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April 24, 2014

Defense Technical Information Center
8725 John J. Kingman Road, Ste 0944
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RE: Grant Award No. N00014-08-1-0341
Principal Investigator: Michael G. Janech, PhD

To Whom It May Concern:

The Medical University of South Carolina is submitting one original and one copy of the enclosed report pursuant to the reporting instructions in Modification No. A00003 of the above referenced award.

Please don't hesitate to contact this office if further information is needed. Thank you.

Sincerely,

Susan A. Greene

Susan A. Greene, CRA
Grants & Contracts Administrator

CC: Michael G. Janech, PhD

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
04/24/2014	Final Technical Report	09/05/2007-01/04/2014

4. TITLE AND SUBTITLE
Identification and Validation of Plasma Biomarkers in California Sea Lions

5a. CONTRACT NUMBER

5b. GRANT NUMBER
N00014-08-1-0341

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Michael G. Janech, PhD

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Medical University of South Carolina
179 Ashley Avenue
Charleston, SC 29425-8908

8. PERFORMING ORGANIZATION REPORT NUMBER
Final

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Office of Naval Research
100 Alabama St. SW
Suite 4R15
Atlanta, GA 30303-3104

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
None

20140501051

13. SUPPLEMENTARY NOTES
This report was also emailed to ONR's Technical Representative, Dr. Laura Kienker at laura_kiender@onr.navy.mil

14. ABSTRACT
This proposal focused on the discovery and validation of serum or plasma biomarkers for domoic acid toxicosis (DAT) in sea lions. Proteomic approaches were utilized to assess whether proteins could classify sea lions as DAT or non-DAT. Commercial cytokine arrays and MALDI-Tof peptide profiling can be utilized as screening tools in the diagnosis of acute DAT, but will only detect about 25% of those sea lions with DAT. 2D gel electrophoresis studies demonstrated that Apolipoprotein E and eosinophil counts combined resulted in a perfect marker of DAT in training set samples. External validation supported this combination as a biomarker with a test sensitivity of 86% and specificity of 85%.

15. SUBJECT TERMS
Domoic Acid Toxicosis, California sea lions, Biomarker, Serum, Plasma, Apolipoprotein E

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Michael G. Janech, PhD
					19b. TELEPHONE NUMBER *(Include area code)*
U	U	U	UU	35	843-792-1083

Final Technical Report

Grant Number: N00014-08-1-0341
Principal Investigator: Michael G. Janech
Performing Organization and address: Medical University of South Carolina, 171 Ashley Ave., Charleston, SC 29425. ENTITY IDENTIFICATION NUMBER: 157-6000-722-A2; DUNS NO:18-371-0748
Cong. District: SC-001
Grant Title: Identification and Validation of Plasma Biomarkers in California Sea Lions
Grant Period: 09/05/2007 - 01/04/2014

2. Abstract/Project Summary

Domoic acid toxicosis (DAT) is a major cause of sea lion stranding and die-offs. This proposal investigated sea lions with and without DAT to determine whether a biomarker of DAT could be determined from serum or plasma in effort to enable the Navy marine mammal program to screen animals for DAT. Several proteomic approaches were utilized and data were modeled using neural networks to assess whether proteins could classify individual sea lions as DAT or non-DAT. Commercial cytokine arrays and MALDI-Tof peptide profiling can be utilized as screening tools offering >90% accuracy in the diagnosis of acute DAT, but will only detect about 25% of those sea lions with DAT. Alternatively, these tools can also exclude a diagnosis of DAT with >98% accuracy, but will only detect 25% (cytokine array) or 60% (MALDI-Tof) of non-DAT sea lions. 2D gel electrophoresis studies of chronic DAT sea lions demonstrated that Apolipoprotein E and eosinophil counts combined in a neural network model resulted in a perfect marker of DAT in training set samples. External validation supported this combination as a biomarker with a test sensitivity of 86% and specificity of 85%. This combination performed the best of all markers in this study.

3. Scientific Technical Objectives

1. Methodological development for high abundance protein depletion from sea lion plasma.

2. Assess the performance of commercial cytokine arrays to predict domoic acid toxicosis in sea lions. Assess the value of Artificial Neural Network analysis to enhance performance of diagnostic tests. Validate biomarkers using investigator-blinded plasma samples from The Marine Mammal Center.
3. Assess the performance of MALDI-ToF mass spectrometry peptide profiling of serum to predict acute domoic acid toxicosis in sea lions. Assess the value of Artificial Neural Network analysis to enhance performance of diagnostic tests. Validate biomarkers using investigator-blinded plasma samples from The Marine Mammal Center.

4. Assess the performance of 2D gel electrophoresis of plasma proteins to predict chronic domoic acid toxicosis in sea lions. Assess the value of Artificial Neural Network analysis to enhance performance of diagnostic tests. Validate biomarkers using investigator-blinded plasma samples from The Marine Mammal Center.

5. **Ancillary Studies:** compare cerebral spinal fluid proteins by tandem mass spectrometry between acute DAT, chronic DAT, and non-DAT sea lions. Compare serum from DAT and non-DAT sea lions using iTRAQ.

4. Approach

1. Methodological development for high abundance protein depletion from sea lion plasma.

California sea lions plasma samples are collected by Dr. Frances Gulland and qualified technicians ate The Marine Mammal Center, Sausalito, CA.. High abundance proteins were depleted from sea lion plasma utilizing several commercially available depletion columns and Proteominer™ ligand library bead columns. Depletion ability was compared using 2D gel electrophoresis to determine the procedure that resulted in the most diverse number of protein spots with least amount of albumin.

2. Assess the performance of commercial cytokine arrays to predict domoic acid toxicosis in sea lions.

Luminex cytokine bead arrays were utilized to estimate the relative quantity of plasma or serum cytokines in sea lions with DAT or without DAT. All data were collated and statistical comparisons were made. Additionally Artificial Neural Networks were trained to discover hidden relationships between cytokine levels across both sea lion groups. Groups of analytes that have the greatest predictive value based on ROC analysis were validated in an investigator-blinded study.

3. Assess the performance of MALDI-ToF mass spectrometry peptide profiling of serum to predict acute domoic acid toxicosis in sea lions.

Serum peptides from different groups of sea lions (N=107): 1) Acute DAT, 2) Diseased but not DAT, 3) Navy Marine Mammal Program sea lions; were purified by c18 zip tip columns and analyzed by MALDI-TOF mass spectrometry. Mass features were aligned, smoothed, and normalized across samples by total ion intensity or glufibrinogen internal control peptide (Progenesis MALDI). Individual masses were assessed as biomarkers using area under receiver area operator characteristic (AuROC) curves. Combinations of features were assess as markers using combinations of artificial neural networks. Best performing features or models were validated using an investigator blinded test set of serum samples from The Marine Mammal Center (N=20).

4. Assess the performance of 2D gel electrophoresis of plasma proteins to predict chronic domoic acid toxicosis in sea lions.

Plasma samples from 20 sea lions were depleted and analyzed by large format 2D gel electrophoresis. Gels were stained with SyproRuby stain and aligned by Same Spots (Progenesis). Differentially abundant protein spots were removed for identification. Spot volumes were further analyzed for individual AuROC curve performance. Proteins in excess of 0.7 were used to train artificial neural networks. Top networks were validated using an external test set (N=10). Attempts to identify all spots utilized in model building were made

by tandem mass spectrometry. Attempts to validate 2D gel results were made using western blotting.

5. Ancillary Studies: compare cerebral spinal fluid proteins by tandem mass spectrometry between acute DAT, chronic DAT, and non-DAT sea lions. Compare serum from DAT and non-DAT sea lions using iTRAQ.

A pilot study to investigate cerebral spinal fluid as a potential source of markers for DAT was undertaken to utilize new technology in the laboratory. CSF proteins were isolated from acute, chronic, and non DAT sea lions. Proteins were identified by tandem mass spectrometry. Protein differences were compared using label-free spectral counting. Assays for quantitypic peptides for 6 CSF proteins were developed. Synthetic peptide standards were customized and performance measures of the assays were calculated. Limit of detection, Limit of quantification, precision and standard response curves were calculated for these peptides for the measurement of sea lion-specific proteins in cerebral spinal fluid.

A second pilot study was undertaken using iTRAQ labeling of serum proteins from sea lions with DAT and non-DAT to compliment the 2D gel project. Serum samples were digested and analyzed by LC/MS/MS. Differences in proteins were estimated using balanced reporter ions.

5. Accomplishments

1. Methodological development for high abundance protein depletion from sea lion plasma.

In plasma biomarkers studies of mammals, albumin, immunoglobulins, and 18 additional proteins comprise 99% of the total plasma protein. This leaves 1% of the plasma proteins relatively invisible to detection by proteomic techniques. In order to investigate a wide range of proteins as biomarkers for California sea lion, many of the abundant protein must be depleted. There are several commercially available columns which have been thoroughly investigated in mice, rats, chimpanzees, and humans; however, these columns remain untested for marine mammals. To resolve the issue of high abundance plasma protein depletion in sea lion, we set out to test commercially available depletion columns and determine their suitability for sea lion plasma protein depletion. Immunodepletion columns are most commonly utilized in plasma and serum proteomic studies, but these columns are usually highly specific for the animal to which the antibodies were raised. Therefore, we also chose to investigate a non-species specific column such as the AURUM albumin/Ig depletion column (Bio-Rad). In December 2007, a new column was released by Bio-Rad called Proteominer Ligand Library bead depletion columns. These columns were not initially selected because they were novel and untested; however, we decided to include these columns in the sea lion study June 2008 following a series of testing of the Proteominer columns in the Nephrology Proteomics Lab at MUSC (The PI is assoc. director of this lab).

Depletion columns were tested on frozen plasma samples collected at The Marine Mammal Center and sent to MUSC on dry ice. Blood samples were collected in citrate tubes to prevent coagulation prior to centrifugation. No protease inhibitors were utilized in this test set. Initially three depletion columns were tested for ability to deplete albumin and immmunoglobulins from sea lion plasma samples. Aurum serum protein mini-kit (Bio-Rad), Proteoprep20 plasma immunodepletion kit (Sigma Chemical), and Proteomelab IgY plasma depletion kit (Beckman) were chosen as the test depletion columns after consulting with companies that have tested these columns on canine and farm animal serum samples. Given sea lions are more closely related to canines, this was our rationale for choosing these products.

