Quantitative Shotgun Proteomics of HD Induced Corneal Injury and Angiogenesis (Briefing Charts)

Dr. John Schlager

Wright Patterson Air Force Base

Clearance (10 March 10): AFMC-2010-0017 / 88ABW-2010-0699

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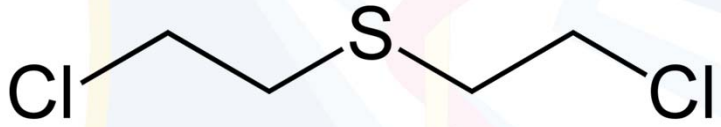
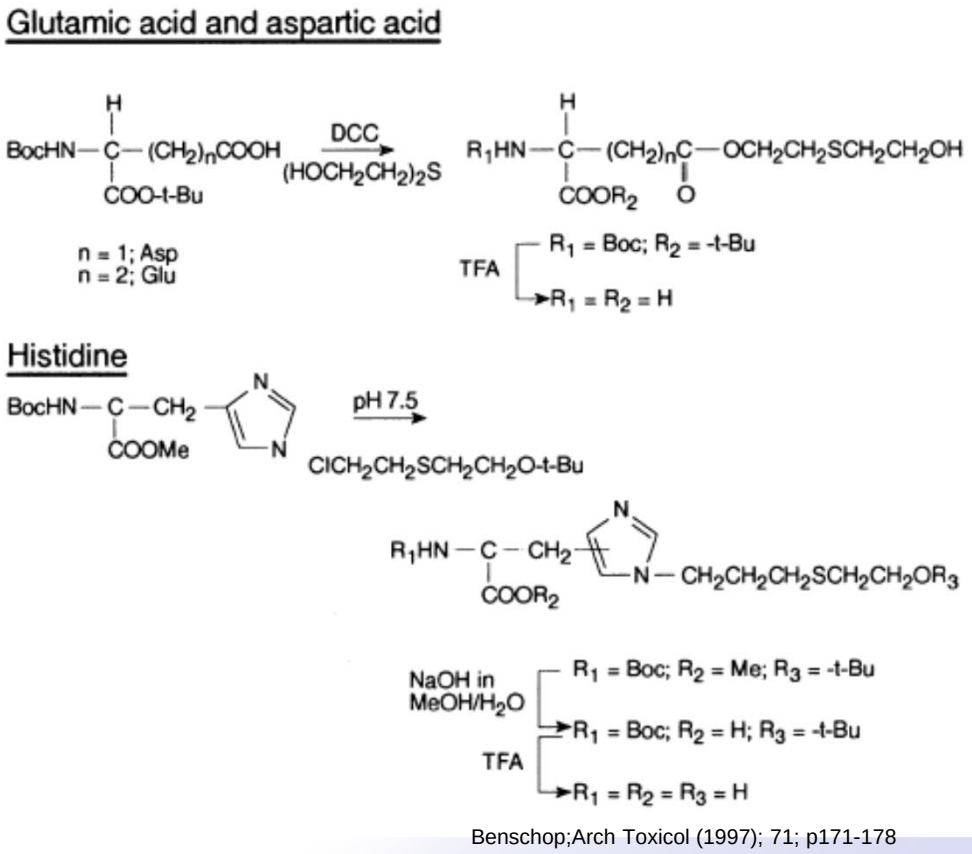
Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

Sulfur Mustard History and Modification Sites



- **Used in multiple conflicts in the 20th century.**
- **Causes cornea to become opaque, rendering blindness.**
- **Attacks DNA and proteins in cells.**
- **Causes blistering to occur on affected area.**
- **Non-lethal, but incapacitating to subject.**



Benschop;Arch Toxicol (1997); 71; p171-178

Mechanism of Sulfur Mustard on Amino Acids

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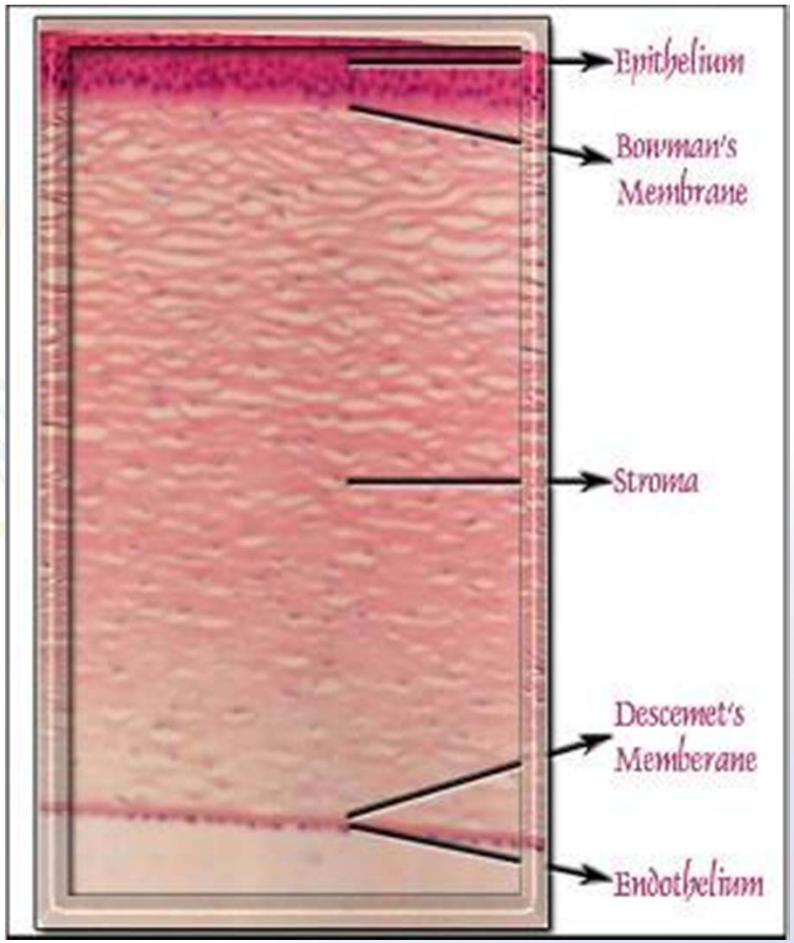
Layers of the Cornea

- **5 Layers: Epithelium (~50µm), Bowman's Layer (~10µm), Stroma (~450µm), Descemet's membrane (~5-15µm) and Endothelium (~5µm)**
- **Responsible for the majority of refraction of the eye**
- **The stroma is composed mainly of collagen fibrils in differing orientations**
- **Although it accounts for 0.1% of surface area, it is 20 to 50x more susceptible to damage**



