Assessment of Protein Stability in Whole Blood

Lisa J. Zimmerman
Jim Ayers Institute *for* Precancer Detection and Diagnosis
Vanderbilt University, Nashville, TN

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Jim Ayers Institute *for* Precancer Detection and Diagnosis

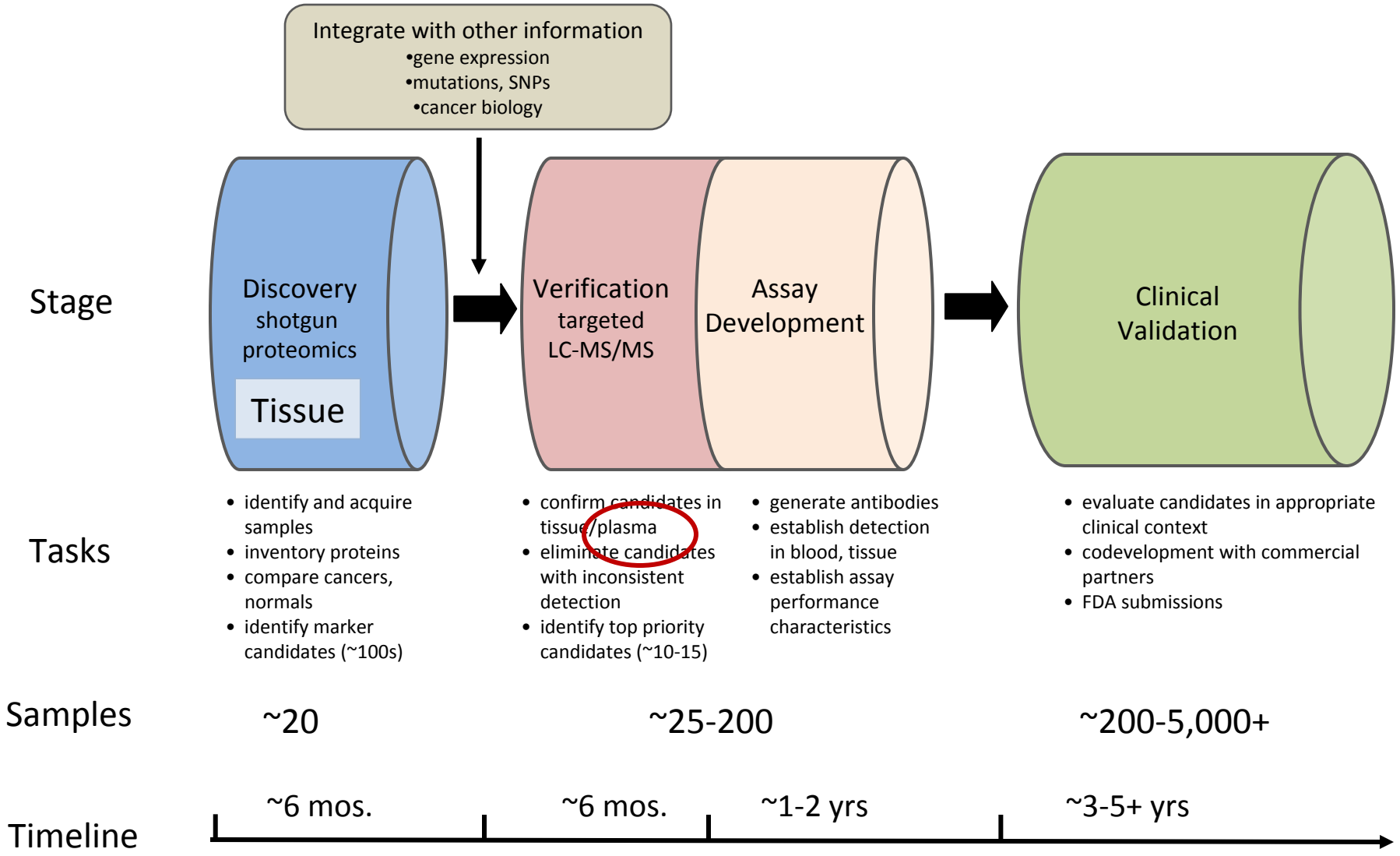
-Established in July 2005 with a gift from Mr. Jim Ayers to develop new diagnostic test to detect cancer in its earliest stages when treatment is most effective.