Plasma samples were prepared by filtration through 0.1μM filters to remove any cell debris and bacteria. Following filtration, 200-500μg protein was depleted according to specific manufacturer protocols. Immunodepletion columns should bind albumin and any high abundance protein to which conjugated antibodies are present in the column. Aurum columns are not immunodepletion columns, but contain a resin which has been shown to bind to albumin and immunoglobulins, irrespective of species. Depleted samples were precipitated in 5vol. acetone and washed with 75% ethanol. Protein concentration was measured by colorimetric assay (Bradford method, BioRad) and 50μg protein was added to a compatible buffer for 2DE (7M urea, 2M thiourea, 2% CHAPS, 0.2% Biolytes, 1% DTT). Proteins were loaded onto an 11 cm IPG strip (pH 3-10) and focused in a Protean IEF cell (Bio-Rad, Hercules, CA) for 100,000 Volt-hours with a maximum voltage of 8000 Volts and a maximum current of 50 μA/strip. After focusing, proteins were separated by SDS polyacrylamide gel electrophoresis on an 8-16% gradient gel using a Criterion Doceca cell (Bio-Rad). Gels were washed with deionized water, fixed with 10% methanol/7% acetic acid, stained overnight in the dark with Sypro Ruby

(Invitrogen Molecular Probes, Carlsbad, CA), destained with 10% methanol/7% acetic acid and imaged on an FX Pro Plus fluorescent imager (Bio-Rad). Images were analyzed using PDQuest software version 7.1. Spots were automatically detected and matched followed by manual editing of spots and spot alignment by an experienced user to improve detection and eliminate artifacts. Spot intensity was normalized to global intensity. **Figure 1** depicts 2D gels from undepleted, Aurum column depleted, ProteomeLab IgY column depleted, and Proteoprep 20 column depleted samples. High abundance spots that corresponded to the size and PI of Albumin were picked by robot and tryptic digested for mass spectrometric identification. Albumin was identified by MALDI-TOF-TOF mass spectrometry using the MASCOT algorithm which matched MS/MS data to canine albumin (score not shown, but was significant). Albumin was then quantified and the normalized intensity compared across gels. For all columns, the ProteomeLab IgY performed the best resulting in 94% fractional depletion of albumin. The other

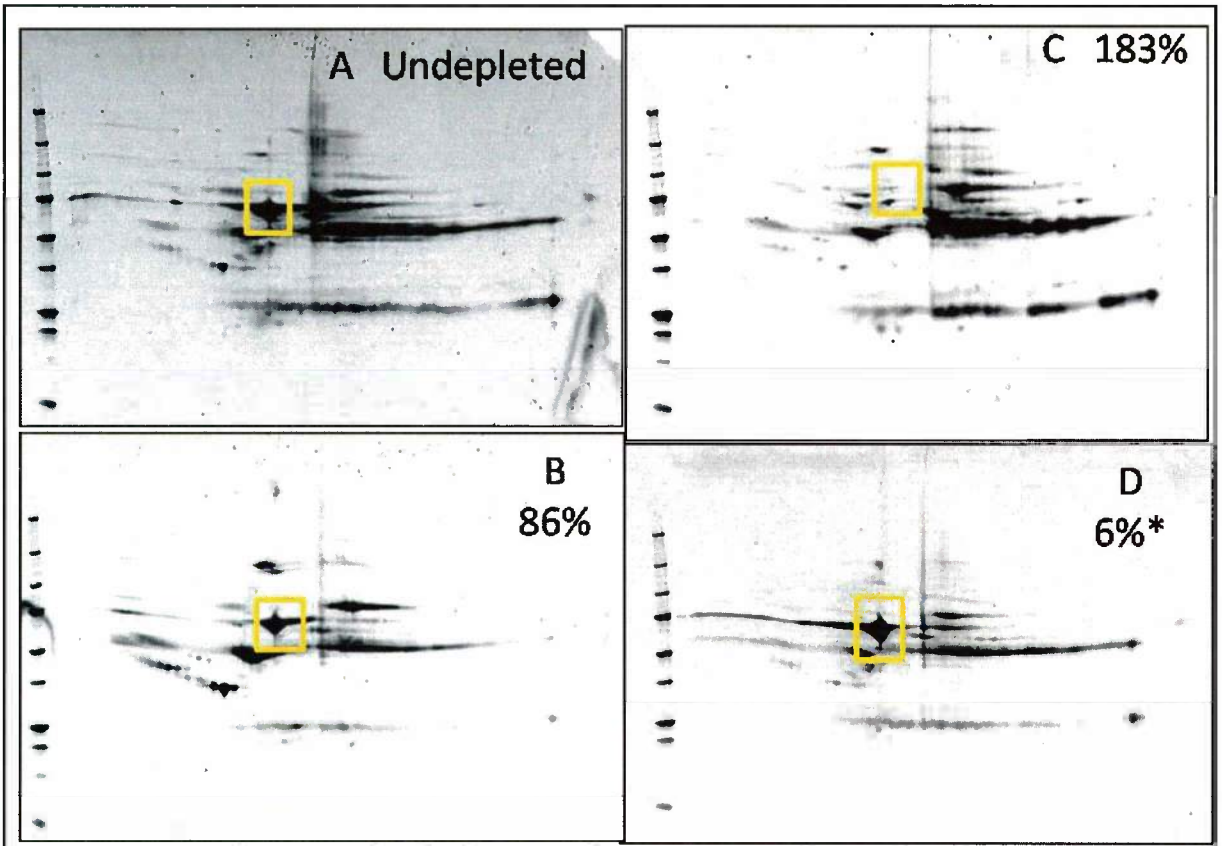

Figure 1. Two-dimensional gels showing plasma proteins from A) undepleted sea lion plasma. B) Aurum column depleted plasma. C) ProteomeLab IgY columns. D) Proteoprep columns. Numbers indicate fractional abundance of albumin from the depleted sample to the undepleted sample (spots normalized to total spot intensity within the gel). Albumin depletion was greatest by ProteomeLab IgY column.

two columns did not deplete albumin as well as the ProteomeLab IgY columns, but did appear to result in fewer immunoglobulin proteins (large horizontal streaks

Although IgY columns appear to be the best choice for high abundance protein depletion for sea lion plasma - there are two major issues with this technique: 1) immunodepletion columns must be reused and are prone to incomplete recharging which results in cross-contamination between samples as the column is reused over and over. 2) Antibodies are proteins and over time, the proteins will degrade leading to incomplete depletion and inconsistent results. In an effort to avoid these problems, we decided to test albumin and IgG depletion using Proteominer ligand library bead columns. Ligand library beads are hexameric peptides conjugated to agarose or sepharose beads. The idea behind ligand library beads is that many plasma proteins bind peptides and protein domains. Instead of creating a group of peptides to which every plasma protein will bind, a random hexamer library was created so that all proteins will have a chance to bind to a specific peptide. Because there is a finite number of ligands, proteins that are in excess will not have a chance to bind and will flow through the column. Proteins that are in lesser abundance will bind completely to the column. After elution, high and low abundance proteins will be more equally represented. One of the best aspects to these columns is that they are single use and therefore not subject to cross contamination and degradation after many uses is no longer an issue.

1mL of plasma (50mg/ml) was loaded onto a Proteominer column and bound proteins eluted according to the manufacturer's protocol. Because the Proteominer elution buffer is not optimal for 2D gel electorphoresis, we were only able to load 15µgs protein onto the 2D gel. **Figure 2** shows a Proteominer depleted plasma sample. Albumin was difficult to observe and the area where albumin should reside is encircled. Proteomic analysis of the top 15 abundant plasma proteins from sea lions using LC/MS/MS pre and post proteominer depletion demonstrated that the Proteominer depletion strategy provided ample reduction in albumin and immunoglobulins. These ligand library bead columns consistently provided the best depletion of albumin and several high abundance proteins compared to three other immunoaffinity columns available through commercial vendors.

2. Assess the performance of commercial cytokine arrays to predict domoic acid toxicosis in sea lions.

This aim underwent several iterations as the project transitioned from the graduate student to the postdoctoral associate. Preliminary findings from 2009/2010 demonstrated that cytokine multiplex analyses of sea lion serum using human bioplex 27 cytokine panels (Bio-Rad) could offer an accurate test to discriminate between sea lions with DAT and those without DAT. Artificial neural networks were utilized to create models which together with 27 cytokines would

be capable of diagnosing sea lions with DAT or non-DAT. Although accurate for classifying sea lions without DAT (negative predicted value = 100%), the test was not specific (18%). We interpreted this test to mean: if the test is negative then the diagnosis was highly confident that the sea lion was negative for DAT, but less than 20% of those non-DAT sea lions would be detected in this manner.

In 2010, we intended to elevate the performance of this test by including more sea lions in the training set of the artificial neural network as well as creating groups of sea lions which were frequency matched so that the sea lion stranding population would be better represented as a whole in terms of gender and age. Prior to this task it was necessary to create an itemized and collated sample database. In this study sera from 110 sea lions [35 acute DAT, 75 nonDAT] were screened using the human 27-cytokine panel (Bio-Rad) using identical methods established by the graduate student in 2009. Samples obtained from the Marine Mammal Center were frequency matched and 20 serum samples from the Navy Marine Mammal Program were included to ensure representation from sea lions that are known not to have a history of domoic acid toxicosis with a high degree of certainty. Sample clinical data are approximately identical for that described in the MALDI profiling experimental methods section below.

Individual cytokines were measured with the assumption that cross-reactivity between sea lion samples would be equal if not accurate given that the antibodies were designed to human proteins. No cytokine was an individual predictor of domoic acid toxicosis as area under receiver operator characteristic (AuROC) curves were less than 0.62.

Artificial neural networks were created to find multidimensional relationships between the data that could be utilized to discriminate between DAT and non-DAT. For this effort, we automated model generating capabilities such that 101 neural networks could be rapidly constructed and tested thereby allow us to compare model performance across a larger number of models. In addition, we noticed that some cytokines were not always detectable in a majority of samples. In reaction to this result, we also created models containing cytokines that were present in 80% and 90% of sea lion samples. Further, we trained models using both raw and quantiled data. Data quantiling was utilized to enhance signal to noise. In all, 606 models were created from the raw and permutated data sets combined. AuROC curves

	Exclusion of DAT		Prediction of DAT
	Quant 9 cytokines	Quant 19 cytokines	Quant 9 cytokines
Model#	33	49	33
AUC	0.94	0.96	0.94
Threshold	1E-15	7E-0.6	0.9999
	Training Performance		
Sens	1.00	0.97	**0.94**
Spec	**0.09**	**0.68**	0.96
PPV	0.34	0.59	**0.92**
NPV	**1.00**	**0.98**	0.97
	Qualification Performance		
Sens	1.00	1.00	**0.30**
Spec	**0.17**	**0.25**	1.00
PPV	0.50	0.53	**1.00**
NPV	**1.00**	**1.00**	0.63

Table 1. Statistical performance measures calculated for the training set and test set from the two best performing models. Model 49 was the best performing model for the exclusion of DAT (100%NPV) based upon the test set. Model 33 was the best performing for the prediction of DAT (100% PPV) based upon the test set.

were utilized to estimate training performance and four thresholds were chosen to calculate statistical performance measures based on optimal threshold, minimal misclassification, highest negative predictive value and highest positive predictive value.

To estimate performance of each model, we utilized serum samples from 20 sea lions that were not utilized to create the model (Test set). Samples were blinded to the investigator and data collected in the identical manner as done for the training set. For trained models using quantiled data, the test data set was converted prior to prediction. Sensivity, specificity, negative predictive value and positive predictive value was calculated for all test set data at each threshold calculated from the training set ROC curves. **Table 1** highlights two 'best' models for the exclusion of DAT or prediction of DAT. For the exclusion of DAT model 49 utilized 19 cytokines which were quantiled. The test-set performance estimates a specificity of 25% with a 100% negative predictive value. The negative predictive value for the training set performance was also high (98%) suggesting that 19 cytokines together with neural network modeling can find sea lions without DAT with high accuracy (>98%), but with very low sensitivity (25%) For the prediction of DAT, model 33 was the 'best' and utilized 9 quantiled cytokines. Statistical performance measures calculated for the test set and training set suggest that this test is also highly accurate and can predict DAT with >92% confidence, but will only detect 30% of those sea lions with DAT.

Overall, we believe that cytokine analysis and artificial neural networks can be utilized as a rapid discriminatory screening tool. Depending upon the model being utilized and the screening need, cytokine profiling can offer a level of confidence to a diagnosis or exclude a diagnosis of DAT, but low sensitivity or specificity limit the utility of this tool in population-wide studies.

3. Assess the performance of MALDI-ToF mass spectrometry peptide profiling of serum to predict acute domoic acid toxicosis in sea lions. Assess the value of Artificial Neural Network analysis to enhance performance of diagnostic tests. Validate biomarkers using investigator-blinded plasma samples from The Marine Mammal Center.

As described in the specific aim 1 of the original proposal, MALDI-TOF peptide profiling was completed for 107 sea lions to estimate whether features in the serum could accurately classify sea lions with DAT. The selection of serum samples were greatly enhanced by the creation of the serum sample database constructed in August/September 2010. This aim is considered complete by the PIs and co-PIs; however, we are currently working towards identifying individual peptides to determine whether any insight can be gained into mechanisms of DAT and whether protein degradation products may reflect potential parent protein biomarkers in the serum. Although we realize that MALDI-TOF profiling may be technically difficult and is not considered a point-of-care diagnostic, we feel that this test can be translated into a centralized laboratory setting for send-out diagnostics. A majority of effort from 2010 was placed on completing MALDI-TOF profiling.

Experimental design and methods.