LC-MS/MS vs. 2-D DIGE

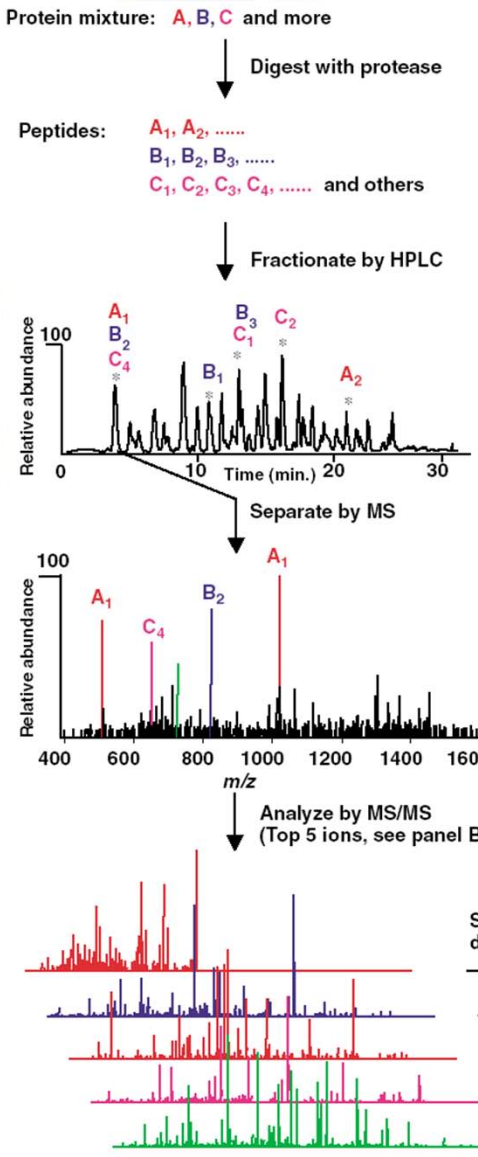
LC-MS/MS

- Requires 1µg of digested mixture
- Analysis time in hours
- Can identify hundreds of proteins in a run
- Up to 8 samples can be analyzed at once

2-D DIGE

- Requires 50µg of each sample
- Analysis time in days
- Spots must be excised and analyzed separately by MS/MS

LC-MS/MS Proteomics



Separate Layers / Lyse cells

Digest Proteins

Label with iTRAQ and combine

Separate on UPLC and analyze peptides with MS/MS

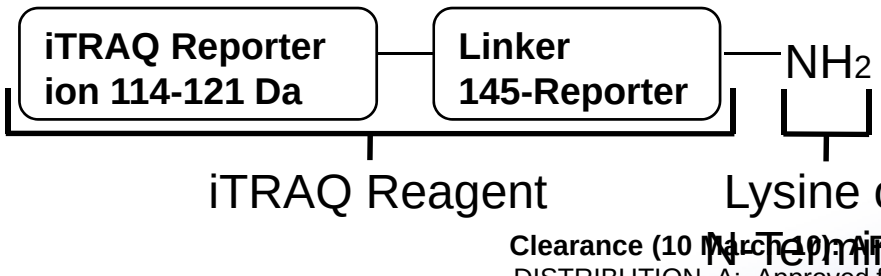
Identify Proteins and Quantitate

Instrumentation

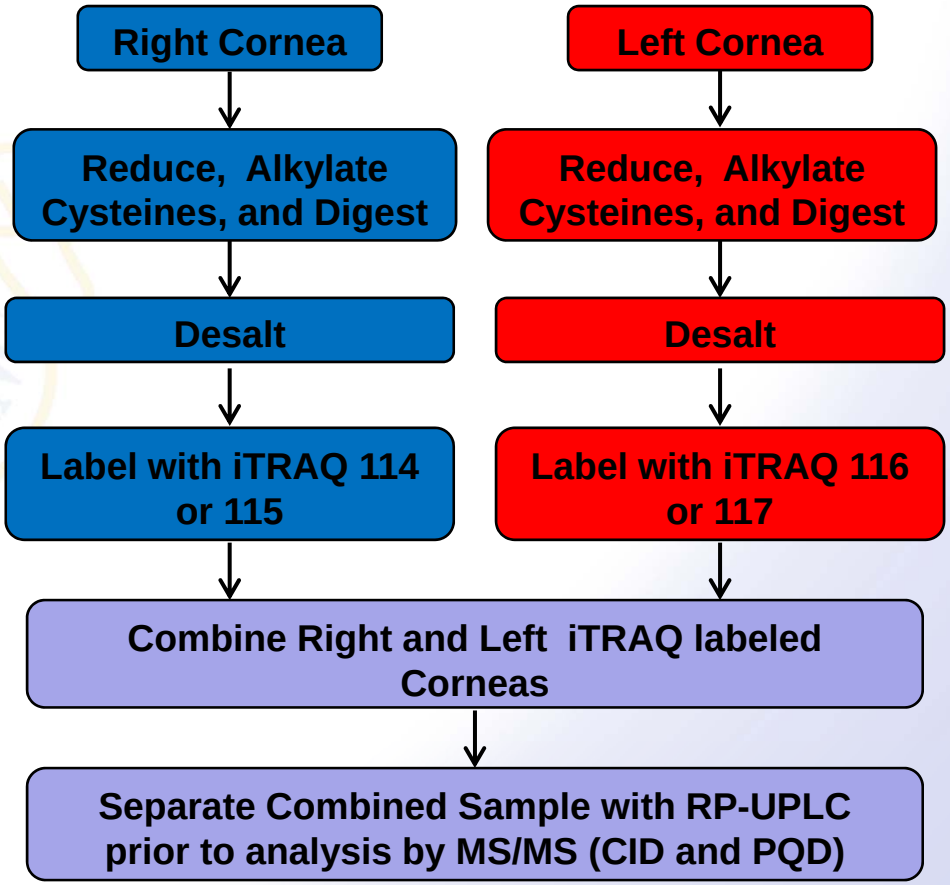
Waters Nano-Ultra Performance Liquid Chromatography
-Ultra high pressure LC system capable of separating complex mixtures over long gradient times with no loss in peak width
-Thermo-Finnigan LTQ XL High Performance Linear Ion Trap
- Collision Induced Dissociation (CID) for generating peptide fragmentation
- Pulsed Q Dissociation (PQD) for generating more fragments and extending the low mass range

