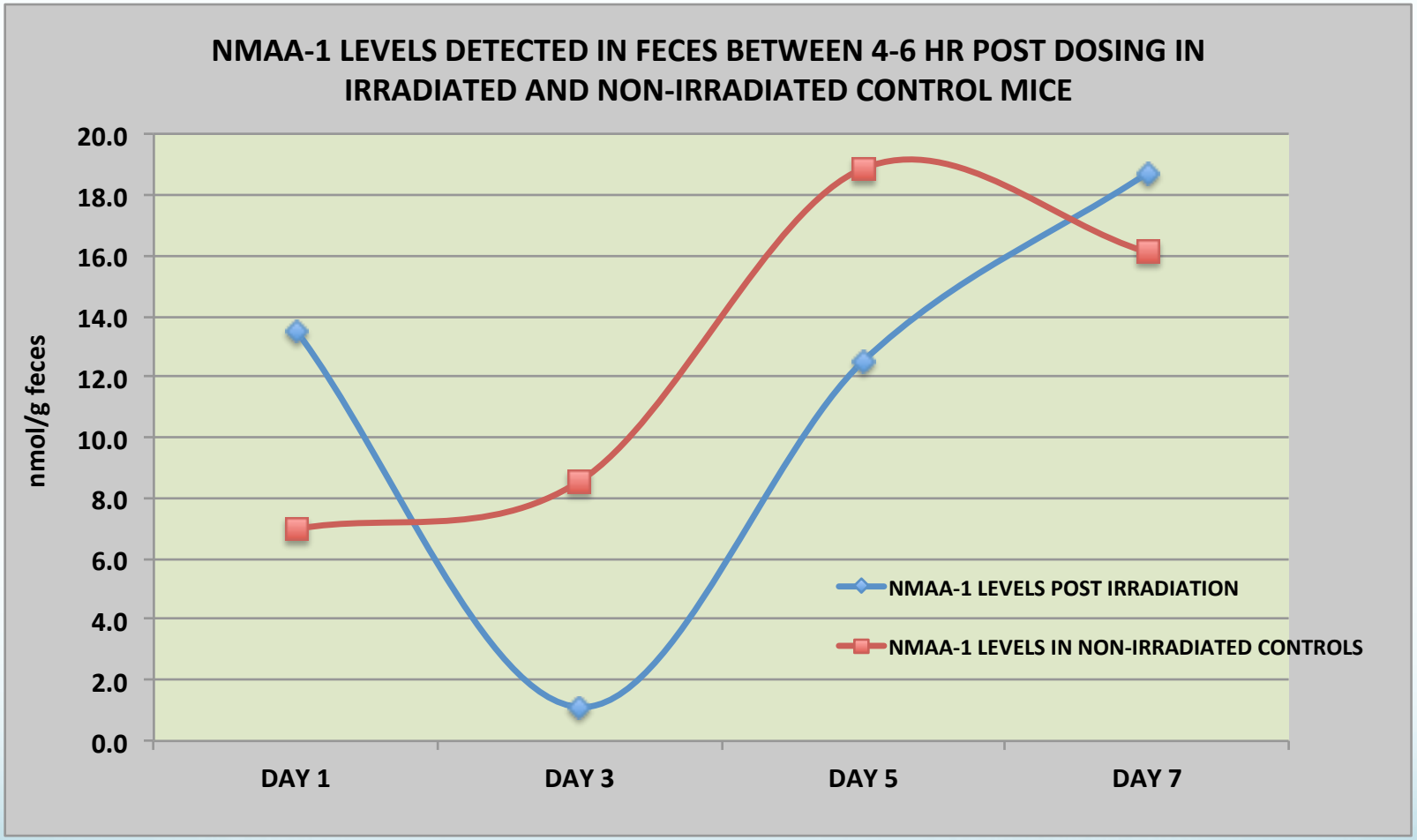
The ability of NMAA-1 to detect H_2O_2 selectively and in a concentration-dependent manner in aqueous solution and in living cells has been reported.