Inclusion Criteria. Serum samples were acquired from the Marine Mammal Center (TMMC; Sausalito, CA) and the U.S. Navy Marine Mammal Program (USNMMP; San Diego, CA). Samples from the USNMMP had no selection criteria applied and were placed in an independent group (NAVY; n=20). These samples were collected between 2000 and 2008, and at the time of

sampling 7 of 20 exhibited clinical signs, 15 of 20 were fasting, and 7 of 20 were initial samples taken upon admission to the USNMMP. Inclusion criteria were applied to available samples at TMMC using available clinical parameters available. We retrospectively identified serum samples collected from 2,343 live California sea lions that stranded along the central California coast between 2005 and 2010. Of these, only sera which were drawn within seven days of admission to TMMC were included, which included sera from approximately 2,000 sea lions. We included sera from both sexes and adult, subadult, juvenile, and yearling age classes while attempting to frequency match these criteria between groups in the training set. Diagnoses were retrospectively confirmed and sera were placed into two groups: those suffering from acute domoic acid toxicosis (acute DAT group), individuals asymptomatic for DAT (non-DAT). Individuals with DAT were identified based on clinical signs such as seizures or neurological symptoms and the presence of domoic acid in bodily fluids (urine, feces, milk, aqueous humor) provided additional DAT confirmation in some cases. Acute DAT cases were differentiated from individuals with chronic DAT or with available brain histology (atrophy indicated chronic DAT). Furthermore individuals placed in the acute DAT group could not progress to chronic DAT. Individuals with acute DAT as well as an additional confounding etiology such as carcinoma or leptospirosis infection were rejected.

The non-DAT group could not have seizures or other neurological problems during their time in rehabilitation (regardless of etiology) or later strand with signs of DAT. Available DA results in bodily fluids were negative, and available histology could not indicate any hippocampal atrophy. This group included those suffering from renal failure associated with *Leptospira interrogans* (leptospirosis sub-group), and individuals without signs of exposure to either domoic acid or leptospirosis (non-DAT/non-leptospirosis group). Blood chemistry was used to confirm leptospirosis such that individuals with BUN > 100, Na > 150, creatinine > 2, and P > Ca were classified as having leptospirosis. Two individuals placed in the leptospirosis sub-group did not meet these criteria (CSL 9332 did not have Na>150 and CSL 7595 did not have creatine>2 or P>Ca), however leptospirosis was suspected. There were 11 individuals that did not have blood work results available to confirm the absence of leptospirosis, but given the absence of indications of leptospirosis they were placed into the acute DAT group and the non-DAT/leptospirosis group. Additionally, individuals not in the leptospirosis sub-group could not have had post-mortem observations characteristic of leptospirosis infection, such as swollen, pale kidneys and poor renicular differentiation at gross necropsy or interstitial nephritis on histology.

Exclusion Criteria. To limit confounding variables, known pregnant females (i.e., those that aborted in rehabilitation or with a fetus in uterus at necropsy) or individuals with significant trauma (e.g., missing limbs or life threatening wounds) were excluded from the study. Sera that were drawn more than 7 days after admission or collected by heart-stick or post-mortem were not included. In addition, serum samples that were not archived at -80°C the day of collection were not used.

Serum Collection and Storage. Sea lions admitted to TMMC had blood drawn into tiger top vacutainers, centrifuged, and the serum was decanted, aliquoted into freezer vials and frozen at -80°C the same day blood was drawn. Serum samples were collected from sea lions in the USNMMP, allowed to clot for 30 to 60 min before centrifuging. Less than 7h passed between clotting and storage at -80°C. Some samples were taken from anaesthetized individuals, 9 of 20 for USNMMP and an undetermined number of samples from TMMC. The internal training

sample set and independent test set were handled independently once received from the USNMMP or TMMC.

Experimental Design. Sera from the training set (n=107) were extracted and analyzed over two days. To limit the effect of interday MALDI-TOF variability, two groups were generated. This was accomplished by first using a randomized list of the 107 sera to separate two groups which were then balanced by the two main groups (DAT and non-DAT), the three non-DAT groups (non-DAT/leptospirosis, non-DAT/non-leptospirosis group, and NAVY), sex, age, year, and outcome (release or euthanasia/death). Day one consisted of 55 sera from 22 males (3 DAT, 5 leptospirosis, 4 other, and 10 NAVY), and 33 females (12 DAT, 2 leptospirosis, 18 other) of which 51% were released. Day two consisted of 53 sera from 21 males (3 DAT, 5 leptospirosis, 3 other and 10 NAVY) and 32 females (15 DAT, 3 leptospirosis, and 14 other) of which 47% were released. The independent test set (n=20) was blinded (identities and diagnoses) to the investigator until after analysis using a number scheme, and was run during one day.

Peptide Extraction. Each sera was diluted to 0.1% (v/v) trifluoroacetic acid (TFA) using 100 µL of 0.15% (v/v) TFA (Thermo Scientific, Rockford, IL). After 5 min incubation at room temperature, 10 µL of C8-magnetic beads (ClinProt™ Profiling Kit, Bruker Daltonics, Billerica, MA) was added, followed by three wash steps of 100 µL 0.1% (v/v) TFA according to manufacturer's guidelines. Peptides were eluted with 20 µL of 50% acetonitrile in manufacturer's stabilization buffer and 15 µL was transferred to a clean tube. Finally, 30 µL of matrix [5 mg mL^{-1} α-cyano-4-hydroxycinnamic acid (Bruker Daltonics) in HPLC grade methanol:acetonitrile:water (5:4:1) containing 25 nM glu-1-fibrinopeptide peptide mass standard (Glu-Fib; Protea Biosciences, Inc., Morgantown, WV)] was added, mixed, and 2 µL of the resulting solution was spotted onto a ground steel target plate (MTP 384 ground steel T F plate, Bruker Daltonics).

Spectra Acquisition. Matrix assisted laser desorption ionization time of flight (MALDI-TOF) spectra were acquired using a Bruker AutoflexIII. The raw spectra were then imported into Progenesis MALDI (Nonlinear USA Inc., Durham, NC). Peak intensities were normalized to the internal standard (Glu-Fib) or Total Ion Current (TIC) and analyzed separately based on normalization procedure. The independent test set was processed the same as the training set, and the training set spectra were used to facilitate alignment of the test set.

Receiver operator characteristic (ROC) Curve Analysis. Samples in the training set were dichotomized such that samples from the DAT group had an input of 1 and samples from the non-DAT had an input of 0, meaning that a positive indicates DAT.

Artificial Neural Network Analysis. The artificial neural network (ANN) algorithm was trained using Glu-Fib or TIC normalized peak data from the training dataset. Three different approaches were used: (i) the training set was divided into a sub-training set and sub-qualification set for cross-validation, (ii) the full training set was used, and (iii) the normalized peak data from the full training set was ranked across samples and expressed as quantiles. These training sets were used independently to train 101 feed-forward ANNs performed by Matlab. The median performing ANN(s) was selected using AuROC, as well as a Combinatorial ANN ($CANN_{101}$) that was the average of 101 ANNs. The external qualification dataset was processed identical to

the training set, with quantiles being determined using the training set rankings. *A priori* threshold values used for qualification were determined differently based on the approach. In the first approach, the internal qualification dataset was used to determine threshold values for either single ANNs or $CANN_{101}$ and the independent test set was run against ANNs trained on the sub-training dataset or the complete training dataset. In the second and third approach, thresholds were determined by ROC analysis of the training set

Table 2. Sea Lion frequency distribution: training set.

	DAT	non-DAT	NAVY
Total	34 (31.8%)	53 (49.5%)	20 (18.7%)
Male	6 (17.6%)	16 (30.2%)	20 (100%)
Female	28 (82.4%)	37 (69.8%)	0
Age			
pup	0	0	0
yearling	1 (2.9%)	8 (15.1%)	5 (25%)
juvenile	2 (5.9%)	6 (11.3%)	7 (30%)
sub-adult	3 (8.8%)	15 (28.3%)	2 (10%)
adult	28 (82.4%)	24 (45.3%)	6 (30%)
Euthansia/Death	21 (61.8%)	33 (62.3%)	0

Findings. Training set serum samples from TMMC (**Table 2**) were collected between 2005 and 2010, with 11.5% (n=10), 6.9% (n=6), 16.1% (n=14), 17.2% (n=15), 35.6% (n=31) and 12.6% (n=11) from 2005 to 2010 respectively. Although we defined 3 subgroups of the non-DAT group, for this study, they are treated as two groups: DAT and non-DAT. Since the majority of DAT samples available were from females, we attempted to frequency match the two groups. Furthermore, individuals from TMMC in the non-DAT group were selected to reflect etiologies common to stranded sea lions admitted to TMMC, which between 1991 and 2000 of 3,379 non-DAT individuals, malnutrition, leptospirosis, trauma, and miscellaneous comprised 35%, 30%, 19%, and 11% of cases respectively with carcinoma present in 3% of cases. In addition to the 107 sera used as a training set, we used an independent test set of 20 sera for qualification, and the identities and diagnoses were blinded to the investigator until after analysis. These 20 sera were from 2007 to 2010 were chosen to include 10 DAT and 10 non-DAT and in general reflect the types of cases seen at TMMC.

Hematological and serum biochemistry data were available for serum collected from 76 of the 87 individuals from TMMC and all 20 of the serum collected at the USNMMP, which corresponds to the draw date of scra used for peptide profiling. Individuals in the DAT group had significantly higher levels of red blood cell counts, hemoglobin, and hematocrit than the non-DAT group (1.1 to 1.2-fold) despite lower levels of BUN and BUN:creatine ratios (-5 and -2 fold, respectively). The DAT group also had lower levels of white blood cells and banded neutrophils (-1.6 and -2.5fold), but increased levels of lymphocytes and eosinophiles (1.3 and 2.4-fold). Levels of Na, Cl, Mg, P and Na/K ratios were lower in the DAT group (-1.1, -1.1, -1.3, -1.6, and -1.1-fold), while K was higher in the DAT group (1.1fold). Lastly, albumin was higher (1.3fold) and triglycerides and sorbitol dehydrogenase were lower (-3.3 and -1.4 fold) in the DAT group.

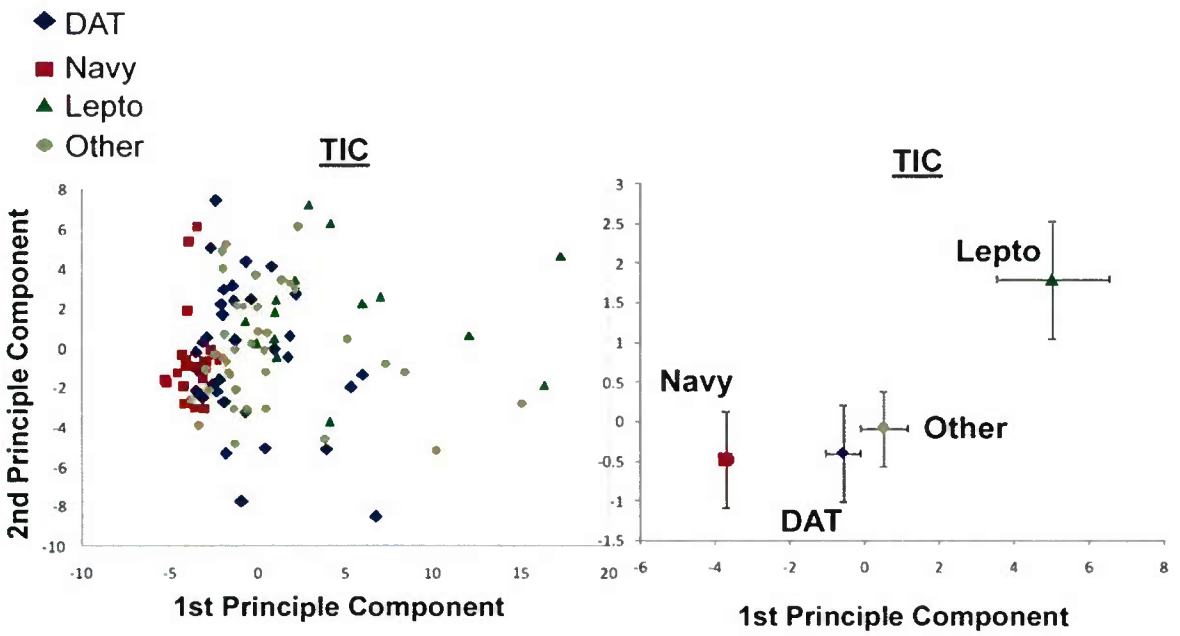

Figure 3. Principal components analysis of 104 MALDI peaks. Animals were designated as DAT, leptospirosis, other (other ailment), or NAVY (marine mammal program) to allow fine discriminatory analysis of the groupings.