iTRAQ Labeling for Protein Expression Quantitation

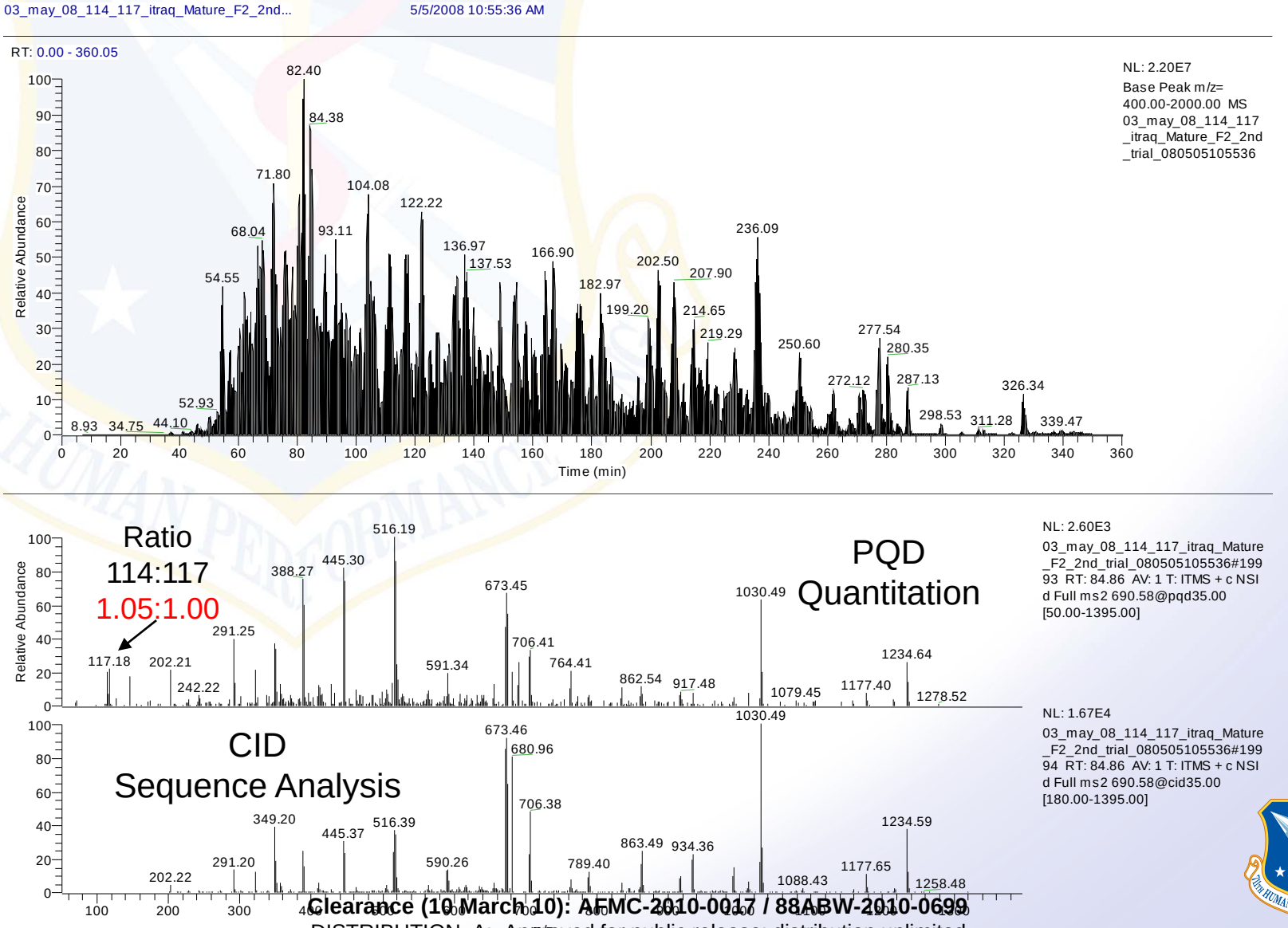
- **iTRAQ labels free amine groups (Lysine and N-Terminus), with a mass tag of 144Da**
- **Peptides elute simultaneously and have the same mass**
- **During MS/MS fragmentation, the iTraq is cleaved and reporter ion is recorded as a mass of 114 to 121**
- **Relative quantitation is achieved by comparing the ratio of the reporter ions**



Workflow of iTRAQ Labeling

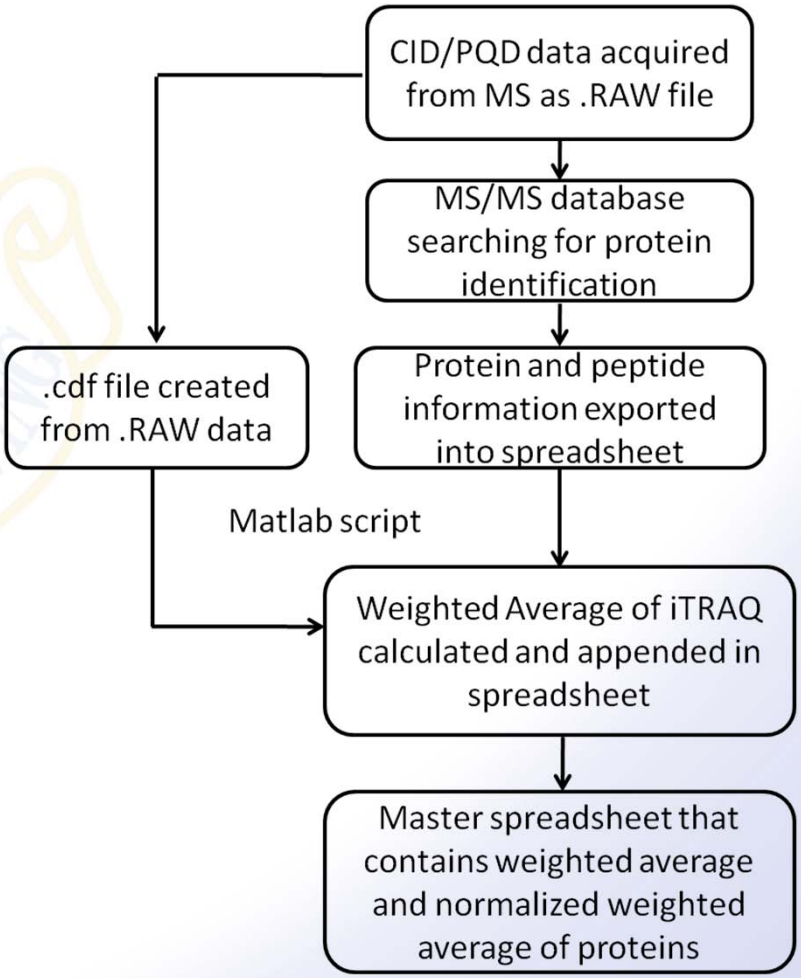


Quantitative Proteomics with Pulsed Q Dissociation and Collision Induced Dissociation



