

Award Number: W81XWH-10-1-0400

TITLE: Glyco-Immune Diagnostic Signatures and Therapeutic
Targets of Mesothelioma

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REPORT DATE: September 2015

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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|--|---------------------------------------|--|---|
| 1. REPORT DATE (DD-MM-YYYY) September 2015 | 2. REPORT TYPE Final | 3. DATES COVERED (From - To) 15Jun2010 - 14Jun2015 | |
| 4. TITLE AND SUBTITLE: Glyco-Immune Diagnostic Signatures and Therapeutic Targets of Mesothelioma | | 5a. CONTRACT NUMBER W81XWH-10-1-0400 | |
| | | 5b. GRANT NUMBER W91ZSQ9305N632 | |
| | | 5c. PROGRAM ELEMENT NUMBER | |
| 6. AUTHOR(S) Margaret Huflejt, Ph.D email: margaret.huflejt@nyumc.org | | 5d. PROJECT NUMBER W91ZSQ9305N632 | |
| | | 5e. TASK NUMBER | |
| | | 5f. WORK UNIT NUMBER | |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES): New York University School of Medicine New York, NY | | 8. PERFORMING ORGANIZATION REPORT NUMBER | |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES): U.S. Army Medical and Materiel Command Fort Detrick, MD 21702-5012 | | 10. SPONSOR/MONITOR'S ACRONYM(S) | |
| | | 11. SPONSOR/MONITOR'S REPORT NUMBER(S) | |
| 12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited | | | |
| 13. SUPPLEMENTARY NOTES | | | |
| 14. ABSTRACT The focus of this grant was to investigate immunoprofiles for serum antibodies to aberrant glycans in human and animal models of mesothelioma. This was accomplished using a one of a kind printed glycan array which is at NYU School of Medicine (NYUSoM). It was hoped that these experiments will allow us to diagnose and prognosticate mesothelioma more accurately in the future. We were severely limited by our ability to start the human mesothelioma glycoprofiles as well as the animal profiles due to delivery and set up times for our one of a kind glycomics laboratory at NYUSoM. We summarize our findings in the final report. | | | |
| 15. SUBJECT TERMS Malignant Mesothelioma; Glycan Array; Immunoprofiles; Robotic Arrayer | | | |
| 16. SECURITY CLASSIFICATION OF: U | 17. LIMITATION OF ABSTRACT: UU | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON USAMRMC |

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1. INTRODUCTION: The overall goal of our investigations is to identify a serum anti-glycan antibody (AGAs)-based immunosignature of human malignant mesothelioma (MM) that would allow for identification of individuals, including military personnel, at high-risk for MM due to their potential long-term exposure to a carcinogenic form of asbestos, in time for an effective early intervention. Since such an immunosignature and the accompanying serum AGA immunoprofile reflect overall health, and more specifically immune health status of a person, both parameters are likely to also provide an insight into biological factors contributing to a susceptibility to this malignancy.

This project is funded in order to investigate immunoprofiles of serum anti-glycan antibodies recognizing Mesothelioma-derived aberrant glycans in human subjects and in animal models of Mesothelioma. We attempted to accomplish this using a one of a kind printed glycan array (PGA), which was developed by us at the New York University School of Medicine (NYU SoM), and was expanded by an addition of 182 novel glycan probes, many of which are Mesothelioma-specific.

2. KEYWORDS: mesothelioma, glycan, printed glycan array, immunoprofiles, diagnosis, rodent models, serum

3. OVERALL PROJECT SUMMARY:

General Comments: This project involved novel technology in which biochemically synthesized glycans were robotically printed on glass slides, hybridized with patient sera, and then analyzed quantitatively by scanning for the presence of these glycans in the serum. We were awarded the grant in July 2010, and in December of 2010, we were able to have our first successful printing of the glycochip using the first order PGA. Over the next two years, as described below, we went through a series of validation steps for the PGA while we were performing the animal experiments. However, in October 2012, Hurricane Sandy essentially stopped all research at our Bellevue laboratory for one year, and forced us to move the Biomek, Printer, and scanners to a newly designed and renovated laboratory which was located off site (the Varick NYU Glycobiology Laboratory). Fortunately, we were able to start up everything again, reprint slides (which took a tremendous amount of trouble shooting due to different lots of the slides), and develop the final **PGA 400** which included more glycans on our chip in anticipation of performing the animal and human experiments. We were fortunate that 1 week before the storm hit in 2012, we harvested the last of the fiber injected rats from Specific Aim 2, since the vivarium at NYU was completely destroyed by the Storm. In essence we were able to do all of the necessary PGA profiling for the humans and the animals between October 2014 to March 2015, and that is what we report here along with the rest of the work that we have performed. Dr. Huflejt was unable to attract further funding for any of her projects and left NYU in April 2015. Moreover, as described in the following write-up, the inability to validate in a blinded fashion differences between mesothelioma and asbestos exposed individuals forced me as the Contact PI, and Head of the Thoracic Laboratory, to put all of our efforts for this project into the completion of Specific Aim II, since we could not expect that the PGA could at this point reach qualifications for GMP.

Specific Aim IA: Validate in a separate set of cohorts that non-cancer bearing asbestos exposed cohorts have distinct anti-glycan antibody (AGA) immunoprofiles from patients with established mesothelioma. PIs in-charge: M.E. Huflejt/H.Pass.

Task 1. Identification of putative immunosignature of Malignant Mesothelioma (MM) on a background of immunoprofiles of asbestos-exposed serum donors

Hypotheses: (1) Profiling of serum anti glycan autoantibodies will differentiate high risk for mesothelioma, asbestos exposed cohorts from mesothelioma cohorts and differences among individuals with MM will reflect in their AGA expression profiles clinical demographics/endpoints including prognosis. (2) Information about expression of “glycogenes” responsible for presentation of glyco-conjugates on surfaces of mesothelioma cells and in circulation will allow (a) identification of biosynthetic pathways of mesothelioma-associated TACAs that will be generated as bio-identical synthetic probes, then arrayed and tested on our experiential diagnostic/ prognostic glycochips, and (b) better characterization and sub-classification of clinical mesothelioma sub-types.