Principle components analysis was utilized to determine whether exploratory analysis could discriminate between groups such as DAT, Leptospirosis, non-DAT without Leptospirosis, and NAVY sea lions (**Figure 3**). Plotting two principle components based on 104 MALDI peaks showed considerable spatial overlap between DAT and non-DAT groups. Only sea lions with leptospirosis and those from the NAVY Marine Mammal Program were distinctly separate, suggesting that features common to sea lions in general are also common to those with DAT. For this reason, supervised learning algorithms were the next logical step to discern whether multidimensional relationships could be informative in the discrimination of DAT from non-DAT.

To determine if a single peak could discriminate between the DAT and non-DAT group, receiver operator characteristic (ROC) curves were generated using normalized peak height. No

single peak had area under the curve (AuROC) > 0.8, therefore none were excellent classifiers of DAT (**Figure 4**). Peaks normalized using Glu-Fib had a mean AuROC of 0.543, ranging from 0.383 to 0.692. TIC normalized peaks had a mean AuROC of 0.538, ranging from 0.396 to 0.754. The top performer from TIC was peak 3017 m/z had an AuROC ± S.E. of 0.754 ± 0.054 (**Figure 4**). Four different thresholds were determined *a priori* from the ROC curve and were qualified with the independent test set. Using a minimum mis-classified threshold we achieved 100% specificity but only 20% sensitivity with 8 of 10 DAT individuals being called incorrectly (Table 3). Further performance analysis of peak 3017 m/z using thresholds which increased sensitivity resulted in a decrease of specificity.

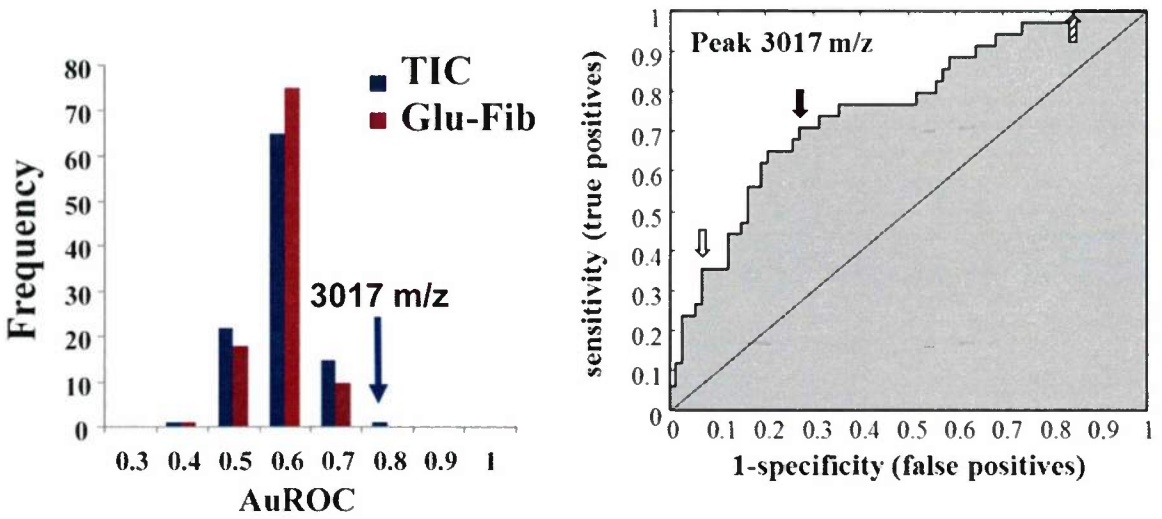

Figure 4. Individual peak AuROC. Peak 3017 M/Z had the highest AuROC = 0.75. No other peak demonstrated a good ability to classify sea lions with DAT.

In addition to evaluating individual peak performance for predicting DAT, we were also interested in whether there were peaks that predicted individuals in the NAVY group. When performance was evaluated for picking NAVY samples versus non-NAVY, 21 TIC normalized peaks had AuROC > 0.8, and the best performer, 1362 m/z, had an AuROC ± S.E. of 0.979 ± 0.02 (**Figure 5**). Interestingly this peak was mostly absent in sera collected at TMMC. Using an optimum threshold, the individuals in the NAVY group were called correctly 18 of 20 times (90% sensitivity), and the two mis-called (#2 and #9) were both initial blood draws and SL#2 showed clinical signs (behavioral; poor performance). Moreover only four non-NAVY group individuals (CSL 6896, 9111, 9271 and 9770) were called incorrectly (95% specificity). Using the same threshold only one individual in the independent test set was called NAVY (data not shown; CSL 9766, an adult female with acute DAT which was released).

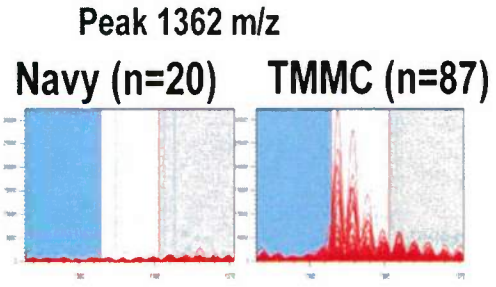

Figure 5. Peak 1362 m/z largely absent in NAVY samples.

Since no single peak was an excellent classifier of DAT (AuROC > 0.8), peak data were modeled using artificial neural network. Glu-Fib and TIC normalized peaks were used separately, and 101 artificial neural networks (ANNs) were trained using three different approaches. The first approach involved splitting the training set into a training set and qualification set for cross-validation, whereas the second approach utilized the complete training set. The third approach quantiled peak height across variables, and these quantiled data were used to train ANNs. Additionally, with all three approaches, two types of ANNs were chosen for qualification: median performing ANNs (based on a median AuROC) or a Combinatorial ANN ($CANN_{101}$). In the first approach, the internal qualification set was used to set thresholds for either single ANNs or $CANN_{101}$ and the independent test set was run against these models or a model(s) trained using the complete training set. In the second and third approach, thresholds for models were determined by ROC analysis of the training set without cross-validation.

Table 3. Qualification results of NRN or ANN models. TIC, total ion current normalized; Glu-Fib, glufibrinopeptide internal standard normalized; minMC, minimal misclassification threshold; optCO, optimal threshold; npvCO, negative predictive value optimized threshold; $CANN_{101}$, combination of 101 ANN models; Median, median ANN model.

		Peak 3017			NRN	ANN	
Patients	Outcome	TIC minMC	TIC optCO	TIC npvCO	TIC	TIC Median	Glu-Fib $CANN_{101}$
	1 = DAT 0 = Non-DAT						
CSL 7507	1	0	0	1	1	1	0
CSL 7778	0	0	0	0	0	0	0
CSL 7177	1	0	0	1	0	1	0
CSL 7813	0	0	0	1	0	0	0
CSL 9006	1	0	0	1	0	1	0
CSL 9023	0	0	0	1	0	1	0
CSL 8964	0	0	0	1	1	1	0
CSL 9278	1	0	0	1	1	1	0
CSL 8868	0	0	0	1	0	0	0
CSL 9058	1	0	0	0	0	1	0
CSL 9790	0	0	0	0	0	1	0
CSL 9771	1	1	1	1	0	1	1
CSL 9747	1	1	1	1	1	1	1
CSL 9353	0	0	0	1	1	1	0
CSL 8963	0	0	0	1	0	0	0
CSL 9810	0	0	1	1	0	0	0
CSL 9250	1	0	0	1	1	1	0
CSL 9280	0	0	0	0	0	0	0
CSL 9751	1	0	0	0	0	1	1
CSL 9766	1	0	0	1	0	1	0
Sensitivity		20%	20%	80%	40%	100%	30%
Specificity		100%	90%	30%	80%	60%	100%
Pos. Pred Value		100%	67%	53%	67%	71%	100%
Neg. Pred. Value		56%	53%	60%	57%	100%	59%

The generated models were qualified using a blinded independent test set of 20 sera, 10 DAT and 10 non-DAT. Using thresholds determined *a priori*, the performance of each model was evaluated by predicting a 1 or 0 for each of the 20 sera (**Table 3**). Compared to the single peak 3017 m/z which gave 100% specificity but only 20% sensitivity, using the different ANN approaches we achieved high specificity (100%) and high sensitivity (100%). Specifically, we found the best performance of Glu-Fib normalized data was 30% sensitivity and 100% specificity which was achieved using a median ANN (Glu-Fib-ANN_{53}). Relative to ANNs generated with Glu-Fib, models made using TIC normalized data achieved higher sensitivity (100% versus 40%) as well as high specificity

(90%). A negative predictive value of 100% was achieved using a median ANN (TIC-ANN_1) which was the highest seen in any model. This model predicted all 10 DAT individuals correctly with four false positives. The four individuals that were predicted incorrectly cannot be explained by sex, age, primary etiology or blood chemistry. Other median TIC ANNs had different performance measures despite the same AuROC, and overall using an optimum cut-off (OC) when different from a minimum mis-classified (minMC) cut-off resulted in higher sensitivity with minimum loss to specificity. For example, in the case of TIC-ANN_{67} and QuantiledTIC-ANN_{88}, the OC improved performance while maintaining the same specificity as the minMC.

4. Assess the performance of 2D gel electrophoresis of plasma proteins to predict chronic domoic acid toxicosis in sea lions. Assess the value of Artificial Neural Network analysis to enhance performance of diagnostic tests. Validate biomarkers using investigator-blinded plasma samples from The Marine Mammal Center.

Thirty plasma samples (known as the training set) were separated by 2DE using large format gels following proteominer depletion. The plasma samples were from 10 sea lions with DAT, 10 sea lions with leptospirosis, and 10 sea lions without DAT or Leptospirosis, but with another ailment. The rationale for choosing this distribution is a reflection of the distribution of disease in the stranded sea lions at the Marine Mammal Center. Second dimension gels were poured by hand; whereas previously we had purchased from Bio-Rad. Because of quality issues from the supplier, we decided to pour gels in-house and rerun several of the samples. Gels were post-stained with Sypro Ruby, imaged, and analyzed by Progenesis Same Spots. In the case where gels did not focus completely, the process was repeated for these samples until we could obtain a match set of high quality images for training downstream models. Statistical analysis was conducted and Receiver operating characteristic (ROC) curves were calculated for each spot to investigate whether one protein spot could be utilized as a biomarker of DAT alone.

Artificial neural network models were developed from the sea lion training set data. Statistical performance measures and area under receiver operating characteristic (ROC) curves were calculated for each of the matched spots (618 spots total). A total of 50 spots were selected for model inclusion and identification based on the ability of the spot to predict an outcome of DAT (area under ROC curve>0.75) or Q-value <0.05.

To validate candidate markers and models, 20 plasma samples blinded to the investigators were sent from the Marine Mammal Center. Each sample was treated the exact manner as the training set including proteominer depletion. Technicians at the Marine Mammal Center were directed to take plasma samples from stranded sea lions with a diagnosis of chronic domoic acid toxicosis. Samples were restricted to protocol criteria that adhered to those set for the training set.

Large format gels were post-stained with Sypro Ruby, imaged, and analyzed by Progenesis Same Spots. In the case where gels did not focus completely, the process was repeated for these samples until we could obtain a match set of high quality images for spot alignment. Test set gels were automatically matched against the training set master and manually validated. Spot numbers were assigned according to the training set master image. Normalized spot volumes were extracted from the test data and exported for marker and model qualification.