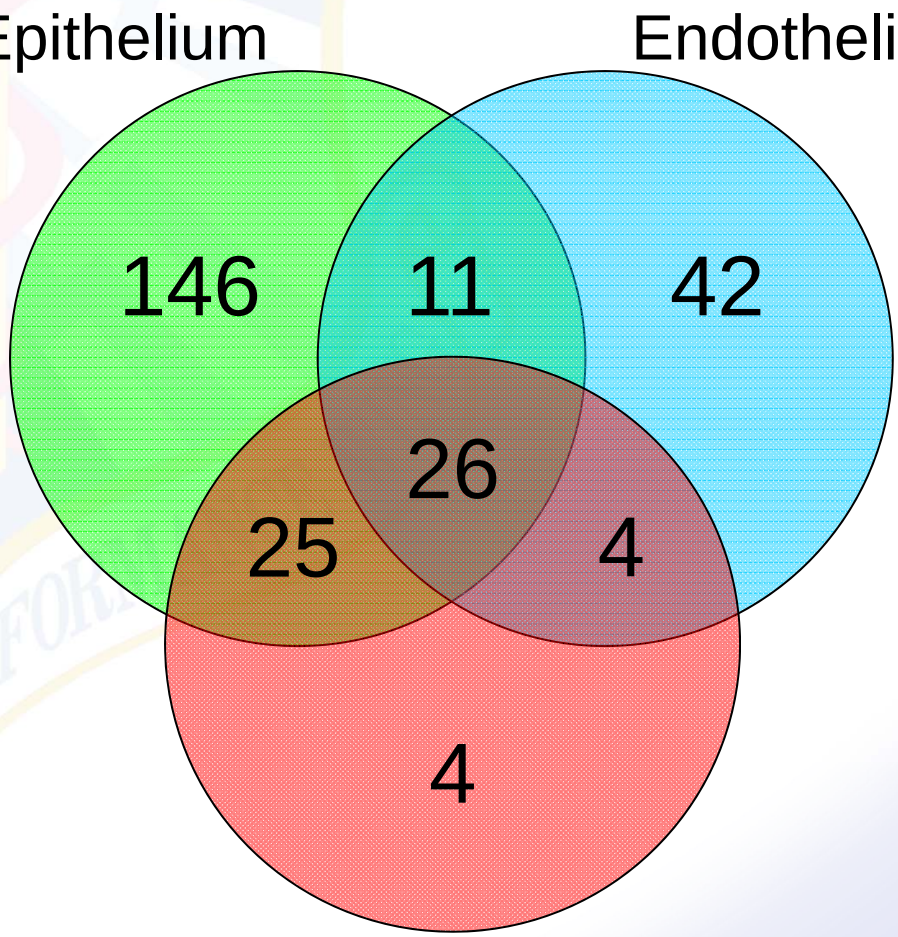
Data Analysis of iTRAQ Labeled Peptides

- **MS/MS data is searched against a database to identify peptides and proteins using SEQUEST in the Bioworks Browser by ThermoFisher.**
- **This data is exported into a new Excel spreadsheet.**
- **We have developed a MatLab script that weights the quantitative data using the raw data and appends the results in the original spreadsheet as a new column.**
- **Excel macros normalize the weighted data and perform statistics as the data is inserted into a master spreadsheet. The master spreadsheet also calculates statistical information for each animal and group.**



Identified Corneal Proteins From Method Development

Identified 208 Proteins in Epithelial Fraction Including Desmosomal Components, Immunity Proteins

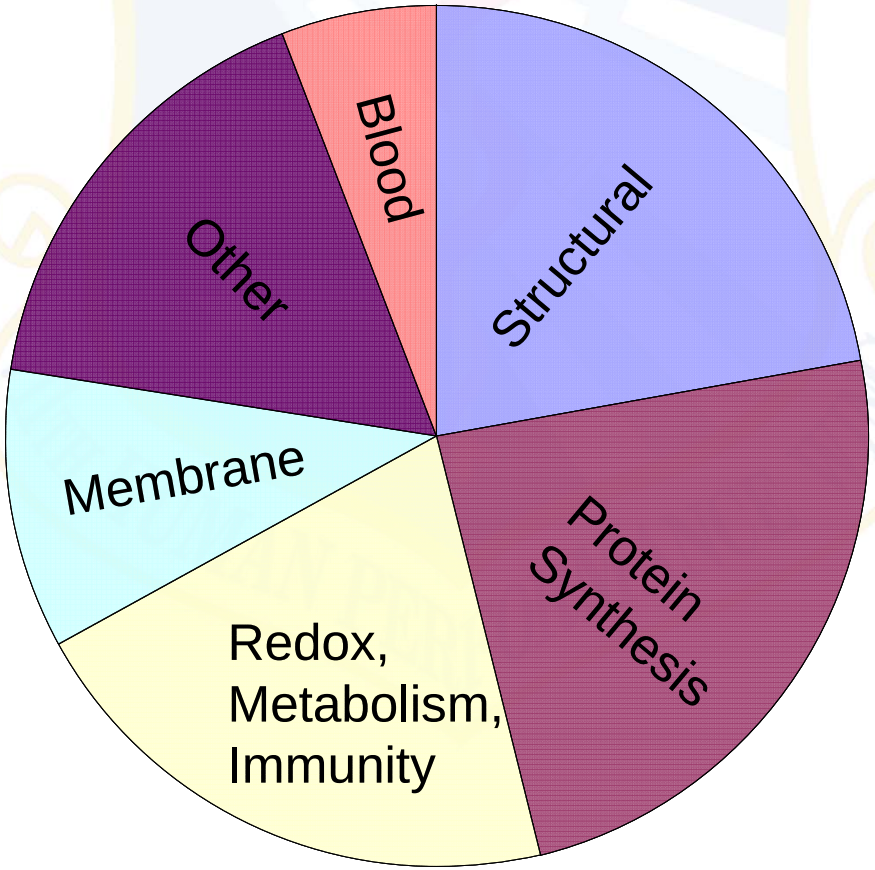


Identified 83 Proteins in Endothelial Fraction Including Angiogenesis Factors

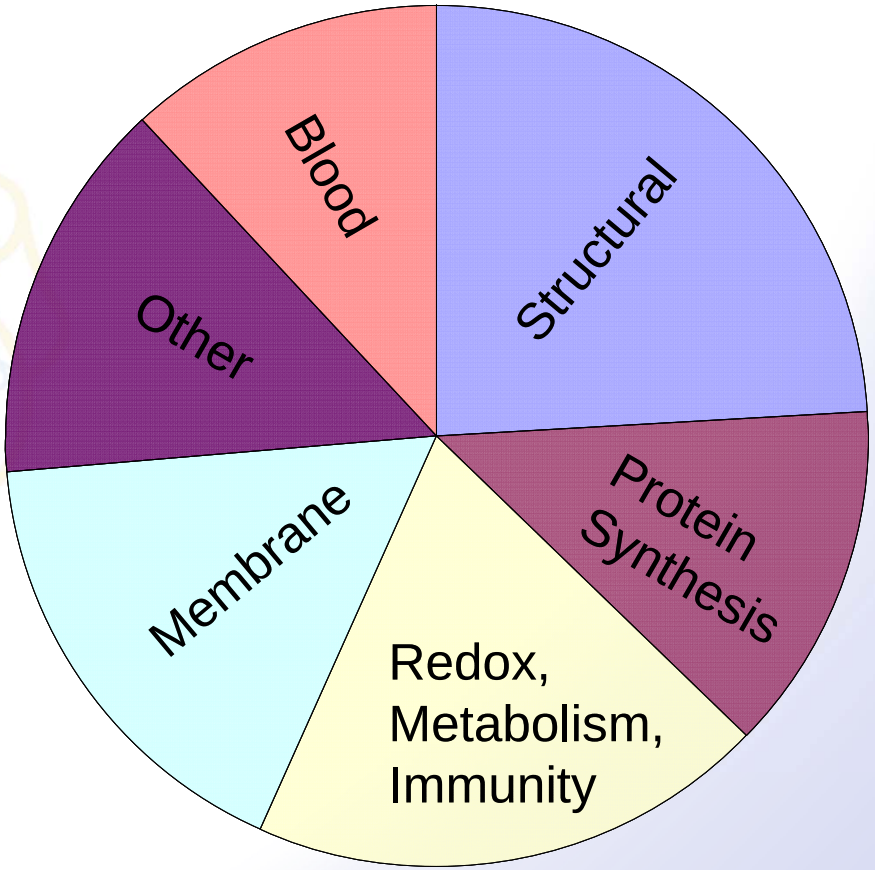
123 New Corneal Proteins Identified

Proteome Science;7:23

Functions of Identified Corneal Proteins



Epithelium



Endothelium

Proteome Science;7:23

Antibiotic and Corticosteroid Treatment Efficacy

Study Design

Group	Group ID	n	Challenge	Treatment	Assessment (days) Harvest times (days)
A	Naïve Control	3	None	None	1, 21, 63
B	SM Control	36	SM	PMBST saline	1, 3, 7, 21, 42, 63
C	Treatment	36	SM	PMBST Pred 1%	1, 3, 7, 21, 42, 63