Development of the PGA 400: Instrumentation and Methods

In the new facilities as described above, Dr. Huflejt was able to expand the glycan library to now include 386 glycans and the platform was known as the PGA-400. (Figure 1) A standard robotic technology for printing a large range of aminefunctionalized glycans on amine-reactive N-hydroxysuccinimide (NHS)-activated glass slides with the surface modified for rapid covalent coupling was utilized. Glycochips were printed at 50 and 10 μM concentrations, at eight replicates of each glycan at both concentrations,



Figure 1: Printing and processing in the NYU GlycoLaboratory, Division of Thoracic Surgery, at Varick Street, NYC.

and quality tested by a set of procedures standardized and optimized for clinical diagnostic research applications. Slides were incubated with serum (1:15 dilution in PBS/3%BSA/1% Tween-20) with gentle rocking for 2 hours at 37°C. Serum IgG, IgM and IgA immunoglobulins bound to printed glycans were visualized simultaneously with the “combo” biotinylated secondary antibodies or with biotinylated secondary antibodies against individual immunoglobulins and streptavidin-Alexa555. Fluorescence signal intensities corresponding to bound antibodies were collected at 90% laser power, and quantified with BioDiscovery/ImaGene software. Total relative fluorescence signal intensity values (range: $\sim 1 \times 10^3$ – 27×10^7 Relative Fluorescence Units, RFU) were used for further analyses. To ensure good quality of data, the quantification was first subjected to visual quality control of scanned images, which is then followed by intra-slide and inter-slide concordance analysis of quantified data. Intra-slide concordance analysis ensures that the coefficient of variation and the overall concordance coefficient across replicates on each slide are within accepted tolerances. The inter-slide concordance implies repeated development of randomly selected sera which are then subject to

pair-wise concordance analysis in order to establish the degree of the reproducibility of quantified data. In addition we will subject each batch of printed slides to a similar inter-slide concordance analysis by using standard pooled sera obtained from a large group of healthy donors.

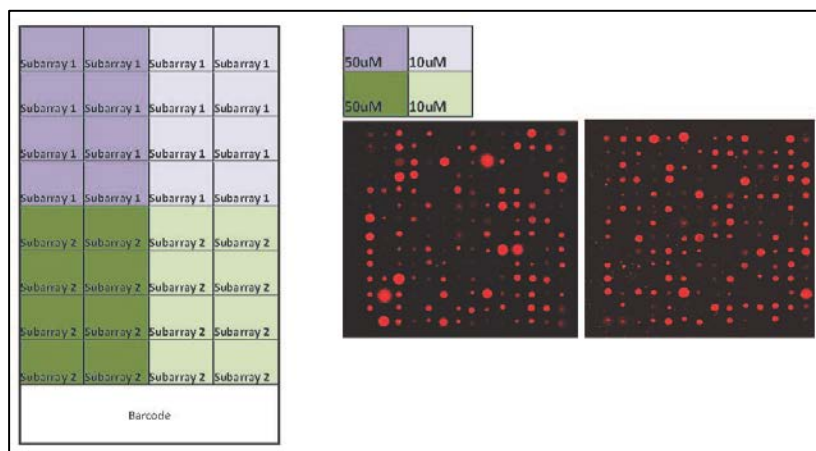


Figure 2: GlyocChip format for observations in human and rodent experiments.

Finally, the data that have passed quality control were tested through our variance component analysis procedures, which result in intra-class correlation (ICC) coefficients for each glycan in our PGA library. This information is a final certification that the data can be trusted in terms of the PGA technology, including printing, development, quantification and data preparation and preprocessing. Requested

volume for specimens to be processed was 100 μ l serum.

Figure 2 shows the new PGA-400 layout (left), and the [50uM Sub-arrays 1 and 2, right] developed with human pooled serum. Each slide had two subarrays performed in quadruplicate and at two concentrations. Moreover, we re-tested our previously described (Vuskovic et al., 2011*) “Karmanos” study population for their immunoprofiles using new NYU PGA-400, which would be a technical validation for the new PGA. Our original results were obtained in 2008 using PGA-200 with the population of Asbestos-exposed (AE) individuals, N=65, and Malignant

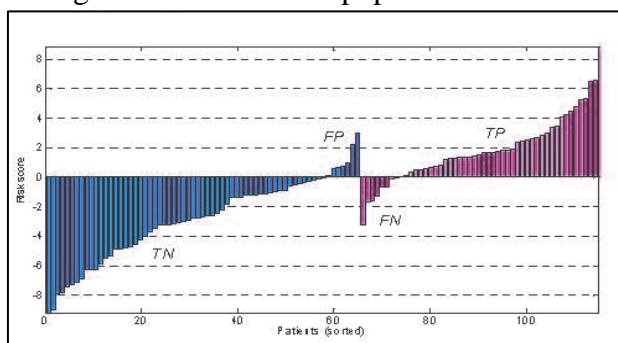


Figure 3: ImmunoRuler where blue bars (controls) and magenta bars (cases) represent discriminant values z_i for each subject in training samples. The diagram was obtained by training the IR with mesothelioma assay (65 asbestos exposed and 50 mesothelioma). Feature selection was performed with forward stepwise feature selection algorithm (FSFS), and the projection vector was obtained with logistic regression with design matrix which contained four interaction terms. The interaction pairs of selected glycan are GID = (311, 121), (189, 507), (328, 121) and (121, 172). This diagram clearly emphasizes the contingency values: number of true negatives (TN), false positives (FP), false negatives (FN) and true positives (TP)

Mesothelioma (MM) patients, N=50. The ImmunoRuler (IR) algorithm has been since further developed by including among others, the “interaction terms” feature that explores diagnostic relationship between expressions of antibodies which are linked by certain mathematical parameter within the entire study population. The results delivered by this iteration of ImmunoRuler are shown in **Figure 3**. We have then determined that the “interaction pairs” of glycans identified by the “ImmunoRuler” are indeed related by their biological activities. Over the last few years, certain serum samples became depleted and our recently tested population included AE, N= 57 and MM, N = 44. In addition to the available study population being smaller than in 2008, these analyses were performed under yet another unfavorable circumstance: a change

in the surface chemistry of Schott Slide H we use to print our glycochips. Due to the change in the chemical formulations on the production site in Schott laboratories, in Jena (Germany)