Objectives of the Ayers Institute

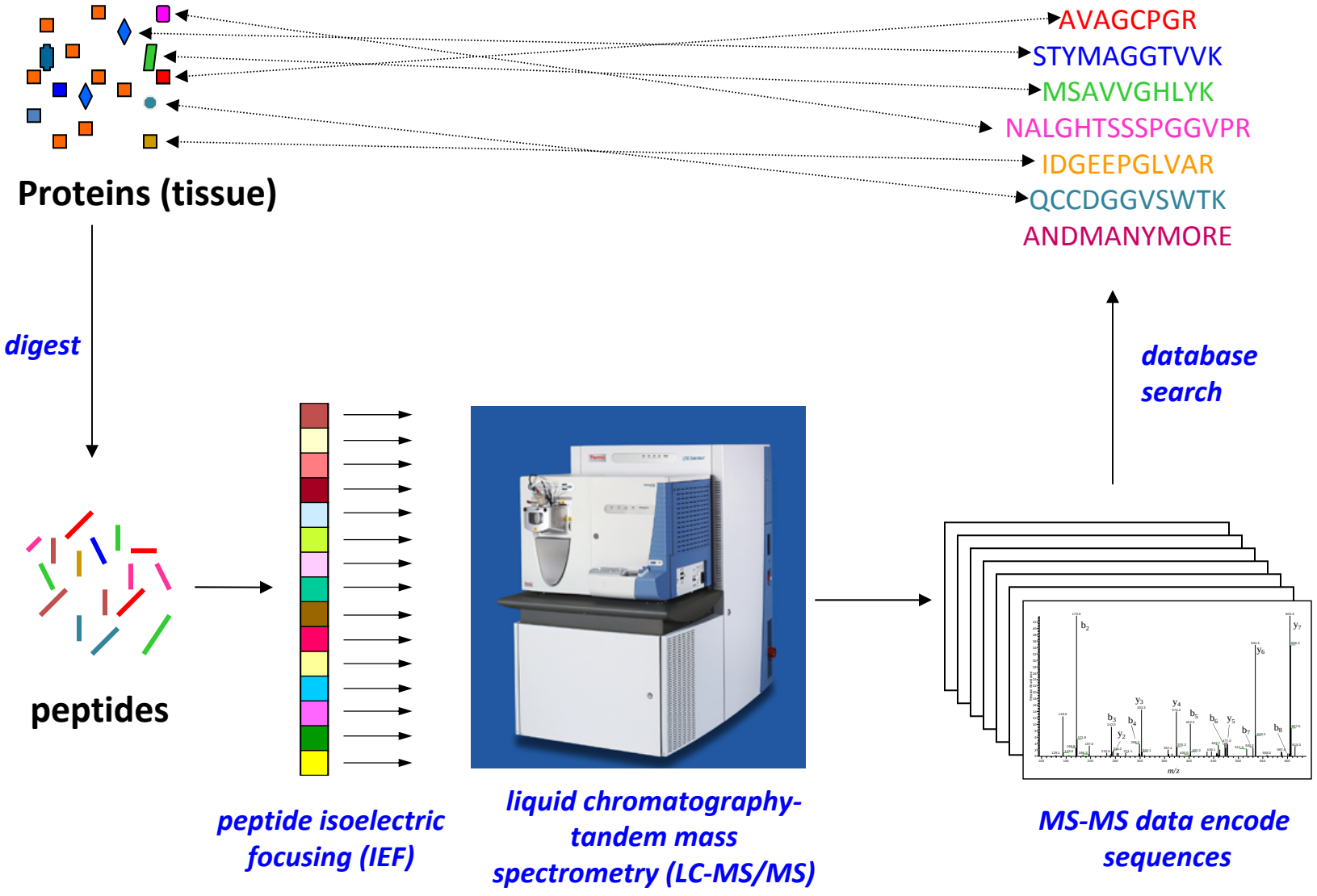
1.To detect cancers and precancers at their earliest stages
2.To develop diagnostic tests to assess prognosis for cancers and to predict responses to cancer treatment
3.To advance the science and technology of cancer detection and diagnosis.