PMBST: Polymyxin B Sulfate and Trimethoprim Ophthalmic Solution

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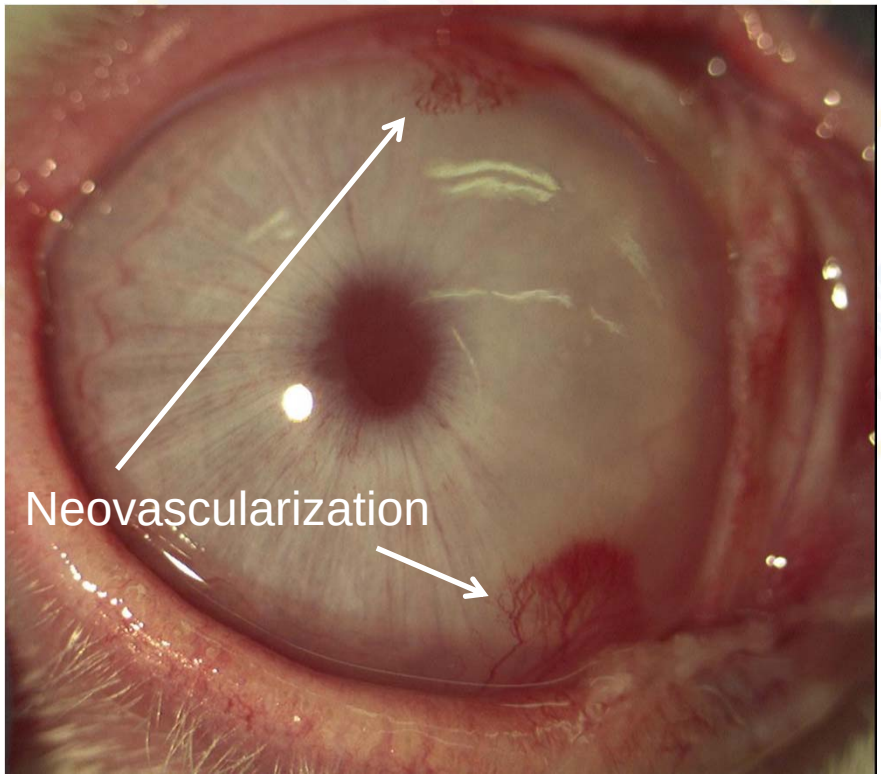
Clinical Results of SM Exposure and Prednisolone Regimen

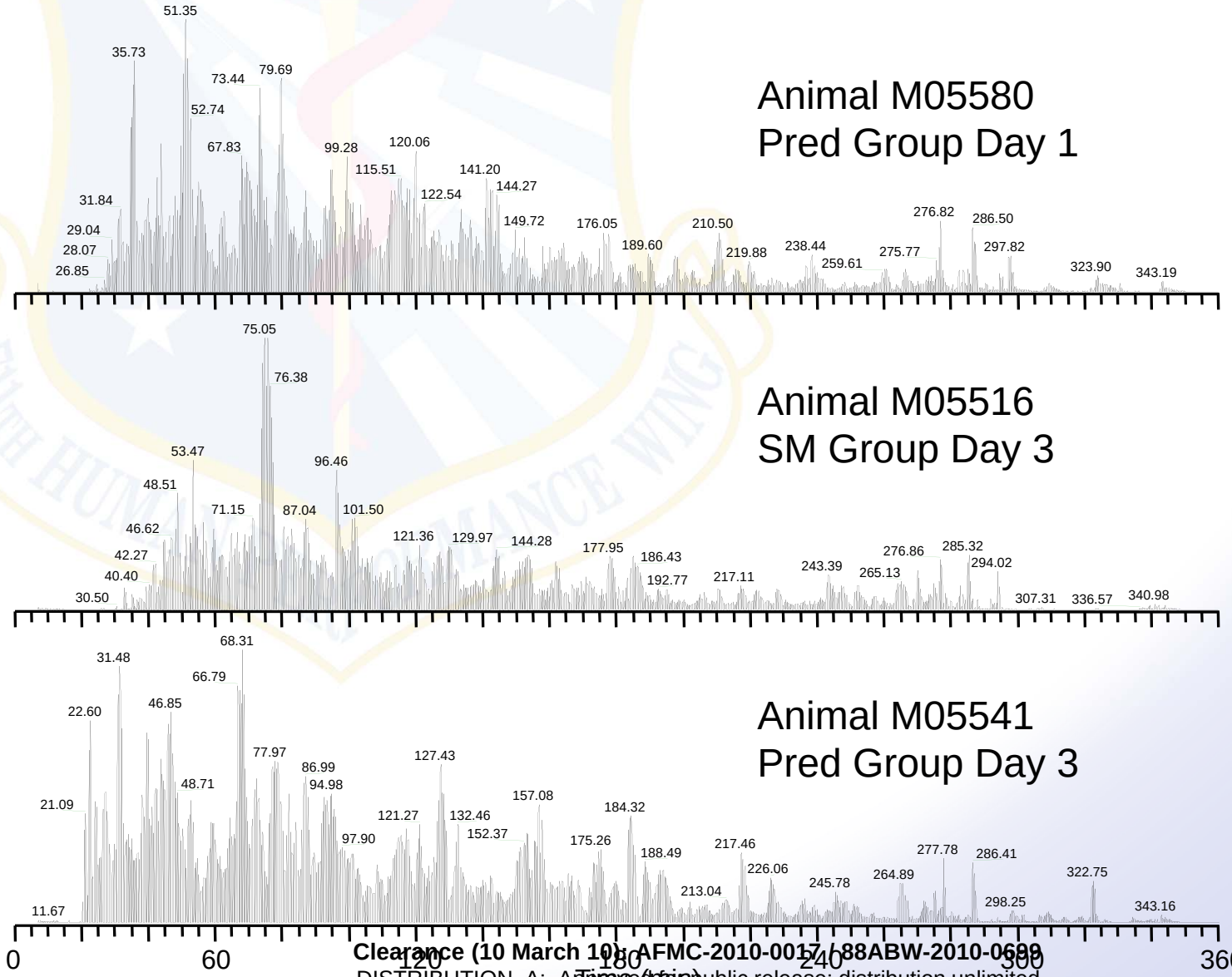


- **NV does not appear in any animal until day 21.**
- **At day 21, 7/18 animals in SM group experience NV, while only 1/18 animals in Pred group experience NV.**
- **At day 42, 5/12 animals in SM group show NV, with only 1/ 12 exhibit NV in Pred group.**
- **At day 63 4/6 in SM show NV, with 1 animal showing extensive NV, while 2/6 show minor NV in Pred group.**
- **Pred group shows significant loss in the corneal thickness compared to SM.**

Experimental Conditions

- Approximately 20-80µg of protein were extracted from the epithelium and endothelium. 350-500µg of protein were extracted from the stroma.
- Labeled Epithelial and Endothelial fractions were combined and diluted to a concentration of ~1µg/µl prior to injection.
- Peptides were separated over a 6 hour gradient of increasing concentration of acetonitrile with 0.1% formic acid.
- The mass spectrometer acquires a full scan, followed by CID and PQD dissociation of the top 8 peaks.
- Dynamic exclusion was enabled to limit the number of times a peptide is dissociated to 2.
- ~90,000 scans are acquired per sample run.