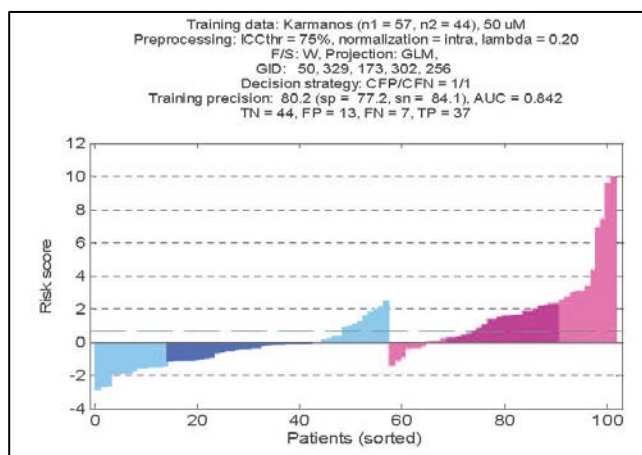


Figure 4: Validation of immunoprofiles using identical serum for the earlier PGA

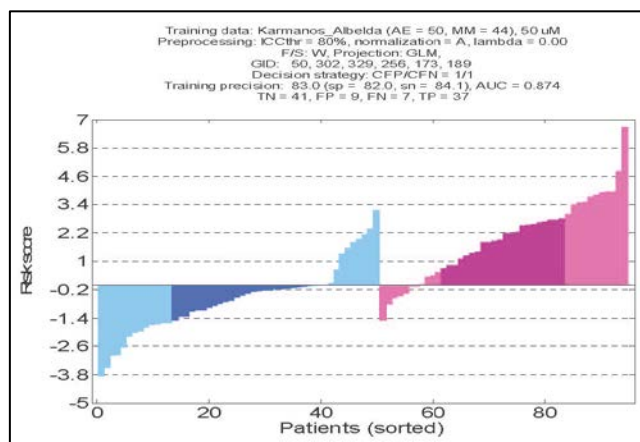


Figure 5: Modification of AE and MPM profiles after followup of patients at Karmanos Cancer Center.

AE, N = 50 and MM, N=44. As shown in **Figure 5**, after removing immunoprofiles of donors not exposed occupationally to asbestos and patients who no doubt had lung cancer developing while donating their serum to this study, Training precision increased from 80.2 to 83.0, Specificity increased from 77.2 to 82.0, Sensitivity remained at 84.1 and AUC increased from 0.842 to 0.874. Finding four occupationally exposed to asbestos, AE donors who died of other (lung) malignancy within two years from serum draw – but who have been classified by IR as “False positive” brings another important question of potential significance of such finding. In addition to quite good agreement between the results obtained for this study population in years 2008 and 2013, we have also found a good agreement between the “diagnostic signature glycans”: within the 4-glycan signature in 2008 and 6-glycan signature in 2013, two glycans differ only in the spacer structure, one remains the same, and one glycan from 2008 is also a fragment of a larger glycan in “Signature 2013”.

Blinded Validation of New York AE and MPM Individuals

Once the PGA was technically “validated”, Dr. Pass (PD/PI) called for a new “blinded” validation of the serum glycan profile which was locked in for the determination of AE vs MM.

surface chemistry of Slide H has been slightly altered what resulted in the reduction of glycan printing efficiency and in overall lowering the quality of our glycochip. During this period we initiated a close collaboration with chemist in Schott labs in Jena – and due to this joint effort, slides were developed slides which were as optimized as possible for immobilization of amino-spacered glycans within all their structural heterogeneity, and peptides/proteins.

The results (**Figure 4**) were obtained with acceptable but still sub-optimal Slide-H-based glycochips. **Figure 4** shows the distribution of “Risk scores” between the AE and MM study populations, with the following: Training precision: 80.2% (sp = 77.2%, sn = 84.1%), AUC = 0.842. However, during an analysis of health status and demographic information available through the Karmanos Cancer Center in Detroit where these samples originated, we determined that within the “AE” population, three serum donors have declared “no known asbestos exposure” and four donors with risk scores placing them in the “false positive” group have died of lung cancer within two years since serum draw. We therefore removed these seven immunoprofiles from further considerations, and constructed a modified immunoprofile which included:

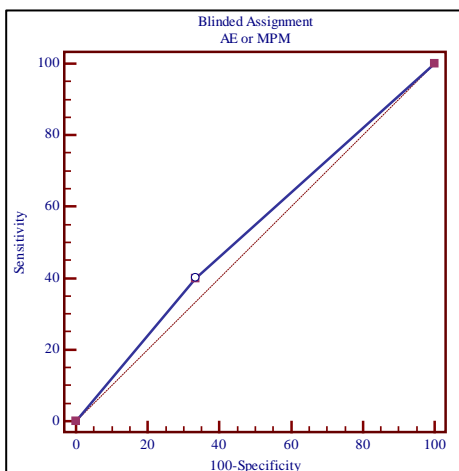


Figure 6: ROC curve for NYU Blinded validation of AE vs MPM

To do this Dr. Pass provided 60 blinded serum specimens to the Glyco Laboratory, 50% of which were mesothelioma and 50% were asbestos exposed individuals from a cohort of insulators who volunteered to have blood drawn for research purposes under our IRB approved biomarker discovery protocol (Protocol 8896). Unfortunately, as seen in **Figure 6**, *the new immunoruler was incapable of separating AE from MM serum*. It is to be noted that in another grant concentrating on lung cancer, the immunoruler also failed to validate in a blinded examination of high risk smokers vs adenocarcinoma. *As such, we felt that further human studies involving the PGA required a major analysis of these failures before proceeding with prognostic implications of the PGA as specified in SOW Specific Aim 1B, and we concentrated on the completion of the*

analyses for the in vivo rat studies.

Specific Aim IIA: Using a rat model of asbestos-induced MM, profile serum AGAs using PGA and define temporal changes in this profile as mesothelial carcinogenesis occurs. Tumor development and growth during 52 weeks will also be followed in animals using high-frequency ultrasound. PIs in-charge: M.E. Huflejt/H.Pass.