Validated protein markers of interest were identified LC/MS/MS. Tryptic peptides were separated by nano-LC and eluted into a 5600 triple TOF mass spectrometer using standard protocols. The top ten masses were selected for MS/MS fragmentation. Data were converted and searched in MASCOT using 10ppm parent ion tolerance and 0.5Da MS/MS tolerances. Oxidation of methionine and carbamidomethylation of cysteine was chosen as modifications. Data were searched against a refined proteomic database constructed in our lab using data downloaded from Swissprot. Species included in the database include, Panda, Dog, Human, Mouse, Sea Lion, Rat, Pig, and Cow in an effort to maximize discoverability, yet minimize false discovery. The criteria for assigning an ID to a protein was met when the MASCOT score for a protein exceeded 80 and at least two peptides matched a mammalian protein in the large database. Identifications (34/50) were supported from the initial gel analysis and were used. One protein spot originally identified as desmoplakin 3 (area under ROC curve = 0.08) was not supported in the second protein identification run and thus was not considered a reasonable identification. At this time, this protein spot is considered 'unknown'. However, an additional protein spot was identified with higher confidence, thus returning our total number of protein identified to 35/50. 15/50 protein spots remain unidentified most likely due to a lack of species specific protein information.

A volcano plot of the training set protein comparison is shown in **Figure 6**. Notably, most proteins did not exhibit a greater than 2-fold change in abundance and only two proteins were statistically different when corrected for false discovery rate (Apolipoprotein E). These proteins were reported in the 2011/12 report along with the statement that these proteins require further qualification as potential markers of DAT to be determined in the investigator-blinded test set. Protein spot volumes in the matched test validation set were separately compared to determine whether this statistical difference held true or was non-reproducible event. Table 1. lists the identified proteins by direction in fold change and an associated p-value for comparison. Numerous protein forms of Apolipoprotein E (APOE) were significantly lower in abundance in the DAT group. The two spots that were statistically lower in the training set were 2159 and 3486, both of which remained statistically lower in the test set providing some validation to the training set relationship. Perhaps confusing is

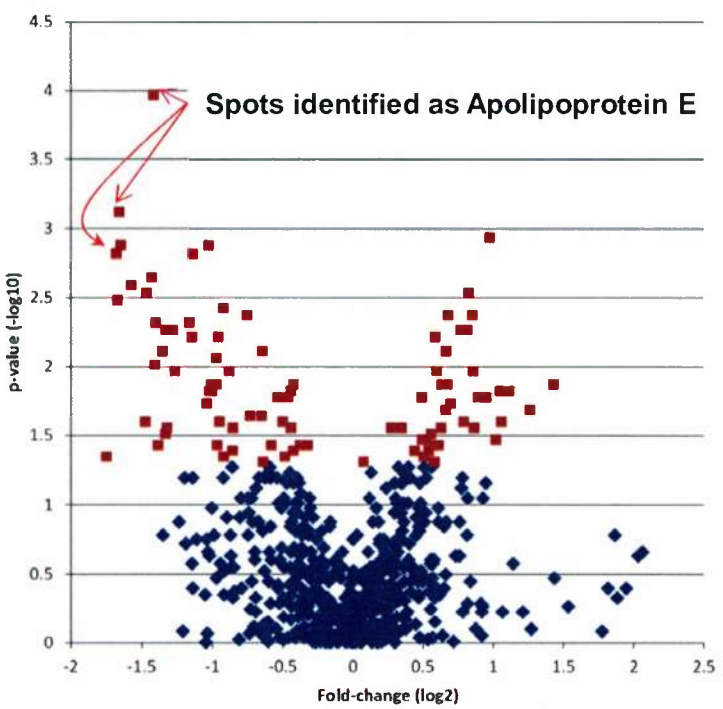

Figure 6. Volcano plot of fold change vs. p-value for protein spot abundance in the training set. Only 2 protein spots (Apolipoprotein E) were significantly lower.

the fact that so many of the spots are identified as APOE and that APOE is found in both the higher abundance group and lower abundance group. Because proteins are separated in 2 dimensions and the migration of the protein is a function of charge and molecular weight, any modification of the protein can affect the distribution of said protein in the gel. In the case of modification, proteins migrate to multiple spots in the gel and for the statistical comparison, each spot is treated as an independent observation. In the case of apolipoprotein E, we know that this protein is o-glycosylated in sea lions[1] and because we know the protein sequence, the identity of this protein is of high confidence. Other protein values listed in **Table 4** were included because they demonstrated predictive ability in the training set i.e. area under the ROC curve was >0.75. Of interest is the fact that 15/21 proteins with negative fold-change values were lipoproteins; whercas the proteins listed in the higher fold-change group tended to be more evenly distributed amongst common plasma proteins.

Higher or Equal in Abundance			Lower or Equal in Abundance		
Protein Name	**Fold-change**	**P-Value**	**Protein Name**	**Fold-change**	**P-value**
ApoE	3.0	0.17	ApoE	-8.8	0.047
Complement C4-A	2.3	0.49	ApoA-IV	-5.1	0.18
Vitronectin	2.2	0.13	ApoE	-5.0	0.03
Fibrinogen gamma chain	1.8	0.22	ApoE	-4.5	0.008
Carboxypeptidase N subunit 2	1.7	0.23	ApoE	-3.7	0.015
Vitamin D-binding protein	1.6	0.71	ApoA-IV	-3.6	0.002
Albumin	1.4	0.04	ApoA-IV	-2.8	0.005
ApoA-IV	1.3	0.01	Clusterin	-2.4	0.003
Antithrombin-III	1.3	0.11	ApoE	-2.3	0.02
Hemoglobin subunit gamma	1.2	0.55	Actin, cytoplasmic 1	-2.2	0.67
Fibrinogen gamma chain	1.2	0.00	Clusterin	-2.2	0.78
EGF-containing fibulin-like ECM protein 1	1.0	0.90	ApoE	-1.9	0.05
ApoE	1.0	0.63	Immunoglobulin J chain	-1.8	0.19
			ApoE	-1.8	0.06
			ApoE	-1.7	0.64
			Similar to Kappa Light Chain	-1.7	0.052
			heavy chain variable region	-1.6	0.11
			Similar to IgJ	-1.4	0.336
			Glutathione peroxidase	-1.4	2E-04
			ApoE	-1.3	0.31
			ApoA-1	-1.2	0.3

Table 4. Fold-change abundance of protein spots in the qualification test set that were statistically different or included in the training set based on area under ROC curve. Proteins higher in abundance in sea lions with DAT are listed in the left-hand column. Proteins lower in abundance in sea lions without DAT are listed in the right hand column. Apolipoproteins E and AIV dominate the population of spots that are lower in abundance.

Artificial neural networks created from the training set data were tested using the test set data. Networks are first created using all 50 spot data and then sensitivity-values (s-values) are tabulated. Networks are cross-validated internally to reduce overtraining, but cross-validation does not remove the possibility of overtraining. Because we realize that a 2D gel is unlikely to be an assay for a biomarker panel, we reduce the features included in the network by extracting the s-values (weighting factors) and iteratively re-computing the networks using only the highest s-values. For example, 21 models are built with only two spot data with the highest s-values. Then 21 models are built with only three spot data with the highest s-values and so on. Area under the curves are plotted and the point at which the models' performance plateaus is the minimal number of spots needed for the maximal predictive ability.

The final model we selected included 8 protein spots. From these 8 protein spot data in the training set we created 101 neural networks and allowed these networks to assign a prediction. Each prediction was then recorded as a 0 = nonDAT or a 1 = DAT. The votes were tabulated and the highest number of votes determined the classification. This was done to remove any investigator bias in model selection. Once completed, we tested our model's ability to predict the diagnosis in the investigator-blinded test set. Once the key was revealed, we noticed that the model tended to severely overestimate the DAT classification to the point that nearly all sea lions were considered to be afflicted with DAT.

Prior to the neural network modeling we had also created a series of smaller models using the two APOE spot data and clinical variables eosinophil count and hematocrit. The rationale for choosing these data were because we had found and published a significant relationship between acute DAT, eosinophil count, and hematocrit[2]. Individual APOE spot data was tested for marker performance in the test set as well as eosinophil count and hematocrit. These variables were further combined using neural networks to establish relationships that could be tested in the test set. Individually, the APOE spots performed below expectation (spot 2159, Sensitivity

Input Data	Sensitivity	Specificity	trAUC
Eosinophil count	71%	92%	0.53
Hematocrit	71%	46%	0.79
2 APOE spots + Eosinophils + Hematocrit	86%	85%	1
1 APOE spot (2159) + Eosinophils	86%	85%	1

Table 5. Statistical performance measures for eosinophil count, hematocrit and combinations of APOE spots with clinical values. Sensitivity and Specificity are based on thresholds determined in the training set. Area under the ROC curve for the training set is given in the column trAUC.

100%, specificity 38%; spot 3486, Sensitivity 86%, Specificity 100%, Sensitivity 62%).

In **Table 5** sensitivity and specificity were calculated for calculated for eosinophil count, hematocrit, APOE spots 2159+3486 + eosinophil count+ hematocrit, and APOE spots 2159+Eosinophil count. Combinations of APOE spot data and clinical data were assimilated into 101 feed-forward neural networks to allow voting as described above. Eosinophil count alone was a poor predictor of chronic DAT in the training set, although appears to be a reasonably good predictor for the test set. Hematocrit was a good predictor of chronic DAT in the training set, but is a poor predictor in the test set. The combination marker of APOE spots and clinical variables were a perfect predictor in the training set and remained very good at classifying the test set. The ability to classify correctly held true even when only a single APOE spot (2159) and eosinophils were utilized in combination. In fact, there was no difference between performance measures between 2APOE spots+eosinophils+hematocrit and a single APOEspot+eosinophils.

We have been attempting to determine whether total APOE levels can be used to discriminate between sea lions with DAT and nonDAT. The directionality of abundance for APOE forms in the 2DE experiments suggested that a majority of the spots are lower in abundance in sea lions with chronic DAT compared to other stranded nonDAT sea lions. Although one APOE protein spot was labeled as elevated with regard to fold-change it was not statistically significant. This suggests that absolute levels of APOE coupled with eosinophil counts may suffice as a marker and would be better positioned for a point of care test than charge and weight form measurements by 2DE. With this rationale in mind, we chose to investigate absolute levels by immunoblotting for APOE. One important question that we wish to answer with the immunoblot is: will the relationship of APOE with DAT exist when we normalize the levels to volume of plasma vs. fraction of protein. This is often overlooked, but extremely important as clinical tests are reported per volume of body fluid or as a ratio of a second measured analyte. They are not reported as per mg protein, although this could be done.

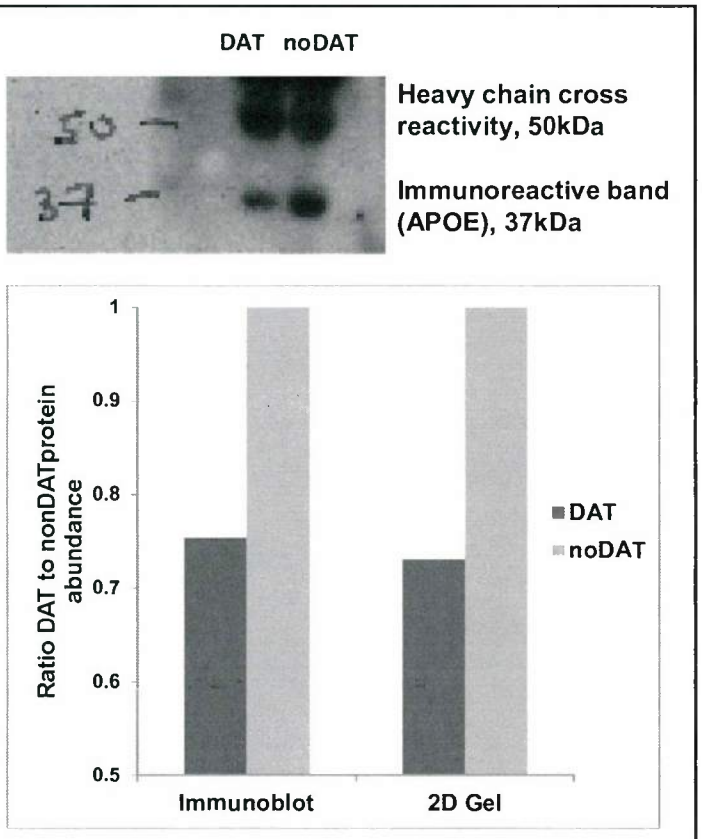

Figure 7. Immunoblot for sea lion APOE in plasma samples from animals with DAT and no DAT. High cross-reactivity with other plasma proteins has confounded the validation of APOE measurements. An immunoreactive band at 37kDa corresponds with the correct size of APOE for sea lion. Although antibody specificity remains a question, it is interesting that this band at 37kDa has the same quantitative relationship as APOE as determined by 2DE (Bar graph).