Base Peak Plots of Selected Epithelial Samples



Quantitative Protein Results of Epithelia

- **Lamin-B1, a protein thought to play a role in the framework of the nuclear envelope, was greatly reduced (0.18 ± 0.03) in Days 1-7 of Prednisolone treated samples**
- **Defensin was elevated to 12.15 ± 2.46 in days 1,3 and 7 of SM group**
- **Keratocan, a protein involved in corneal transparency, is higher in NV animals and than in non-NV animals.**
- **Eukaryotic translation Initiation factor was found to be increased in Pred group NV animals, while remaining at equal levels in all other animals**
- **Alpha 1-antiproteinase, a plasma protein involved in protection from inflammation, is consistently higher in Day 63 than any other time point**

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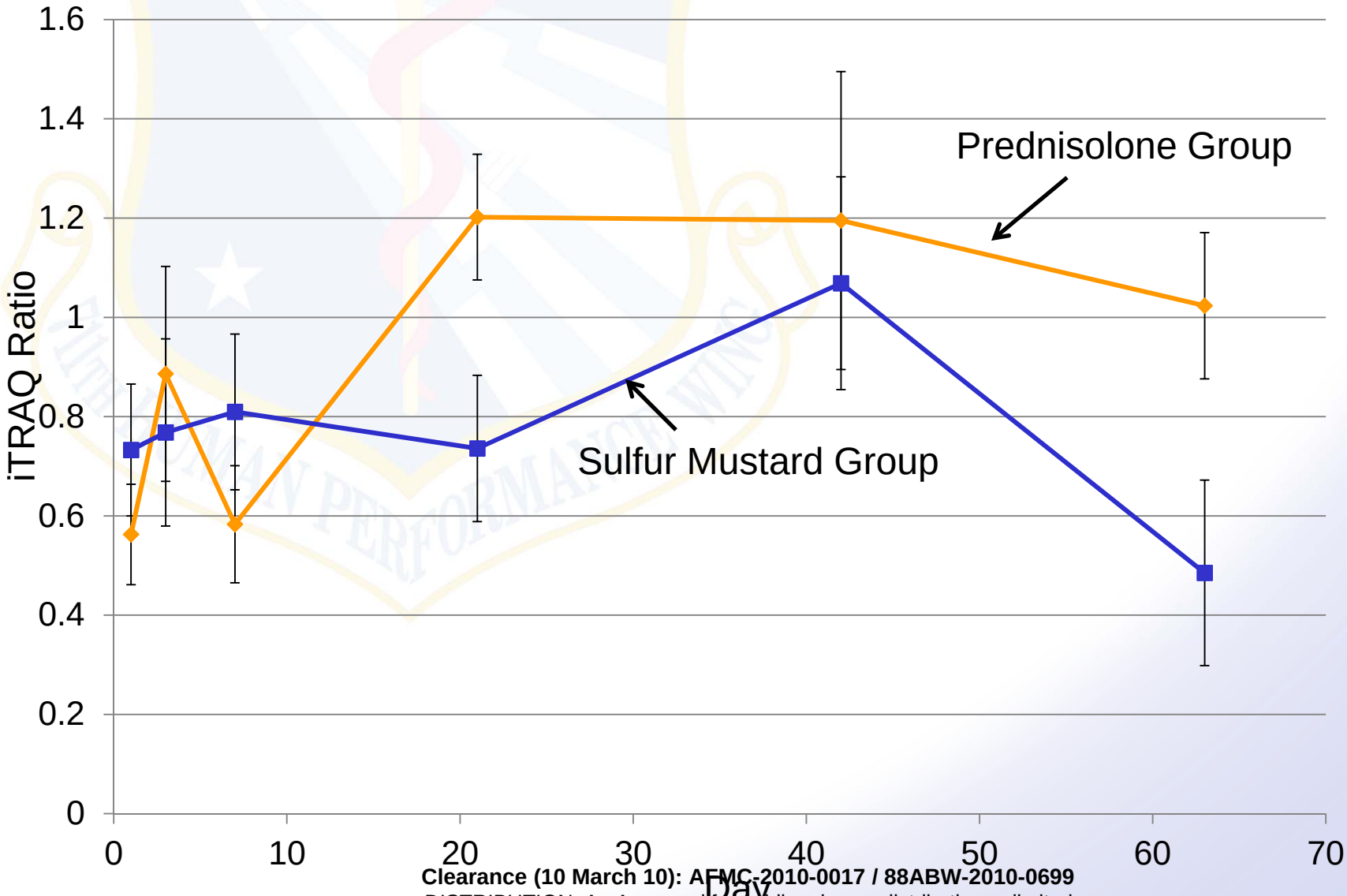
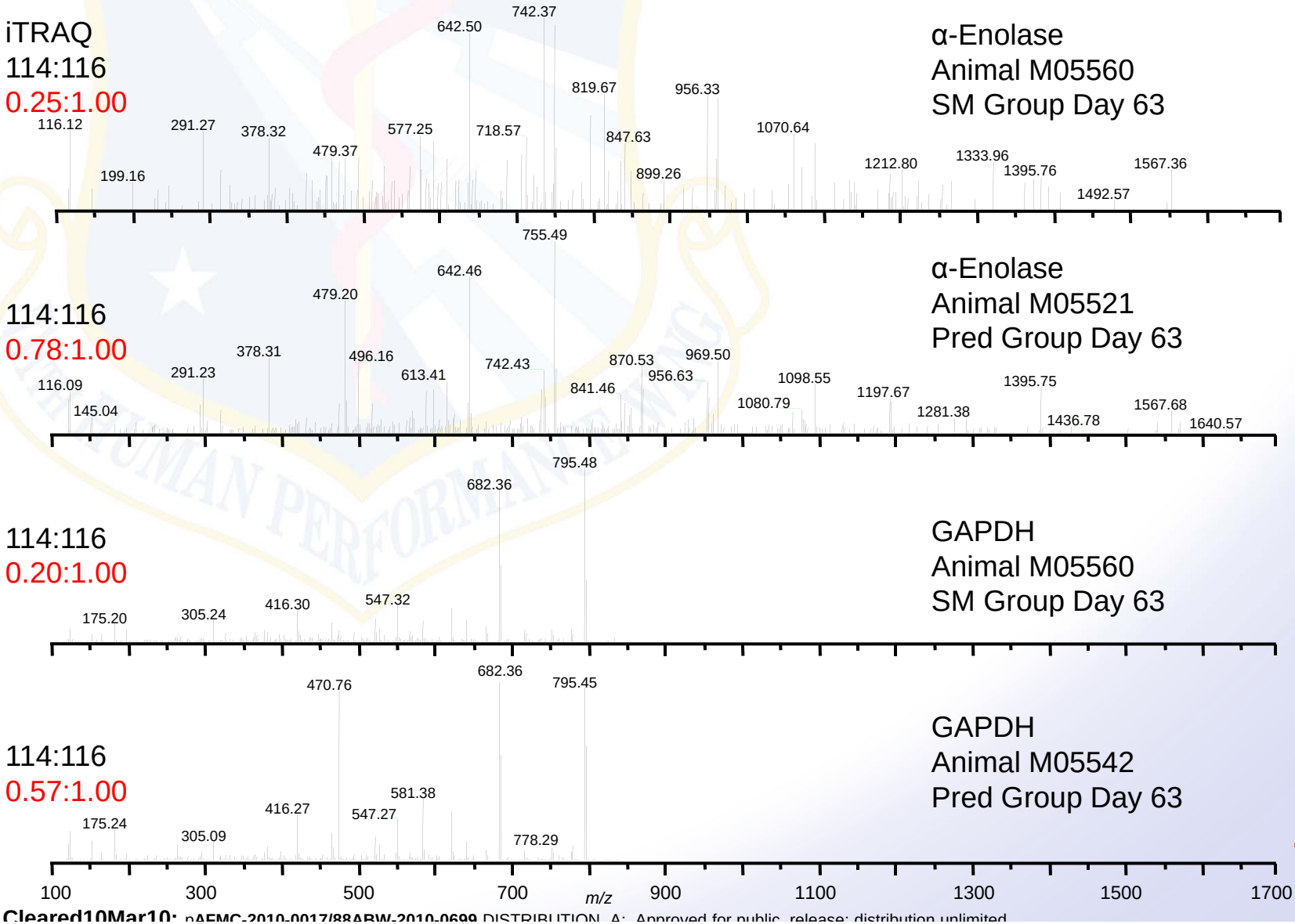
Pyruvate Kinase Protein Expression of SM and Pred Groups