Development of MPMs in the Rat Model

The study was carried out using three groups of Fischer 344 female rats: (i) 32 animals exposed to intraperitoneal (IP)-applied asbestos/crocidolite as MM-inducing agent; (ii) 32 animals exposed to silica fiber as an IP-applied control for asbestos/crocidolite exposure, and (iii) 8 animals, for a one-time sham saline IP injection – as a control for age-related changes in AGA immunoprofiles. At the experimental end-point, animals were sacrificed according to the recommendations of IACUC. If animals exhibited symptoms of ill health, stress or fatigue prior to the study’s endpoint, they were euthanized and necropsied before the study endpoint at the



Figure 7: IP asbestos rat with development of extensive abdominal mesothelioma at autopsy

“humane point”. End-point necropsy procedures included: detailed observations of the internal organs of each individual experimental animal, including photographic records of selected cases, and the collection of tumors and other tissues, including serum from each experimental animal.

There were variable responses to the peritoneally injected asbestos among the 32 experimental animals: 25 animals developed mesothelioma (78%), and 7 animals were found disease-free at the endpoint necropsy. *Among the 25 animals that developed*

mesothelioma, 13 animals reached the study end-point: 7 animals had fully developed peritoneal mesothelioma, and 6 animals had minimal disease in the form of miliary tumors. Twelve animals did not reach study end-point: 6 animals were found dead of the disease and 6 were euthanized at the later stages of the experiment due to the animals' rapidly deteriorating health, which was the result of quickly progressing mesothelioma. This observation is very significant since it implies distinctly different individual responses to a carcinogen, similar to humans. These different biological responses to a carcinogen are also indirectly manifested as differences in the weights of individual animals. In extreme cases, such differences are the result of "wasting" or the accumulation of large volume of ascites. During the experimental end-point necropsies out of 32 animals injected with silica, 31 animals were found disease-free and one animal was found to have developed a sarcoma tumor. All control saline-injected animals were found disease-free at the experimental end-point necropsies, and control saline-injected animals and silica-injected animals survived until experimental end-point.

Anti-Glycan Antibody Serum Profiles

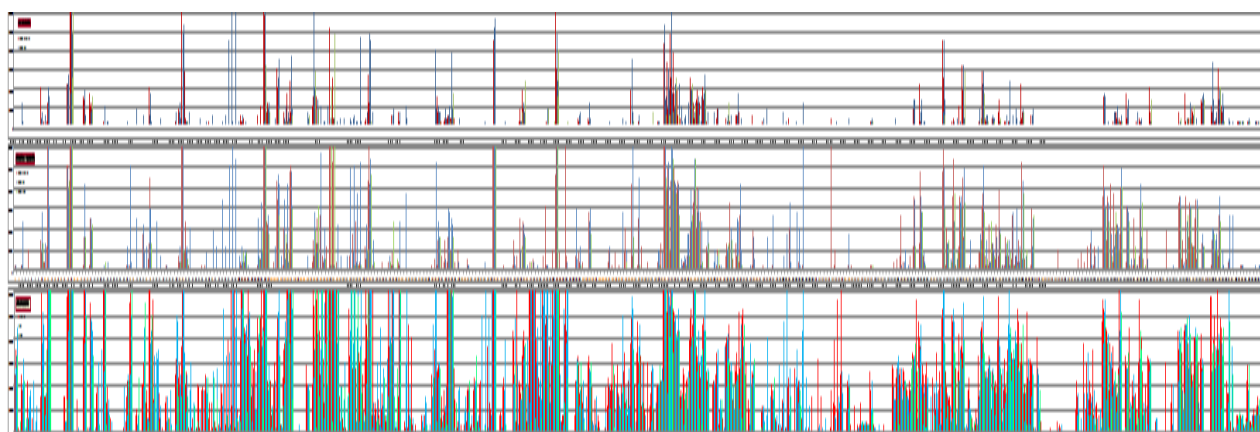


Figure 8: Development of anti-glycan antibodies in rats after saline injection, silica, or asbestos IP. See text for details.

We were able to obtain sera from all three groups longitudinally. For the purposes of the AGA analysis we compared the sera of the 13 animals that reached the endpoint in the asbestos group to the 8 saline injected and 31 silica injected animals at endpoint. AGA "pre-injection" and "1 month post—injection" immunoprofiles were obtained from the pooled sera of three to five animals. Sera of these animals were pooled due to the low volume of blood collected from the tail vein of animals still young and small at early experimental time-points. The endpoint AGA immunoprofiles are obtained from the sera of individual animals. **Figure 8** shows three sets of bar-graphs presenting fluorescence intensities of rat serum anti-glycan antibodies binding to glycan probes present in our Printed Glycan Array NYU PGA-400. The **top bar-graph shows pre-injection AGAs** for all three experimental animal groups: "asbestos", "silica" and "saline". In all three bar graphs, asbestos-injected animals are colored red, silica-injected animals are colored blue, and saline-injected animals are colored green. **The middle bar-graph shows one-month post-injection AGAs for all three experimental groups**, and the **bottom bar-graphs shows AGAs at the study endpoint for all three experimental groups**. The bar-graphs have been aligned in a way that allows us to observe changes in the individual AGAs over time between the experimental animal groups. For instance, appearances of specific AGAs in response to the asbestos injection are detectable in the "1 month post-injection" immunoprofiles. Significant and distinct differences between serum AGAs intensities in "asbestos" vs. "silica" vs. "saline" rats at the study endpoint are also immediately noticeable.

Important Results of the AGA Profiling of Rat Induced Mesotheliomas

Fiber exposure, whether silica or asbestos, caused significant changes in antiglycan antibody production in the rat. A significant number of these antibodies were elevated in both silica and asbestos exposed rats and the translational relevance of these findings need to be explored in other funding mechanisms.; however, major increases in **GlcNAc1-4GlcNAc-Asn (GID 115)**, **6-Bn-Gala1-4(6-Bn)GlcNAc-sp (GID 126)**, **GlcNAc1-3Galb1-4GlcNAc-sp2 (GID 167)**, were seen selectively in asbestos rats vs silica rats, and these are generally referred to as the “GlcNAcs”. Moreover, **6-Bn-Gala1-4(6-Bn)GlcNAc-sp** and **GlcNAc1-3Galb1-4GlcNAc-sp2** were also found to be significantly elevated in asbestos exposed and MPM bearing humans when compared to normal individuals. The GlcNAcs have also been associated with the separation of adenocarcinoma from mesothelioma(1) and recently have been associated with modulation of epithelial mesenchymal transition(2). **Le^x1-6’(Le^C1-3’)Lac-sp4 (GID 538)** was also found to be elevated in asbestos exposed rats. Le^x1-6’(Le^C1-3’)Lac-sp4 is involved in the pathway which transfers fucose to N-acetyllactosamine polysaccharides to generate fucosylated carbohydrate structures. It catalyzes the synthesis of the non-sialylated antigen, Lewis x (CD15), and CD15 has been reported as a distinguishing feature between mesotheliomas and pulmonary adenocarcinomas(3).