Immunoblots of APOE for sea lions has been published [1] on delipidated and

semi-pure apolipoprotein fractions. The PI contacted Dr. Steve Young to acquire an aliquot of the antibodies utilized in the 1991 paper [1]. Unfortunately Dr. Young was unable to located the antiserum utilized 22 years prior. We then took advantage of the high similarity between the c-terminal domains of human and sea lion APOE proteins and ordered a commercial antibody from Abcam for immunoblot analysis. The cross-reactivity of the antibody was such that it was primarily reactive against human APOE, but was listed to cross react with mouse and rat APOE as well. **Figure 7** illustrates a typical immunoblot for APOE in sea lion plasma. To test the antibody's ability to cross-react with a protein of similar molecular weight to APOE, lanes were loaded with a volume of plasma equivalent to 0.3µl which equals approximately 21µg protein/lane. Two different samples were loaded, one sample was from a sea lion with DAT and the other was from a sea lion without DAT. The APOE protein in sea lions is approximately 37kDa in weight, but has been reported to migrate as two separate bands due to o-glycosylation. The molecular weight is significantly higher than human APOE. Our results demonstrate that a protein of 37kDa is detected with the commercial APOE antibody; however, as is typical with immunoblotting of sea lion plasma, there is a high cross-reactivity with other plasma proteins. This problem has been described in previous reports from our laboratory and does not appear to be due to the choice of secondary antibody as we have exhaustively screen secondary antibodies from commercial vendors and have utilized different dilutions and blocking reagents. Sea lion plasma immunoblots are consistently dirty and data gained using this technique to validate identifications and markers was inconsistent.

We further compared the APOE 2DE results from spot 2159 with the immunoblot measured by densitometry. Setting the non-DAT protein abundance data to 1, the protein abdundance data from the sea lion with DAT was set relative to the non-DAT sample. In both the immunoblot and 2DE comparison, the abundances correlate very well and were almost identical. This result suggested that the immunoblot method may be a workable method from which to validate the APOE results for sea lions. The question that still remains is: Is this 37kDa band really APOE or is this an artifact of some non-specific protein that varies in a similar direction as APOE and is of a similar molecular weight. Before addressing this question we decided to run a larger set of plasma samples to determine whether the relationship is consistent. 52 sea lion plasma samples were run as described above (12% Bis-Tris PAGE). The gels were blotted and membranes probed for "APOE". The results demonstrated that the 37kDa band was detectable in about 50% of the samples. Because we loaded a standard reference on both sides of the gel, we were able to determine immediately that the problem with detection was due to inefficient transfer. We have spent the last two months attempting different transfer apparati, buffers and running conditions to no avail. This coupled to the fact that we are not certain that the antibody is specific has created a conundrum. At this point we are currently discussing whether to continue the validation by immunoblot or completely redirect the validation toward parallel reaction monitoring (quantitative tandem mass spectrometry) assays that we have developed for cerebral spinal fluid proteins. We are leaning toward the later because specificity of detection is very high confidence with mass spectrometry as parent and product ions for sea lion APOE are detected in undepleted plasma.

5. Ancillary Studies: compare cerebral spinal fluid proteins by tandem mass spectrometry between acute DAT, chronic DAT, and non-DAT sea lions. Compare serum from DAT and non-DAT sea lions using iTRAQ.

Tandem Mass Spectrometry and cerebral spinal fluid biomarkers.

In August 2010, the grant N000140810341 was reviewed. In the review, the referee suggested that the project should consider the analysis of cerebral spinal fluid (CSF) in the effort to discover biomarkers of DAT. Due to major limitations in CSF sample availability we had not previously considered this fluid for analysis. Based on the reviewer's recommendation we reassigned effort from the initial aim 3 proposed in the grant proposal to initiate a proteomic investigation of cerebral spinal fluid from sea lions. Due to sample limitations at The Marine Mammal Center, we were limited to 12 CSF samples for the initial analysis. A detailed description of the samples and rationale was provided in the 2010/11 Annual report.

To summarize our label-free proteomic findings from cerebral spinal fluid analysis, There were five CSF proteins that were considered differentially abundant **(Figure 8)** based on Fisher's Exact test: immunoglobulin lambda 6C (Igλ6-C), Gelsolin (GSN), Dickkopf-3 (DKK3), Neuronal Cell Adhesion Molecule 1 (NCAM1), and Oligodendrocyte myelin glycoprotein (OMG). Individually, all the proteins appear to have some value as biomarkers to discriminate DAT from non-DAT. To show that the proteins together can completely separate both

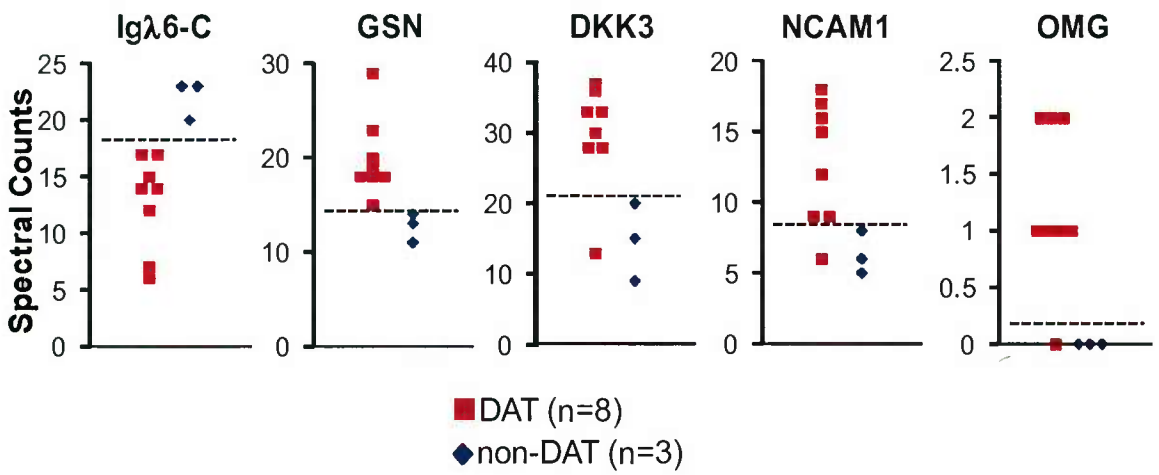

Figure 8. Spectral count data for each individual sea lion respective of 5 significant proteins identified using tandem mass spectrometry. Dashed line represents a threshold value that can best discriminate between DAT and nonDAT.

populations, we used principal components analysis to group proteins in an unsupervised manner. Based on these 5 proteins alone, two groups of sea lions could be easily discerned **(Figure 9)**.

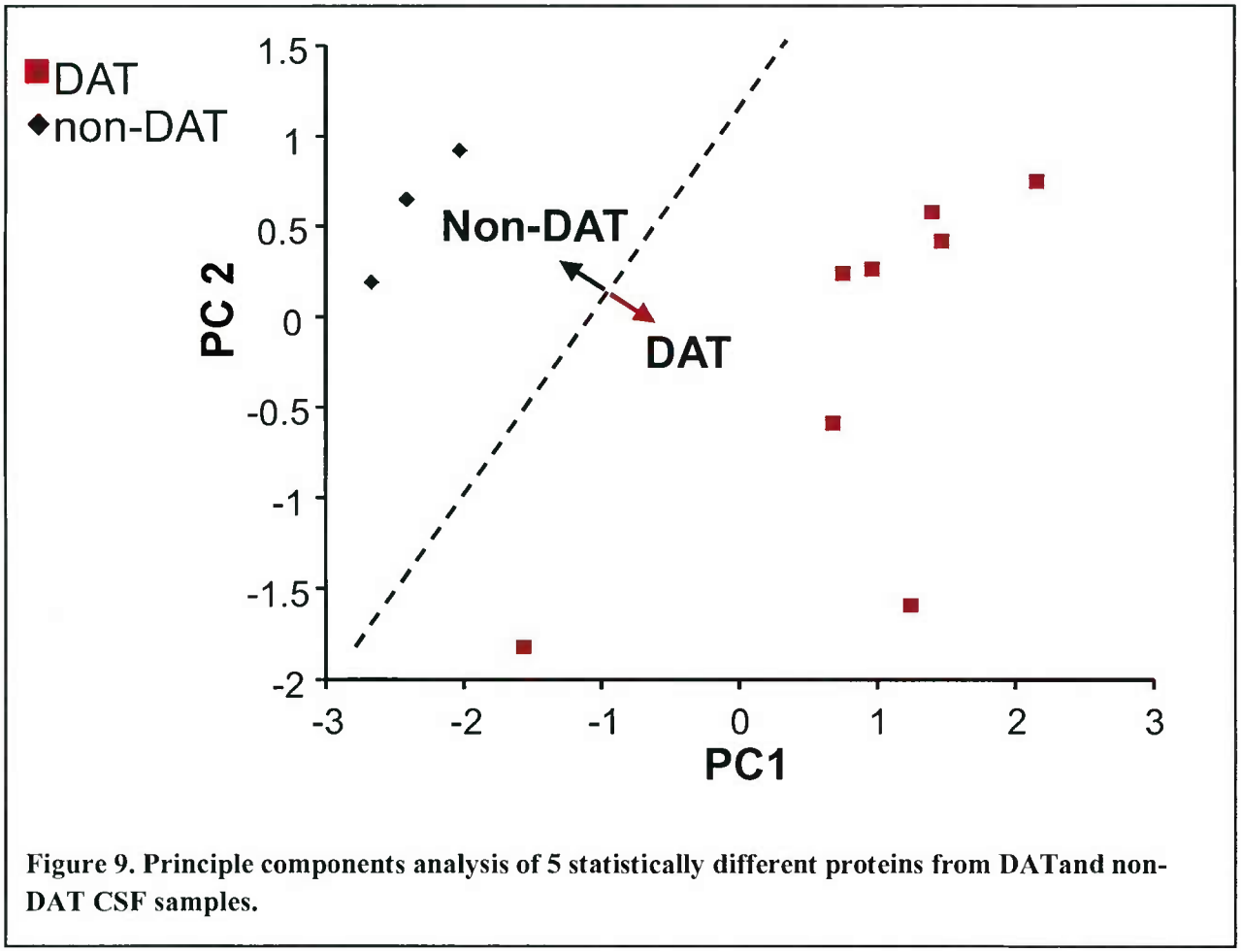

Figure 9. Principle components analysis of 5 statistically different proteins from DATand non-DAT CSF samples.

We attempted to validate four out of five proteins in Figure 3 using Western blot analysis, but commercial antibodies that may have cross-reacted with sea lion proteins did not provide sufficient evidence for appropriate cross-reactivity based on a positive control sample of rat brain. Although bands were visualized by western, marked differences predicted molecular weight gave us pause in believing the data at least until we can verify the complete sea lion protein sequence.