Epithelial Proteins Trends from Neovascularized Animals

- Many proteins were found to be reduced when compared with the control cornea
- Proteins involved in glycolysis were reduced across all animals
- Laminin Receptor 1 protein was reduced to 0.45 ± 0.19. This protein functions plays a role in cell adhesion and activation of signaling transduction pathways
- ATP Synthase was also reduced to 0.28 ± 0.10 in SM animals. The protein was 2.10±0.44 in prednisolone
- Immunoglobuilns were found to be increased to 1.56±0.20 in all NV animals
- Transferrin was increased to 1.83 ±. 56, not including 3 animals that showed no transferrin in the control cornea
- Heat Shock Protein 70kDa was also found more elevated on animal M05560 than any other animal, including moderate NV animals.

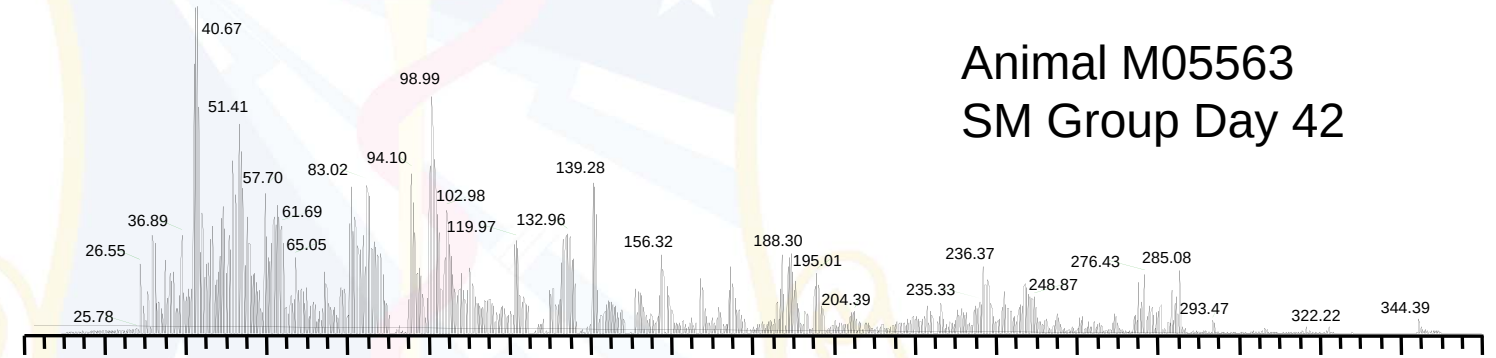
PQD Spectra of α-Enolase and GAPDH from NV Corneas



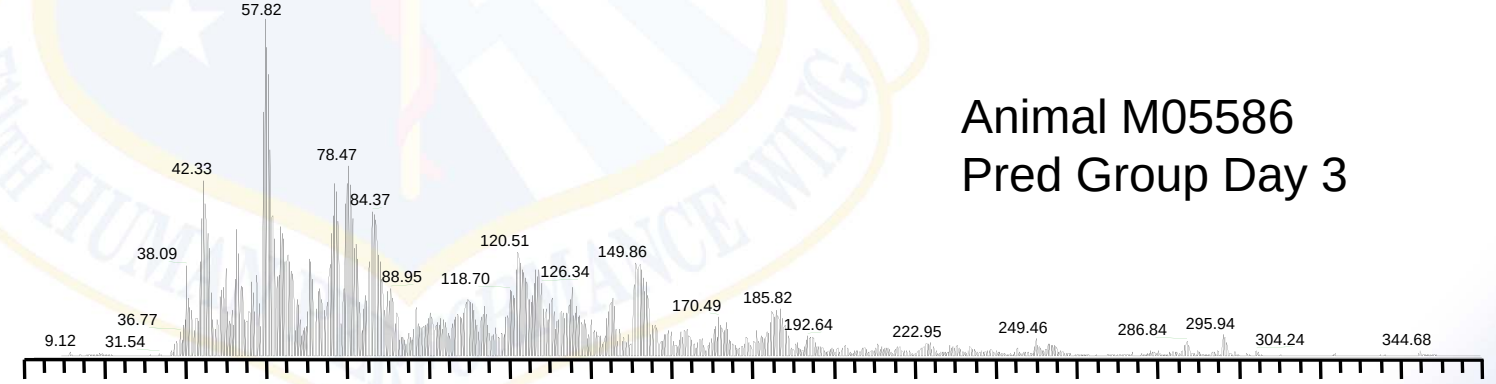
Protein Averages of NV Epithelia

Protein Name	Accession Number	Avg. SM Group	Avg. Prednisolone Group
Pyruvate Kinase	206205	0.49±0.12	1.29±0.27
α-enolase	2661039	0.49±0.09	0.80±0.13
GAPDH	31645	0.41±0.11	0.85±0.20
Phosphoglycerate Kinase	4505763	0.23±0.11	0.42±0.24
Aldehyde Dehydrogenase	2183299	0.47±0.08	0.77±0.11
Lactate Dehydrogenase	187074	0.45±0.12	1.07±0.42
Desmoplakin	1147813	0.78±0.38	0.53±0.27
Junction Plakoglobin	15080189	0.38±0.10	0.91±0.19
Beta-Actin	4501885	0.91±0.27	1.02±0.14