Specific Aim II B: Use the syngeneic II-45 cell line xenograft in rat model of asbestos-induced mesothelioma to correlate mesothelioma tumor growth with rat serum anti-glycan antibodies (AGA). PIs in-charge: M.E. Huflejt/H.Pass

The goals of this experiment were (i) to identify glycans showing the dynamics of anti-glycan antibodies during outgrowth of syngeneic mesothelioma tumors, implanted intraperitoneally (IP) or subcutaneously (SC), by comparing immunoprofiles of saline-injected control rats to rats with the implanted tumor cells, and (ii) to identify glycans showing the dynamics of anti-glycan antibodies in response to the chemotherapy drug Gemzar by comparing immunoprofiles of Gemzar-injected rats with the immunoprofiles of saline-injected control rats. Gemzar is an anti-cancer drug often used in MM treatment, and is known to have immunomodulatory effects. In this experiment we investigated whether this immunomodulatory effect is detectable on the level of the AGA dynamics in healthy, non cancer bearing animals.

Methods: Fresh stocks of syngeneic rat mesothelioma II-45 cells were expanded, and testing performed testing for a panel of animal pathogens, with specific focus on rat pathogens. As determined by Charles River Research Animal Diagnostic Services, our II-45 cell line was pathogen-free, and was ready for injections as proposed in the second arm of the study.

Experimental Design and Schedule are shown in **Table I** below.

Table I: Schedule for Specific Aim II B

| Group | | 6/13/14 | 6/27/14 | 7/1/14 | 7/11/14 | 7/15/14 | 7/24/14 | 7/25/14 | 7/29/14 | 7/31/14 |
|------------------|---------------|----------|---------|-----------|-----------------------------------|---------|-----------------------------------|---------|-----------------------------------|------------------|
| | | Day - 18 | Day - 4 | Day 0 | Day 13 | Day 14 | Day 23 | Day 24 | Day 28 | Day 30 |
| Control | 6 IP Females | Bleed | Bleed | Injection | Sacrifice: bleed | Bleed | Sacrifice: bleed | Bleed | Sacrifice: bleed | Sacrifice: bleed |
| | 6 SC Females | Bleed | Bleed | Injection | Sacrifice: bleed | Bleed | Sacrifice: bleed | Bleed | Sacrifice: bleed | Sacrifice: bleed |
| II-45 Cell Lines | 12 IP Females | Bleed | Bleed | Injection | Sacrifice: bleed & harvest tumors | Bleed | Sacrifice: bleed & harvest tumors | Bleed | Sacrifice: bleed & harvest tumors | NA |
| | 12 SC Females | Bleed | Bleed | Injection | Sacrifice: bleed & harvest tumors | Bleed | Sacrifice: bleed & harvest tumors | Bleed | Sacrifice: bleed & harvest tumors | NA |
| Gemzar | 12 TV Females | Bleed | Bleed | Injection | Sacrifice: bleed | Bleed | Sacrifice: bleed | Bleed | Sacrifice: bleed | Sacrifice: bleed |

Prior to the first blood draw, 3-4 week old Fischer F344 female rats with tattooed tails featuring unique identifying numbers were acclimatized for 18 days. After 3 weeks of acclimatization, the experiment began. In accordance with NYUSM DLAR blood drawing policy, blood draws were performed by tail nicking in order to obtain 200-500 μ L of blood per draw.

On day 0, 1×10^6 syngeneic rat mesothelioma cells were injected into the rats' dorsal flanks in 0.2 mL of HBSS via a subcutaneous (N=12) or intraperitoneal (N=12) injection. Control animals were injected with 0.2 mL of HBSS via a subcutaneous (N=6) or intraperitoneal (N=6) injection. A 0.2 mL solution of Gemzar (40 mg/kg body weight) in HBSS was injected into "Gemzar" rats (N=12) via a tail vein. Animals were observed daily by DLAR staff and at least two times per week by research associates participating in the project. Tumor growth was monitored over a 4 week period using a digital caliper.

To characterize an "early" stage of tumor growth, on day 13 tumors were removed from euthanized animals in the subcutaneous (SC) and intraperitoneal (IP) cell line group. Resected tumors were examined for their pathological features, and stored in formalin for further analysis. For comparison, 6 animals from the subcutaneous (SC) and intraperitoneal (IP) control group, and 3 animals from the Gemzar group were also sacrificed on day 13. All animals were sacrificed according to the recommendations of IACUC.

At days 24 and day 28, tumors from the subcutaneous (SC) group and intraperitoneal (IP) cell line group were harvested and stored in a similar manner. The remaining control and Gemzar animals were sacrificed on day 30 at the conclusion of the experiment. End-point necropsy procedures included: detailed observations of the internal organs of each individual experimental animal, including photographic records of selected cases, and the collection of tumors and other tissues, including serum from each experimental animal. Saline-injected rats had no health problems observed, and no tumors were found at the necropsy. AGA immunoprofiling of serum specimens was then performed at Study Endpoint

Results

Development of MPMs in the Rat Model

Disease free, study endpoint, N=0;

- Minimal disease, study endpoint, N=0;
- Fully developed disease, study endpoint, N=13;
 - SC: N=9;
 - IP: N=4;
- Fully developed disease, humane endpoint, N=7;
 - SC: N=3;
 - IP: N=4;
- Fully developed disease, found dead, N=4;
 - IP: N=4;
 - Found dead on 7/23/14, N=3;
 - Found dead on 7/28/14, N=1;