-Initial focus was on colorectal cancer; however, efforts on detecting biomarkers for other cancers
such as lung, gastric and pancreatic cancers has taken place over the last few years.

Ayers Institute Biomarker Pipeline



Shotgun Proteomics for Biomarker Candidate Discovery



Development a Quality Control Metric to Assess Plasma Integrity

-*Question*? Can we develop a metric to assess whether a plasma sample is "*good*" or "*bad*" and whether to it is viable for proteomic analyses?

-Reasons:

1.Candidate markers identified in tissue will be verified in plasma (blood-based diagnostics)

2. Vanderbilt BioVU DNA Databank –research resource providing a "View into biology"

-Repository of DNA extracted from discarded blood samples (de-identified)
-As of 2009, over 50,000 DNA samples were in the biobank (700/week)

MALDI-MS Based Platform to Asssess Pre-analytical Variability

- Many previous studies focused on LMW proteome following some type of enrichment strategy (e.g. C8, C18, MWCO spin filter)

ADSGEGDFLAEGGGVR
DSGEGDFLAEGGGVR
SGEGDFLAEGGGVR
GEGDFLAEGGGVR
EGDFLAEGGGVR
GDFLAEGGGVR
DFLAEGGGVR
FLAEGGGVR
LAEGGGVR
SSSYSKQFTSSTSYNRGDSTFESKSYKMA
SSSYSKQFTSSTSYNRGDSTFESKSYKM
SSSYSKQFTSSTSYNRGDSTFESKSY
SSSYSKQFTSSTSYNRGDSTFESKS
SSSYSKQFTSSTSYNRGDSTFES
SSYSKQFTSSTSYNRGDSTFE
SYKMADEAGSEADHEGTHSTKRGHAKSRPV
DEAGSEADHEGTHSTKRGHAKSRPV

fibrinogen

SSKITHRIHWESASLLR
SSKITHRIHWESASLL
SKITHRIHWESASLL
KITHRIHWESASLL
ITHRIHWESASLL
THRIHWESASLL
HRIHWESASLL
RIHWESASLL
IHWESASLL
HWESASLL
SSKITHRIHWESASL

Complement C3f

GLEEELQFSLGSKINVKVGGNSKGTLKVLR
GLEEELQFSLGSKINVKVGGNSKGTL
GLEEELQFSLGSKINVKVGGNS
GLEEELQFSLGSKINVKV
NGFKSHALQLNNRQIR
NGFKSHALQLNNRQI
NGFKSHALQLNNRQ
NGFKSHALQLNNR
NGFKSHALQLN

Complement C4 precursor

QAGAAGSRMNFRPGVLSSRQLGLPGPPDVPDHAAYHPF
QLGLPGPPDVPDHAAYHPFR
MNFRPGVLSSRQLGLPGPPDVPDHAAYHPF
PGVLSSRQLGLPGPPDVPDHAAYHPF
GVLSSRQLGLPGPPDVPDHAAYHPF
SSRQLGLPGPPDVPDHAAYHPF
SRQLGLPGPPDVPDHAAYHPF
QLGLPGPPDVPDHAAYHPF
GLPGPPDVPDHAAYHPF
PGPPDVPDHAAYHPFg
GPPDVPDHAAYHPF
HAAYHPF

ITIH4

Koomen, J. M., et al. *J. Proteome Res*. **4**, 3, 2005, 972-981.