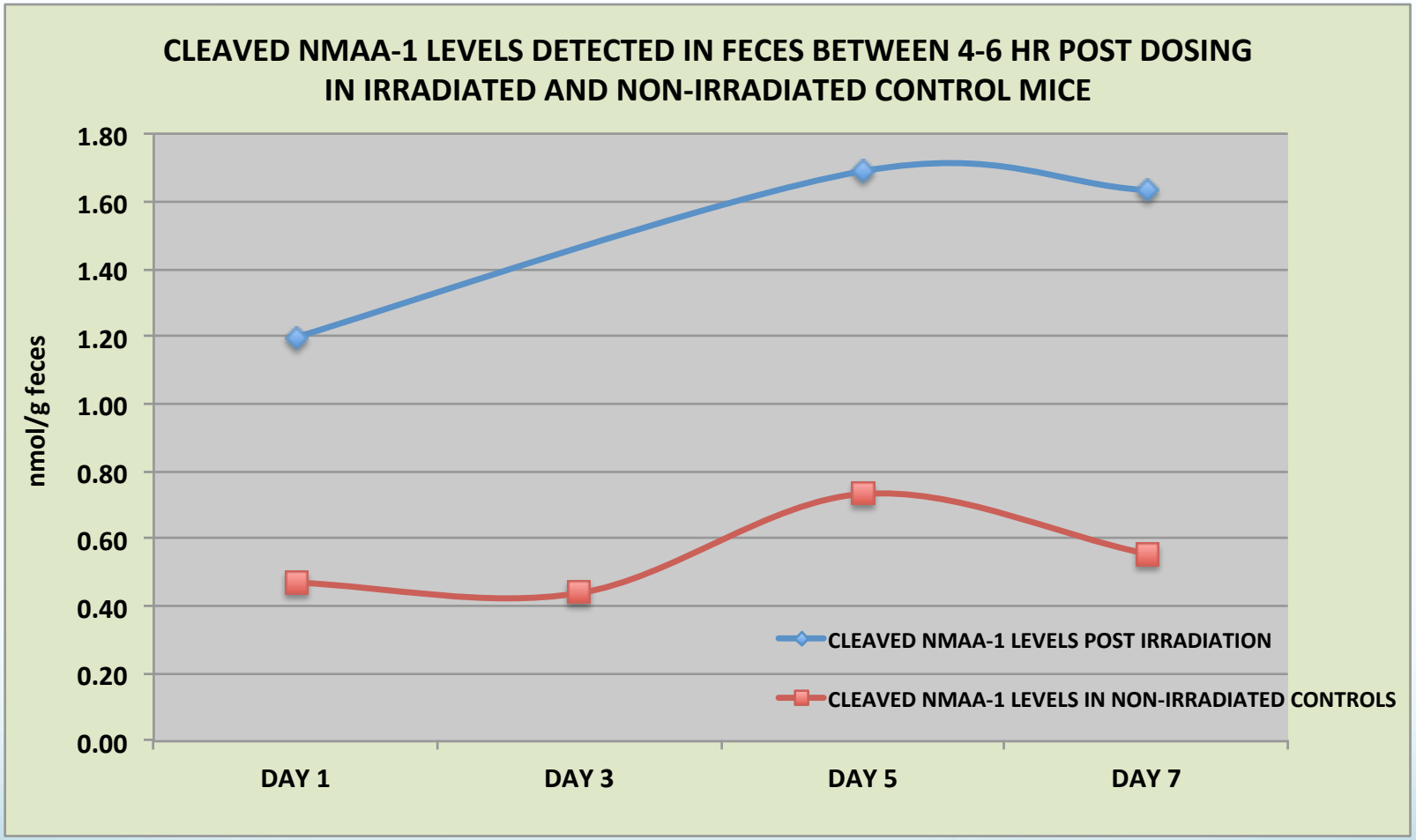
The same selectivity is being utilized to detect NMAA-1 and cleaved NMAA-1 in feces of irradiation-induced GI damage in mice.

STUDY PLAN

Non-irradiated C57/BL6 mice were dosed with NMAA-1 orally and feces were collected at 4, 6 and 8 hr post dose. A separate set of mice was irradiated with a total body irradiation of 13 Gy. Subsequently after 24 hr of irradiation these animals were dosed with NMAA-1 and again feces were collected at 4, 6 and 8 hr and analyzed for the presence of NMAA-1 and cleaved NMAA-1 using a LC-MS/MS system.

NMAA-1 as a novel biomarker of GI injury




CLEAVED NMAA-1 LEVELS DETECTED IN FECES BETWEEN 4-6 HR POST DOSING
IN IRRADIATED AND NON-IRRADIATED CONTROL MICE
nmol/g feces
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
DAY 1
DAY 3
DAY 5
DAY 7
CLEAVED NMAA-1 LEVELS POST IRRADIATION
CLEAVED NMAA-1 LEVELS IN NON-IRRADIATED CONTROLS

Concluding remarks

- A significant improvement in the dynamic range and sensitivity of the assay was achieved with the MSD method for calprotectin as compared to the ELISA method.
- I-FABP and L-FABP are being used as markers of GI toxicity in different in vivo studies. Species specific antibodies that could be good for further establishment and optimization of these methods are being investigated. The calprotectin and I-FABP MSD methods are currently being utilized to assay fecal samples from NHP (Rhesus) and mouse irradiation studies.
- An up to three-fold increase in the level of the oxidized NMAA-1 marker was noted in the fecal samples collected between days 5 to 8 in mice irradiated at 13 Gy as compared to the control levels, as measured by LC-MS/MS analysis. NMAA-1 has proved to be very distinctive in its role as an indicative marker for free radical presence in the GI during GI damage due to chemical or radiation based toxicity. This promises to be an extremely important diagnostic tool for such condition and further optimization is ongoing.
- Further optimization and multiplexing of the MSD (**calprotectin, lactoferrin, L-FABP and I-FABP**) and LC-MS/MS (NMAA-1 and citrulline) methods are being undertaken to provide more sensitive assays for these fecal and serum biomarkers of gastro-intestinal inflammation, injury, and recovery in animal models.