Anti-Glycan Antibody Serum Profile Results

General Observations

We note that the immune response to the syngeneic mesothelioma cell (SMC) implantation as evaluated by the increase in specific AGAs varied markedly between individual animals. The immune system of rats is very similar to human immune system, and this observation may illustrate the variations in human responses to various carcinogenic / immunogenic factors. As in our earlier experiments with rats exposed to asbestos or silica, expressions of anti-glycan antibodies (AGAs) are at the very low levels in animals at the age of several weeks, and then gradually increase with the age of animals; this very low expression level of AGAs in young animals is best demonstrated in young control rats which have been injected with saline only at the beginning of the experiment. Implantation of SMCs results in the marked increase in specific anti-glycan antibodies already within two weeks after the procedure, suggesting activation of the immune response. Intraperitoneal implantation of SMCs induces much stronger expressions of AGAs as compared with the subcutaneous SMC implantations. Gemzar has been shown to stimulate immune defense in certain anti-cancer treatment settings; here we observe that *a single bolus injection of Gemzar alone was able to markedly increase expressions of certain specific anti-glycan antibodies, many of them known to target tumor-associated antigens*. Interestingly, this increase in Gemzar-induced AGA expressions was highest within the first two weeks following the drug injection, and within the next two weeks, AGA expressions started to decrease. It is possible, that this decrease in AGA expression resulted from the Gemzar activity wearing off. Question remains – whether the administration of Gemzar on a defined schedule would continue stimulation the production of anti-cancer anti-glycan antibodies, and therefore contribute to the control of malignancy.

Specific AGA Responses in the SMC Implantations

Our results from the measurement of AGAs in response to SMC were classified by their overexpression compared to the control saline injected animals. **Table II** summarizes these findings while **Figure 9** graphically details the individual animals and their responses as recorded on the PGA. Our first observation is that there is intense stimulation of antibodies that are associated with innate immunity involved in pathogen control by dendritic cells, including viruses (GIDs 97-99). In addition to selective elevation of these antibodies, we also detected elevation of antibodies associated with tumors (GIDs 295 and 301) which have been associated with tumors. Two of the antibodies elevated in the SMC experiment were also associated with exposure to asbestos and tumor development (GID 20, L-a-Rhamnose and GID 130, 6'Bn Lewis^c; **green background Table II**).

Table II: Overexpressed Anti-glycan antibodies in rodents receiving saline, SC syngeneic mesothelioma cell line, IP syngeneic mesothelioma cell line, or intravenous Gemzar.

| GID | Spacer form of Saccharide | Designation | Biological Association | Structure | Comments |
|-----|--|--|--|------------------|---|
| 097 | Galb1-4GlcNAcb-sp3 | LacNAc | Present on inflammatory dendritic cells; Ligand for siglec-8 and certain viruses; | Disaccharides | Elevated in Cancer Bearers IP; |
| 098 | Galb1-4GlcNAcb-sp5 | LacNAc-C8 | Present on inflammatory dendritic cells; Ligand for siglec-8 and certain viruses; | Disaccharide | Elevated in Cancer Bearers IP; |
| 099 | Galb1-4GlcNAcb-sp8 | LacNAc-PEG | Present on inflammatory dendritic cells; Ligand for siglec-8 and certain viruses; | Disaccharides | Elevated in Cancer Bearers IP; |
| 162 | 6-O-Su-Galb1-4GlcNAc | 6'-O-Su-LacNAc | Present on inflammatory dendritic cells; Ligand for siglec-8 and certain viruses; | Disaccharides | Elevated in Cancer Bearers IP; |
| 264 | Galb1-4Galb1-4GlcNAcb | Galb1-4 LacNAc | Potential tumor-associated antigen | Trisaccharides | Elevated in Cancer Bearers IP; |
| 295 | Neu5Aca2-6Galb1-4Glc | Neu-6 Lactose | Antigen associated with tumors, and certain stimulated cells | Trisaccharides | Elevated in Cancer Bearers IP; |
| 301 | Neu5Aca2-6Galb1-4GlcNAcb | Neu-6 LacNAc | Antigen associated with tumors, and certain stimulated cells | Trisaccharides | Elevated in Cancer Bearers IP; |
| 020 | Rhaa | L-a-Rhamnose | Pathogen-associated; including Mycobacterium | Monosaccharides | Elevated in Cancer Bearers IP and SC |
| 024 | GlcNAca | a-N-Acetylglucosamine (GlcNAc) | Pathogen-associated | Monosaccharides | Elevated in Cancer Bearers IP and SC |
| 026 | Rhab | b-Rhamnose | Pathogen-associated; including Mycobacterium | Monosaccharides | Elevated in Cancer Bearers IP and SC |
| 081 | Gala1-4GlcNAcb | a-N-Acetyl-D-Lactosamine (LacNAc) | Mimicry-carbohydrate, antibodies markedly elevated in human tumors | Disaccharides | Elevated in Cancer Bearers IP and SC |
| 125 | (6-Bn-Galb1-4)GlcNAcb | 6'Bn-N-Acetyl-D-Lactosamine (LacNAc) | Mimicry-carbohydrate, antibodies markedly elevated in human tumors | Disaccharides | Elevated in Cancer Bearers IP and SC; increased with Gemzar vs Saline |
| 130 | (6-O-Bn-Galb1)-3GlcNAcb | 6'Bn Lewis ^c | Mimicry-carbohydrate, antibodies markedly elevated in human mesothelioma and other tumors, and in rats exposed to asbestos | Disaccharides | Elevated in Cancer Bearers IP and SC; increased with Gemzar vs Saline |
| 388 | Galb1-4GlcNAcb1-6(Galb1-3)GalNAca | 6 LacNAc TF Thomsen-Friedreich antigen | Tumor-associated antigen | Tetrasaccharides | Elevated in Cancer Bearers IP; increased with Gemzar vs Saline |
| 390 | (GlcA1-4) ₂ | maltotetraose | Microorganism-associated | Tetrasaccharides | Elevated in Cancer Bearers IP; increased with Gemzar vs Saline |
| 507 | GalNAca1-3GalNAcb1-3Galb1-4Galb1-4GlcB-sp3 | Forsman antigen | Tumor-associated antigen | Penta accharides | Elevated in Cancer Bearers IP and SC; increased with Gemzar vs Saline |

Important Results of the AGA Profiling of Syngeneic MM Rodent Tumors

We have described the appearance of antiglycan antibodies with growth of these tumors, and curiously some of the glycans targeted are those associated with infectious pathogens while others are specifically related to malignancy. The PGA platform appears to have some consistency in that we see commonality of glycans involved with both asbestos induced tumors from Specific Aim IIA and this SMC. This should only make sense since the F344 II-45 tumor model is exactly the one that was generated originally from asbestos implantation IP in newborn rodents. The development of antibodies by Gemzar against a cohort of overexpressed glycans that are expressed by the tumor is interesting, and needs further exploration in animals with tumors implanted. Unfortunately this will necessitate new funding.