Development a Quality Control Metric to Assess Plasma Integrity

- Easiest approach –MALDI-MS based analysis; Spectra can be distinguished according to time of processing (TOP), temperature or both; and relevant peaks can be identified that differentiate between the above conditions.

-Blood collected from volunteers on the **Yul Brynner Head and Neck Cancer Screening Day**.

-MALDI-MS spectra from plasma of 41 normal *volunteer* human subjects were used to test hypothesis (total of 375 spectra)

Blood

Place immediately on ice; invert with lavender top plastic tube (EDTA).

1.5 mL
Transferred to
Eppendorf tube

Step 1. 100 µL blood aliquotted into 96-well plate for each sample.

Parameters examined: time prior to centrifugation and temperature.

Time	Temperature
0	
4hr	4C and RT
24 hr	
72 hr	
1 week	

Step 2. Remaining blood is spun down @ 1,500 x g for 15 minutes and plasma stored at -80C.

Designed at Time “0”.

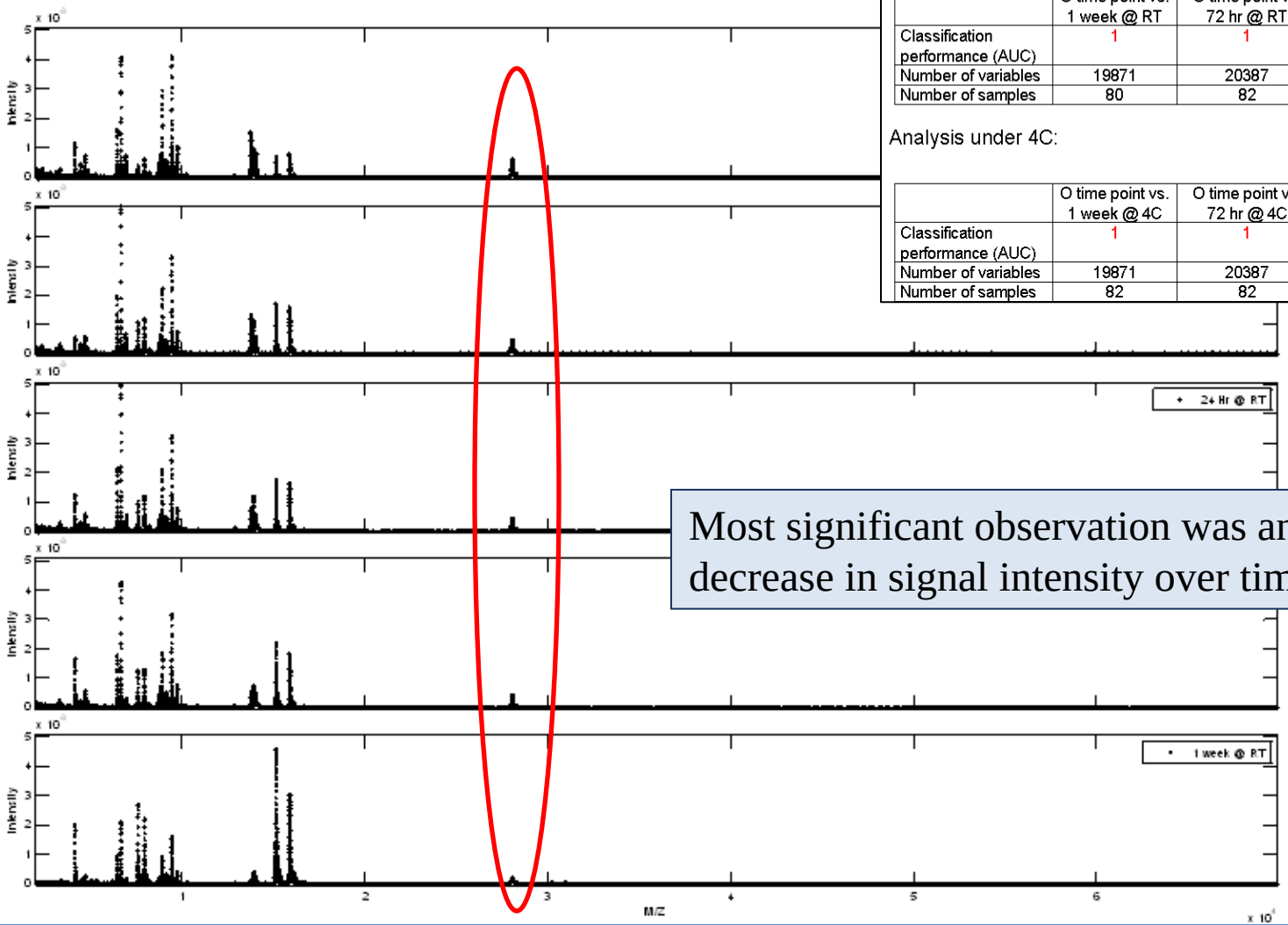
Example @ Fixed temperature at RT, vary time