A sixth protein called, Reelin, was identified by tandem mass spectrometry and was numerically lower, but did not show a statistical change in abundance ($P<0.12$). Reelin is an interesting protein that is depressed in schizophrenia, Alzheimer's disease, and epilepsy. Reelin serves as a ligand to the ApoE receptor 2 and very low density lipoprotein receptors and has been shown to inhibit granular cell dispersion in the hippocampus of mice dosed with kainite (analogous to domoic acid). Due to the relevant nature of this protein to neurological disorders that parallel domoic acid toxicosis, we decided to validate the directionality of Reelin abundance and determine whether modest differences may exist that are not detectable using label-free tandem mass spectrometry quantification which is inherently variable. We utilized a western blotting approach using an antimouse-Reelin antibody. The anti-Reelin antibody gave better than expected results and showed cross-reactivity with a low molecular weight Reelin protein of

expected size (180kDa). Non-specific cross-reactivity was noticeably absent as compared with sea lion plasma.

We then loaded a 4-12% polyacrylamide gel with the same samples used for mass spectrometry and ran the gel under denaturing conditions. Lanes were loaded according to volume CSF (10µl CSF) rather than total protein to reproduce a clinical unit measure similar to what is utilized in a diagnostic assay e.g. mg/dL vs. mg/g protein. In this case, because sea lions with DAT had higher total CSF protein concentration on average, the amount of protein loaded onto DAT lanes exceeded that of control sea lions. Based on western blot results, Reelin protein in CSF of sea lions with DAT was about 1.4-fold lower ($P<0.001$) compared to control animals (**Figure 10**). These data suggest that domoic acid toxicosis in sea lions shares a common mechanism with other mammalian neurodegenerative diseases in part through Reelin signaling. Secondly, the western blot data support the mass spectrometry identification and quantitative directionality i.e. low spectral counts are equivalent to less protein. Thirdly, control animals were nonDAT, but one animal did have encephalopathy suggesting that differences in Reelin abundance may indeed be much larger if healthy animals or animals without brain injury were compared.

Method	Protein	Fold Δ	p-value
MS/MS	Reelin	-1.6	0.121
Antibody	Reelin	-1.4	<0.001

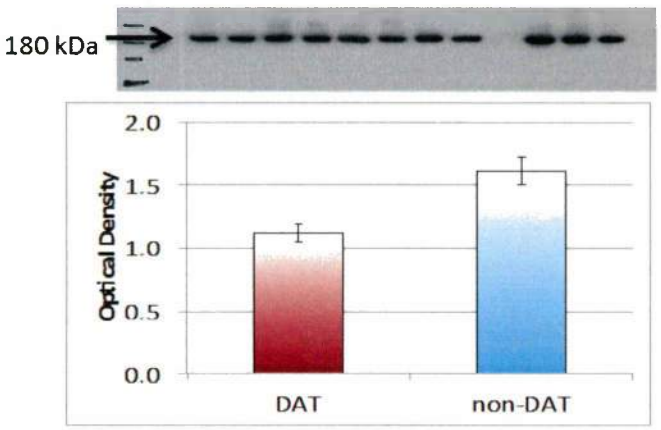

Figure 10. Reelin abundance measured by tandem mass spectrometry and western blotting. Although not statistically different by tandem mass spectrometry analysis, Reelin was lower measured by Western blotting ($P<0.05$, T-test).

The utility of Reelin alone as a biomarker for DAT is not exceptional [Sensitivity 62%, Specificity 50%, Positive predictive value 100%, Negative Predicitive Value 50%] but may also reflect other neuronal injury than DAT. Together with the 5 protein panel of CSF candidate markers, it may be able to lend more confidence to a classification. Additionally, the changes in Reelin offer important mechanistic insight into the mechanism of DAT in sea lions. Establishing common patterns of protein abundance between sea lions and other mammalian laboratory animal models provides the opportunity to draw parallels between tightly controlled laboratory experiments and wild sea lion populations that under the marine mammal protection act cannot be utilized for comparative experimentation.

Based on the interesting results from the CSF protein marker study, we wanted to determine whether a panel of protein markers could be constructed for this fluid that would allow us to create a diagnostic platform for the measurement of these proteins in sea lion CSF. As mentioned prior, western blots only worked for the protein Reelin, so antibody-based assays are

not a likely option for determining sea lion protein CSF protein abundance for the candidate markers listed. Additionally, it is important that measurements be made and reported in SI units because standardized diagnostics are rarely conducted using relative values (exceptions being cDNA array data for cancer diagnostics and immunohistochemistry) and the effects of matrices can skew mass spectrometry data if internal standards are not available for estimating these effects. To that end, we constructed mass spectrometry assays similar to select reaction monitoring (also referred to as select ion monitoring) assays using our ABSciex 5600 Triple TOF instrument for all six proteins. Quantification by ion monitoring involves a stable isotope internal standard that is chemically identical to the tryptic peptide selected for monitoring with the exception that one of the amino acids in the synthetic peptide contains $^{13}C/^{15}N$ thereby making this peptide slightly heavier in mass. For all practical purposes, the peptide will elute and fragment the same as a native peptide, but because it is slightly heavier, will be detected at +8 or +10amu if lysine or arginine is labeled. Therefore, the mass spectrometer is able to visualize both peptides at the same time and fragment the parent ion into b and y ions for identification. Measurements are made on the fragment ions which offers an additional level of specificity to the assay in the case other parent ions overlap in mass at a specific elution time. Secondly, by doing this type of standardization, we are able to validate our peptide identifications based on comparisons of elution time and fragment ion spectra.

An example data profile is shown in **Figure 11**. Five microliters of sea lion CSF is tryptic digested and 6 internal peptide standards, synthesized and quality controlled by New England Peptide, were spiked into the a CSF sample at known concentration prior to peptide isolation by solid phase extraction. Peptides + standards are injected onto a nano c18 column and data acquired across an elution gradient for 30 mins. Product ion scans are made for specific parent ion masses relevant to the sea lion peptides we are monitoring and fragment spectra are recorded for each ion with a mass within 1amu of the entered mass. Fragment ion masses specific for the peptides of interest and their associated standards are extracted and intensity plotted across time (**Figure 11B**). Area under the extracted ion chromatograms for both the standard and native peptide are compared and the ratio between the two are used to calculate endogenous concentration. In Figure 6B we show the quantification of sea lion NCAM1 and the linear range of this this peptide in **Figure 11C**. As proof of concept we measured all six candidate markers in a single sea lion CSF that had DAT to determine whether the standards could be used to measure tryptic peptides in sea lion. All standard peptides and endogenous tryptic peptides displayed identical elution profiles and fragment ion spectra (shifted +8 or +10 for the standard) confirming the identity of the peptide. A measurement for each candidate protein concentration is compiled in **Table 1**. The multiplexed protein assay provided reliable numbers that is specific for sea lion proteins, can be compared across many sea lion CSF samples, and importantly can be directly linked to a standard peptide.

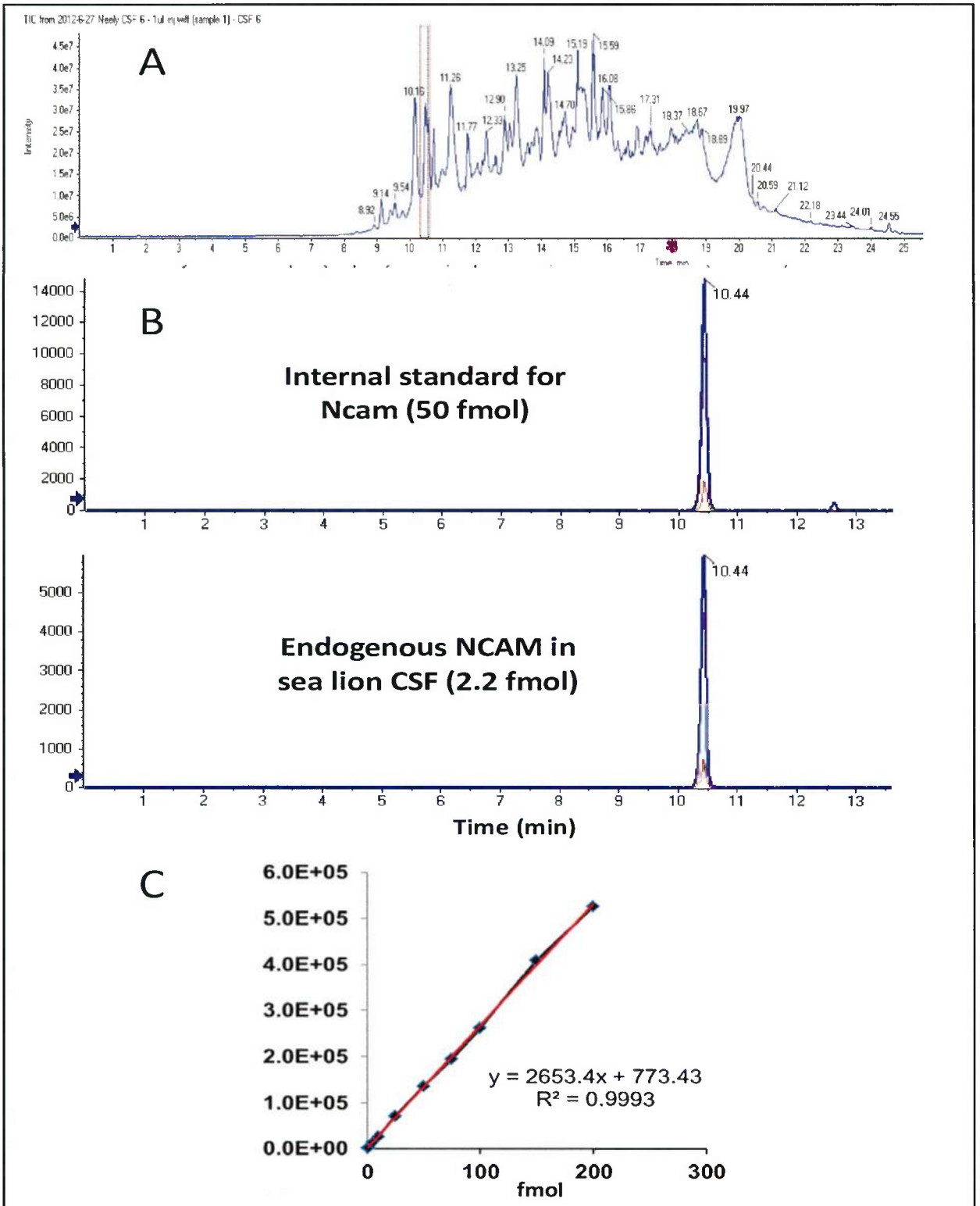

Figure 11. Reaction monitoring assay for sea lion NCAM1. A) total ion current of tryptic CSF peptides. NCAM1 peptide elutes at 10.44min (red box). B) Extracted ion chromatograms for NCAM 1 sea lion peptide. The internal standard containing $^{13}C/^{15}N$ elutes at the identical time point as sea lion native NCAM1. C) Dilution curve showing linearity of NCAM1 from 1 fmol to 200 fmol.

Table 6. Six CSF proteins measured by mass spectrometry reaction monitoring using the assay developed in our laboratory. Protein abundance can now be reported in SI units instead of relative spectral counts making this a standardized measurement specific to sea lions.

CSF Protein Name	fmol	fmol/µl CSF
Gelsolin	4.91	8.2
Oligodendrocyte Myelin Glycoprotein (OMG)	24.7	41.2
Reelin	13.6	22.6
IgG lambda 6-C	8.1	13.6
NCAM (CD56)	2.2	3.7
Dickkopf 3 (DKK3)	1.3	2.1

At this point, it is important to understand whether these candidate markers using standardized measurements, but also to ask the question whether this information can guide therapeutic trials for studies of intervention. One of the major hurdles to therapeutic intervention is knowing which mechanisms parallel published studies in rodent models thereby creating precedent for informed clinical studies to proceed. Several studies have suggested that domoic acid causes temporal lobe epilepsy in humans, rats, and sea lions. Temporal lobe epilepsy is partially characterized by a widening of the dentate gyrus granular cell layer known as granular cell dispersion (GCD). However, in sea lions there is an acute necrosis of granular cells that is not characteristic of excitotoxic induced injury or epilepsy. Scientists have speculated that a "sensitivity" due to limbic seizures is due to a possible adaptation to hypoxia; however it is still not known as to why granular cells in sea lions become "necrotic" following excitotoxic injury.