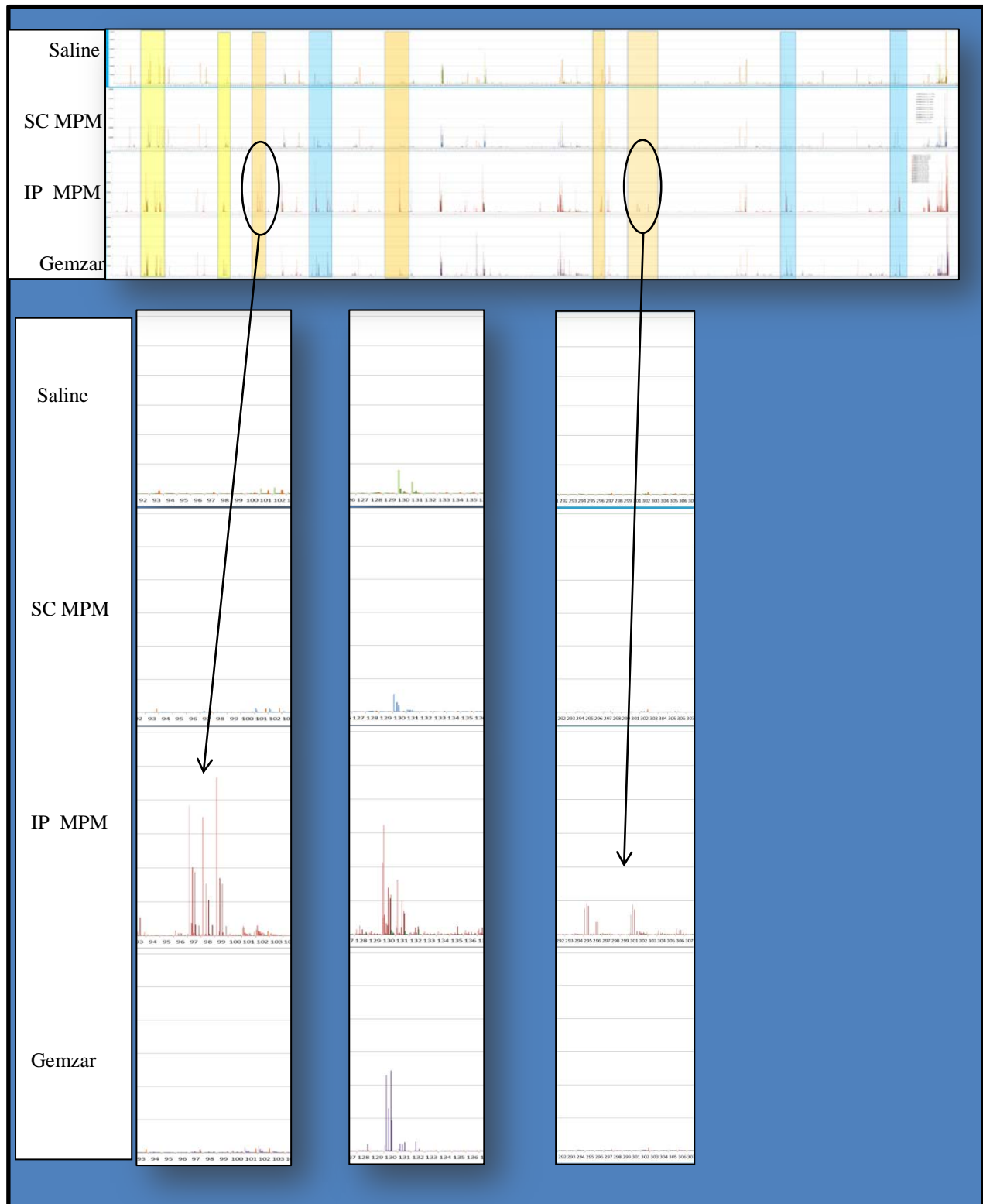


Figure 9: Development of AGAs in rodents receiving saline, SC syngeneic mesothelioma cell line, IP syngeneic mesothelioma cell line, or intravenous Gemzar. Upper panel: Complete set of the PGA 400 data. Box colors correspond to AGAs described in Table II. Lower panel: Selective upregulation of AGAs after IP SCM magnified view. Gemzar in some instances also upregulates the same AGAs that are upregulated by the presence of tumor.

5. KEY RESEARCH ACCOMPLISHMENTS

- We have demonstrated that the Printed Glycan Array 400 can be technically validated when using the same specimens measured at different times. However, the profiles generated, at least for mesothelioma vs asbestos exposed individuals from New York did NOT have the same immunoruler results as those from another geographic site. We hypothesize that there were differences in the populations with respect to their asbestos exposure, specifically the type of fiber (crysotile, NYC vs crocidolite, Michigan). It is extremely unlikely that there was a difference in the processing or procurement of the specimens since they were all harvested using Early Detection Research Network NCI SOPs. Potentially the stages of the mesotheliomas between NYU and Michigan were different, but we have checked that and there are no significant differences in stage groupings. Nevertheless, this demonstrates that the immediate feasibility for moving the PGA 400 further Phase III biomarker trials in humans is low.
- As a research tool however, the PGA 400 demonstrated that
 - Cancer and non cancer associated AGAs generate antibodies in the presence of asbestos or silica in rodents.
 - These AGAs are generated early in the course of development of mesothelioma in these models
 - Syngeneic tumors in rodents will also demonstrate the development of AGAs of which some are identical to those developed by exposure to asbestos. This could have implication in the future with a more consistent PGA in order to find early AGAs in humans.
 - Chemotherapy, specifically Gemzar, also through potential immune modulating mechanisms also generated the production of AGAs and some of these are identical to those generated by asbestos exposure.