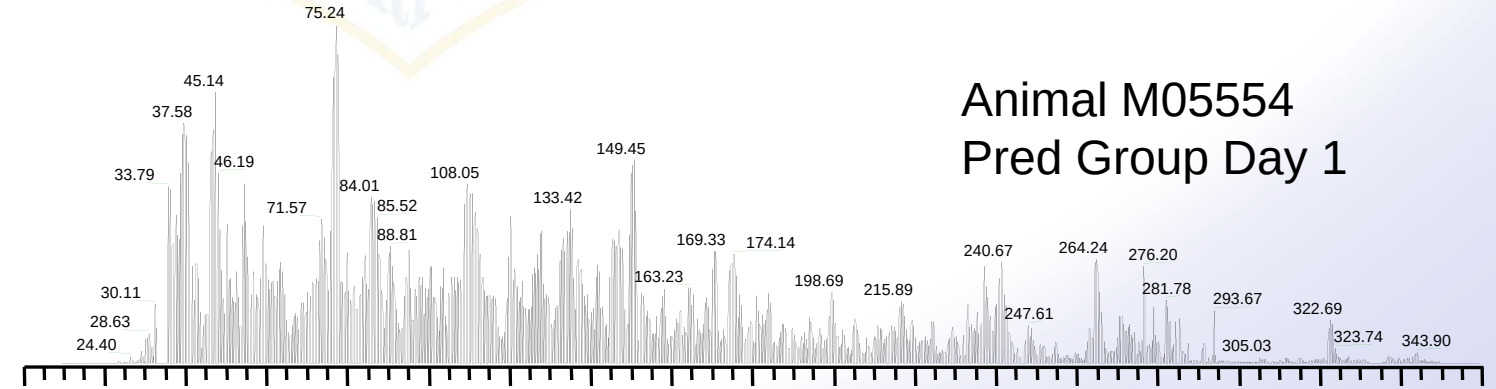
Base Peak Plots of Selected Endothelial Samples



Animal M05563
SM Group Day 42



Animal M05586
Pred Group Day 3



Animal M05554
Pred Group Day 1

Quantitative Proteomic Results of Endothelia

- Endothelial analysis shows that there was less deviation form the normal than in epithelium
- Lactate Dehydrogenase, a metabolic protein, was found to be reduced in Day 1 (avg= .32) versus later time points (avg= .82)
- Nidogen 1, a structural protein was found to be 1.76 in severe NV, versus normal levels for moderate NV
- Lysyl oxidase like 4, a protein that modulates collagen formation, was present only at later time points
- Cartilage Acidic Protein 1, a protein involved in cell differentiation, was increased at later time points vs. early time points

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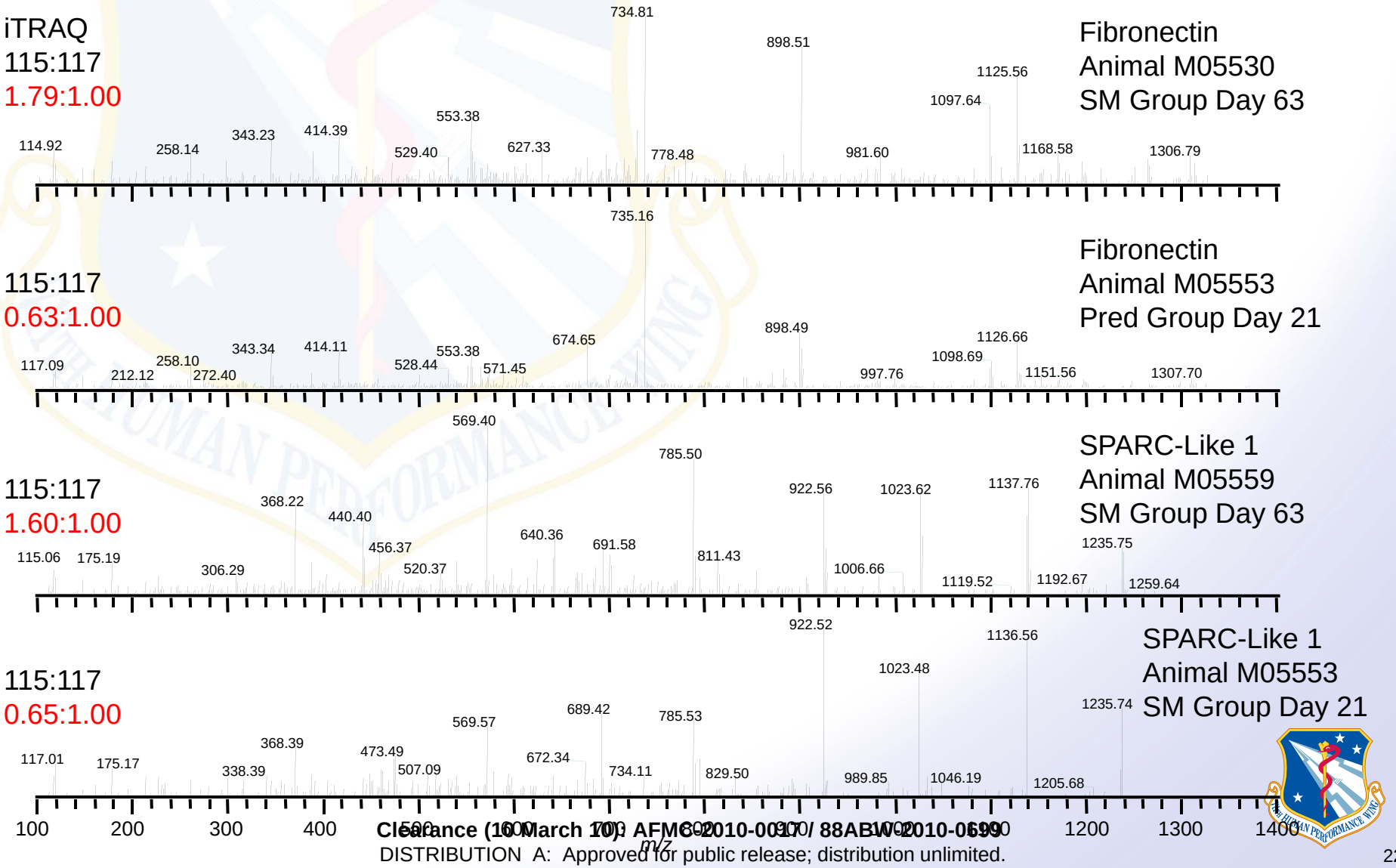
PQD Spectra of Fibronectin and SPARC-Like 1 from NV Corneas



Protein Averages of NV Endothelia

Protein Name	Accession Number	Avg. SM Group	Avg. Prednisolone Group
Keratocan	5901992	3.07±0.92	3.31±0.33
Fibronectin	31397	1.45±0.14	0.46±0.11
Fibulin-5	19743803	0.83±0.10	0.52±0.10
SPARC-Like 1	21359871	1.26±0.18	0.44±0.07
Lysyl Oxidase Protein Like 4	20177956	2.23±0.38	1.13±0.47
Serum Albumin	126723746	1.88±0.24	1.95±0.31
Transferrin	6175087	2.00±0.41	2.25±0.45
Immunoglobulin	165398	2.54±0.73	2.93±0.25
Thrombospondin-1	40317626	1.77±0.47	0.95 (in only 1 sample)

Conclusions

- **Removal and separate analysis of the epithelium and endothelium accompanied with long separation times expands the number of identified proteins for quantitation**
- **Quantitative proteomics using iTRAQ labeling has the ability to profile protein expression across the proteome in the cornea**
- **Metabolic proteins in the epithelium, including those involved in glycolysis, were found to be more reduced in SM only corneas than prednisolone treated corneas**
- **Endothelial proteins involved in wound healing and anti-angiogenic proteins were increased across all NV animals**
- **Prednisolone was found to moderately improve neovascularization of the cornea compared to solely washing in saline**

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QUESTIONS????