The protein phenotype in the CSF of sea lions with domoic acid toxicosis does not fit neatly into expression profiles of temporal lobe epilepsy and may be specific to excitotoxic injury in sea lions or denote some novel mechanistic feature of domoic acid toxiciosis that has not yet been revealed.

iTRAQ Analysis of Plasma Proteins

Although not specifically listed in the grant proposal, we decided to apply advance proteomic techniques to the study of plasma proteins from sea lions with DAT. Two-dimensional liquid chromatography tandem mass spectrometry (2D LC/MS/MS) is commonly utilized in biomarker discovery programs for human clinical studies as a targeted approach.

For this study, we acquired 8 plasma samples from age/sex-matched sea lions (adult females) stranded in 2009. Plasma samples were drawn within a few days of stranding in the DAT animals. For non-DAT plasma samples, animals were allowed to recover from injury and plasma was collected just prior to release. We did this because we were more interested in understanding

which plasma proteins were changing in animals with acute DAT versus a relatively healthy sea lion without DAT. Plasma samples were thawed at MUSC and normalized using Proteominer beads to decrease albumin and immunoglobulin abundance. Proteins were digested using trypsin and peptides labeled using isobaric tags (iTRAQ reagent, Applied Biosystems). Peptides were combined and separated by strong cation exchance spin column prior to separation on a c18 column. Fractions were spotted to a MALDI target and data acquired using an ABI 4800 TOF-TOF mass spectrometer. Proteins were quantified based on size tags and identification assigned using MASCOT against the canine protein database. Protein quantification was collated using iQUANTITATOR and in-house program designed by Dr. John Schwacke at MUSC.

Before discussing protein results, it is noteworthy to point out that of 4645 spectra acquired by mass spectrometry, 4260 were not assigned to proteins. This fact largely points to a need for a sea lion genome database for any downstream LC/MS/MS intensive studies. Small differences in amino acid sequence will not always permit peptide matching and protein identification. Because dogs are most closely related to sea lions, we speculated that this database would provide the best possible match against the sea lion peptide spectra.

From the iTRAQ experiment, 86 uniquc proteins identified by at least a single peptide were quantified. Only ten proteins were identified by two or more peptides. Analysis of expression change magnitude values (normalized fold change) indicated only one protein was significantly different between sea lions with DAT and sea lions without DAT. Serum amyloid A was statistically higher in DAT sea lions; however, did not perform well as a biomarker as only two sea lions with DAT had levels that were distinguishable from those sea lions without DAT.

6. Conclusions

Utilizing a suite of targeted and non-targeted proteomic approaches coupled with machine learning tools, there is no perfect biomarker for diagnosing domoic acid toxicosis in sea lions; however, there are markers that do perform well in combination with other data.

Objective specific conclusions:

1) Proteominer Ligand Library depletion strategies for high abundance protein depletion performed best for removing albumin. Protein diversity was elevated following depletion and these columns are a reasonable choice for future studies requiring protein depletion, although Proteome Lab IgY depletion also appears to perform well and could be suitable alternative choice.

2) Commercial cytokine arrays developed for human measurements can be utilized as a screening tool offering >92% accuracy in the diagnosis of acute DAT, but will only detect 30% of those sea lions with DAT. Alternatively, these panels can also exclude a diagnosis of DAT (predict non-DAT) with >98% accuracy, but once again will only detect 25% of the truly non-DAT sea lions.

3) MALDI-ToF peptide profiling of acute DAT demonstrated that no single feature was an excellent classifier of acute DAT. The best performing feature was located at a mass of 3017 m/z. External validation of this feature as a marker produced results similar to the commercial cytokine kit for the diagnosis of DAT. Neural network modeling of the MALDI features produced excellent models for DAT diagnosis in the training set, but the validation performance was limited in that only 30% of DAT sea lions would be detected with 100% accuracy. Once again, this would offer a high level of confidence to a suspected diagnosis of DAT if the test was positive, but a negative result would not offer no confidence to exclude DAT. On the other hand, one neural network model performed well for exclusion of DAT with 100% accuracy for 60% of the non-DAT sea lions. This model could offer a high level of confidence when screening sea lions.

4) 2D gel electrophoresis studies of chronic DAT sea lions demonstrated that Apolipoprotein E was the major classifier for DAT vs. non-DAT sea lions. Low levels of this protein correlated with DAT, but alone were only good biomarkers. When Apolipoprotein E levels and eosinophil counts were combined in a neural network model or by decision tree, the AuROC was equal to 1 in the training set. External test set validation supported this combination as a biomarker with a test sensitivity of 86% and specificity of 85%. This combination performed the best of all markers in this study.

5) Proteomic analysis of cerebral spinal fluid resulted in the identification of 6 proteins that could classify acute or chronic DAT and non-DAT perfectly in a very small number of animals. These data were not validated due to lack of samples from sea lions. Notably, the protein Reelin was depressed in sea lions with DAT which fits a phenotype found in neurological disorders and could be a therapeutic target in future studies.

iTRAQ analysis of serum proteins using tandem mass spectrometry was limited in its ability to discriminate sea lions based on DAT vs. non-DAT. Serum amyloid A was statistically elevated but validation of this protein as a marker was unsuccessful.

7. Significance

Domoic acid toxicosis is a major cause of death in sea lions along the Pacific coast. The toxin responsible for this toxicosis is excreted by the body very quickly making this a very difficult disease to diagnose. The Navy marine mammal program maintains sea lions in localities where domoic acid is prevalent; therefore, monitoring of toxicosis related to domoic acid is important.

Data presented indicate that protein biomarkers for domoic acid toxicosis in minimally invasive samples (plasma or serum) do exist, but that most of these markers have either low sensitivity or specificity therefore making these tests limited in application. Apolipoprotein E together with eosinophil count appears to be the best performing marker with very good sensitivity and specificity. Alone, eosinophils or apolipoprotein E do not perform as well when analyzed alone versus when analyzed in combination. For a disease like domoic acid toxicosis, where variations in dose and timing are highly variable, it appears that combining routine clinical data with target protein abundance is the better method by which to classify sea lions with a highly variable disease course. Large scale assay validation for apolipoprotein E could provide an effective method to screening sea lions along with a common clinical laboratory value.

Although not the main intention of the study, it is interesting that both plasma Apolipoprotein E and cerebral spinal fluid Reelin levels were found to be lower in sea lions with domoic acid toxicosis. From a mechanistic point of view, both proteins share a common receptor in ApoE receptor 2 which is known to inhibit granular cell dispersion in the hippocampus of mice treated with a drug similar to domoic acid. Treatments that elevate both proteins could be relevant targets for therapeutic intervention.

8. Publications

a) Refereed Journals

Funk JA, Janech MG, Dillon JC, Bissler JJ, Siroky BJ, Bell PD.. Journal of the American Society of Nephrology. 2014 Feb 8. [Epub ahead of print]

Neely BA, Soper JL, Greig DJ, Carlin KP, Favre EG, Gulland FM, Almeida JS, and Janech MG. Serum profiling by MALDI-TOF mass spectrometry as a diagnostic tool for domoic acid toxicosis in California sea lions. Proteome Sci 10: 18, 2012.

a1)Planned submissions

Neely BA, Ferrante, JA, Soper JL, Gulland FM, Almeida JS, and Janech MG. Candidate plasma biomarkers of domoic acid toxicosis discovered by two dimensional gel electrophoresis in California sea lions.

Neely BA, Soper JL, Gulland FM, and Janech MG. Proteomic analysis of cerebral spinal fluid from California sea lions reveals differences associated with domoic acid toxicosis.

b) Non-Refereed Significant Publications

None

c) Books or Chapters

None

d) Workshop/Conference abstracts, presentations, posters, or papers.

MG Janech. California Sea Lion Proteomics. Symposium of California Sea Lion Carcinoma: Current Knowledge and Future Directions, Sausalito, CA September 12-13 2012.

BA Neely, Soper JL, Gulland FMD, Arthur JM, Janech MG. 2012. Proteomic analysis of cerebral spinal fluid from California sea lions (*Zalophus californianus*) with domoic acid toxicosis. 43rd Annual International Association for Aquatic Animal Medicine Conference. May 2012.

BA Neely, Soper JL, Gulland FMD, Arthur JM, Janech MG. 2012. Proteomic analysis of cerebral spinal fluid from California sea lions (Zalophus californianus) with domoic acid toxicosis. Dept. of Medicine Research Day December 2011, Charleston SC.

BA Neely, Soper JL, Gulland FMD, Arthur JM, Janech MG. 2012. Proteomic analysis of cerebral spinal fluid from California sea lions (Zalophus californianus) with domoic acid toxicosis. MUSC Student Research Day Novermber 2011, Charleston SC.

BA Neely, Jennifer Soper, Elizabeth G. Favre, Frances M. D.Gulland, Jonas S. Almeida, John M. Arthur, Michael G. Janech AN ASSESSMENT OF Serum peptide profiling by MALDI-TOF as a DIAGNOSTIC TOOL FOR domoic acid toxicosis in California sea lions. Dept. of Medicine Research Day. 2011 Charleston, SC

BA Neely, Jennifer Soper, Elizabeth G. Favre, Frances M. D.Gulland, Jonas S. Almeida, John M. Arthur, Michael G. Janech AN ASSESSMENT OF Serum peptide profiling by MALDI-TOF as a DIAGNOSTIC TOOL FOR domoic acid toxicosis in California sea lions. International Association for Aquatic Animal Medicine. 2011 Las Vegas, NV

JA Ferrante, B Neely, E Favre, JM Arthur, FMD Gulland, E Andrews, J Soper, and **MG Janech**. Haptoglobin as a candidate biomarker of domoic acid toxicosis in California sea lions. ISAAH 2010, Tampa Bay , FL.

MG Janech. "Omics in Medicine", Workshop on 'omics approaches for investigation of responses to marine toxin, contaminant, and pathogen exposures in sentinel marine mammals. July 28-29, 2010, Charleston, South Carolina

MG Janech. "Proteomics and Sea Lions, Biomarkers for Domoic Acid Toxicosis" Workshop on 'omics approaches for investigation of responses to marine toxin, contaminant, and pathogen exposures in sentinel marine mammals. July 28-29, 2010, Charleston, South Carolina

MG Janech "Proteomics: Medical and Diagnostic Applications". Navy Marine Mammal Program /ONR Clinical Research Workshop, Hubbs-Sea World Research Institute, March 2010.

JA. Ferrante, JM. Arthur, FM. Gulland, B Hill, J Almeida, and **MG. Janech**. Serum cytokines as predictors of domoic acid exposure in California sea lions, Zalophus californianus. Society of Marine Mammaology Annual Meeting, Montreal, Canada, 2009.

JA Ferrante, JM Arthur, FM Gulland, J Almeida, MG Janech. Plasma cytokines as predictors of domoic acid exposure in California sea lions, Zalophus californianus. Southeast and Mid-Atlantic Marine Mammal Symposium, Univ. North Carolina – Wilmington, 2009.

JA Ferrante, F M. Gulland, J Arthur, F Van Dolah, **MG. Janech**. Depletion of high abundance proteins from California Sea Lion (Zalophus californianus) plasma using commercially-available depletion columns. Southeast and Mid-Atlantic Marine Mammal Symposium, Charleston, SC, 2008.

9. Patent Information

No patents were generated from this award.

10. Technology Transfer

None to report

11. Honors/Awards

Postdoctoral Associate- Benjamin Neely
1st place student presentation at the 43rd Annual IAAAM Conference, 2012
1st place student presentation at the 42nd Annual IAAAM Conference, 2011
2nd Place Winner Postdoc/Resident/Fellows II /MUSC Student Research Day 2011
1st place poster at Department of Medicine's 5th Annual Research Day, 2010

Masters Student – Jason Ferrante
Awarded Slocum-Lunz Foundation student grant for IL-10 assay qualification in sea lions. 2010
Entered Ph.D. program in Veterinary Science at the University of Florida in 2009, expected to graduate Fall 2014.