5. REPORTABLE OUTCOMES;

Nothing to report

6. CONCLUSIONS

There is no doubt that asbestos exposure as well as exposure to other fibers will generate anti glycan antibodies. Some AGAs are present in animal who develop mesothelioma and are different from those with silica exposure which did not generate mesotheliomas. These AGAs could have implications for early asbestos carcinogenesis. The PGA 400 is not ready for prime time with regard to separating asbestos exposed individuals from those with malignancy. Future studies, if funded, must investigate why the platform failed with the human studies and whether it was a technical, specimen, or cohort problem.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

- Vuskovic MI, Xu H, Bovin NV, Pass HI, Huflejt ME. Processing and analysis of serum antibody binding signals from Printed Glycan Arrays for diagnostic and prognostic applications (2011). *Int J Bioinf Res App.* 2011; 7: 402-426. PMID: 22112531.
- Vuskovic, MI, Fang, H., Pass, HI, and Huflejt, ME. (2011). Optimal Combination of Glycan-Based Serum Diagnostic Markers Which Maximize AUC. *Proc. of the 15th World Multi-Conference on Systemics, Cybernetics and Informatics (WMSCI 2011) Vol. II:182- 187.* Copyright by the International Institute of Informatics and Systems, 2011 ISBN -978- 1-936338-30-6 (Vol. II).
- Bovin N, Obukhova P, Shilova N, Rapoport E, Popova I, Navakouski M, Unverzagt C, Vuskovic M, Huflejt M. (2012). Repertoire of human natural anti-glycan immunoglobulins. Do we have auto-antibodies? *Biochim Biophys Acta.* 2012; 1820(9):1373-82. PMID: 22365885.

- 2009: Glyco-Immune and Glyco-Genomic Diagnostic and Prognostic Features of Malignant Mesothelioma. Mesothelioma Working Group Meeting; Early Detection Research Network - NCI, September 2009; Bethesda, MD.
- 2009: A link Between Profiles of Serum Anti-Glycan Antibodies and Changes in Glyco-Gene Expressions: Diagnostic and Prognostic Implications. 3rd Annual World Congress of Gene-2009, Foshan, China, December 1-7, 2009. (Invited presentation; Session Chair).
- 2010: Glycomics Identifies Immuno-Therapeutic Targets of Malignant Mesothelioma. Mesothelioma Applied Research Foundation International Symposium on Malignant Mesothelioma, June 12th, 2010, Washington, DC. (Invited Presentation).
- 2010: Glycomics Identifies Diagnostic Biomarkers and Immuno-Therapeutic Targets of Malignant Mesothelioma. International Mesothelioma Interest Group; 10th International Conference; September 1st, 2010, Kyoto, Japan (Invited Presentation).
- 2012: Glycomics as a Source of Biomarkers in Malignant Mesothelioma. International Mesothelioma Interest Group; 11th International Conference; September 11st, 2012, Boston, MA. (Key Note Lecture, and Invited Presentation).
- 2012: Glycomics in Malignant Mesothelioma. Lung and Mesothelioma Working Group Meeting; Early Detection Research Network - NCI, October 2nd, 2012, San Antonio, TX.

7. INVENTIONS, PATENTS AND LICENSES:

- **PCT/US2011/030005 International application. (WO 2011/119967, September 2011). System, Method and Computer-Accessible Medium for Evaluating a Malignancy Status in At-Risk Populations and During Patient Treatment Management.**
 - **Inventors: M.I. Vuskovic, M.E. Huflejt H.I. Pass.**

9. OTHER ACHIEVEMENTS

- **Nothing to report**

10. Reference List

- (1) Kayser K, Bohm G, Blum S, Beyer M, Zink S, Andre S, et al. Glyco- and immunohistochemical refinement of the differential diagnosis between mesothelioma and metastatic carcinoma and survival analysis of patients. *J Pathol* 2001;193:175-80.
- (2) Lucena MC, Carvalho-Cruz P, Donadio JL, Oliveira IA, de Queiroz RM, Marinho-Carvalho MM, et al. Epithelial mesenchymal transition induces aberrant glycosylation through hexosamine biosynthetic pathway activation
1. *J Biol Chem* 2016.
- (3) Mohammad T, Garratt J, Torlakovic E, Gilks B, Churg A. Utility of a CEA, CD15, calretinin, and CK5/6 panel for distinguishing between mesotheliomas and pulmonary adenocarcinomas in clinical practice. *Am J Surg Pathol* 2012;36:1503-8.

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|------------------------------|---|
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| Contribution to Project: | <i>Actively involved in all tasks proposed under Specific Aims, supervises research assistants, coordinates research activities and together with Dr. Huflejt works closely with consultants on data analyses and the interpretation of the study results.</i> |
| Funding Support: | <i>U01 EDRN The North American Mesothelioma Consortium: Biomarker Discovery Laboratory, U01 Identifying non-coding RNAs for early detection and prevention of lung cancer</i> |
| | |
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| Nearest person month worked: | <i>3.00</i> |
| Contribution to Project: | <i>Printing and development of PGA slides, immunoprofile analyses, auto antibody signal quantification, biological and mathematical data evaluation and interpretation. Preparation of affinity-purified rat anti-glycan antibodies to be used as a component of therapeutic protocol. Works together with Dr. Pass on data analyses and the interpretation of the study results.</i> |
| Funding Support: | <i>. U01 EDRN The North American Mesothelioma Consortium: Biomarker Discovery Laboratory</i> |
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| Contribution to Project: | <i>Performed majority of work with experimental animals, and involved in immunoprofiling and analyses of animal sera using PGA, as well as signal quantification, and statistical data pre-processing. maintenance documentation and record keeping, and participate in all meetings and discussions related to data evaluation and the interpretation of the study results</i> |
| Funding | <i>No other grant support</i> |

| | |
|------------------------------|---|
| | |
| Name: | <i>Jordon Preiss</i> |
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| Nearest person month worked: | <i>1.80</i> |
| Contribution to Project: | <i>Performed majority of work with experimental animals, and involved in immunoprofiling and analyses of animal sera using PGA, as well as signal quantification, and statistical data pre-processing</i> |
| Funding Support: | <i>U01 EDRN The North American Mesothelioma Consortium: Biomarker Discovery Laboratory, U01 Identifying non-coding RNAs for early detection and prevention of lung cancer</i> |