Analysis of degradation of predictivity with time

	O time point vs. 1 week @ RT	O time point vs. 72 hr @ RT	O time point vs. 24 hr @ RT	O time point vs. 4 hr @ RT
Classification performance (AUC)	1	1	1	0.8357
Number of variables	19871	20387	19471	34424
Number of samples	80	82	82	82

Analysis under 4C:

	O time point vs. 1 week @ 4C	O time point vs. 72 hr @ 4C	O time point vs. 24 hr @ 4C	O time point vs. 4 hr @ 4C
Classification performance (AUC)	1	1	1	0.9750
Number of variables	19871	20387	19471	20387
Number of samples	82	82	81	82



Most significant observation was an overall decrease in signal intensity over time

Several classifiers that differentiated samples on the basis of MALDI spectral analysis were identified. Predictivity (red) of classifiers are shown which separate spectra produced at different TOP's. Predictivity is measured with area under the ROC curves and thus 0.5 = random predictivity, 1= perfect predictivity.

Assessment of Plasma Stability Using Analysis Using Shotgun Proteomics

A. Collection Times (Time prior to centrifugation) and Temperature

-0, 4, 24, 72 hr and 1 week at either 4C or RT
-10 from each time point were pooled and aliquotted into 3 processed replicated

B. Freeze-Thaw Cycles

-Three plasma samples (designated A, B, and C) were collected from EDTA tubes.

-Seven 200 uL aliquots were prepared for each; each aliquots was subjected to 0, 1, 2, 3, 5, 10, or 25 freeze-thaw cycles.

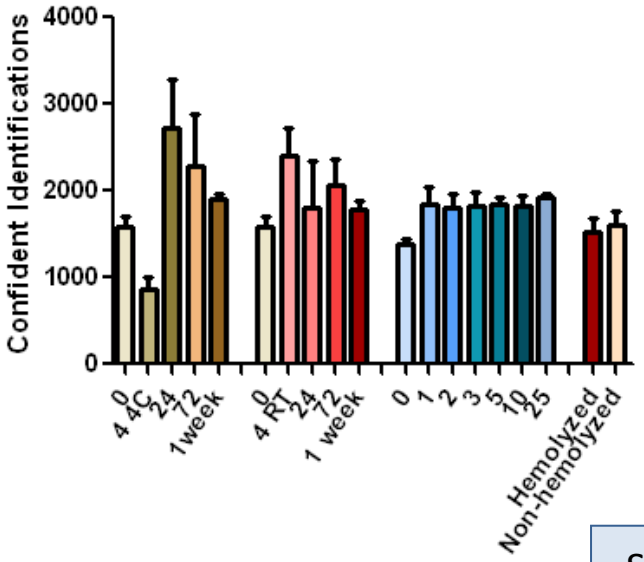
Digestion and LC/MS/MS Analysis

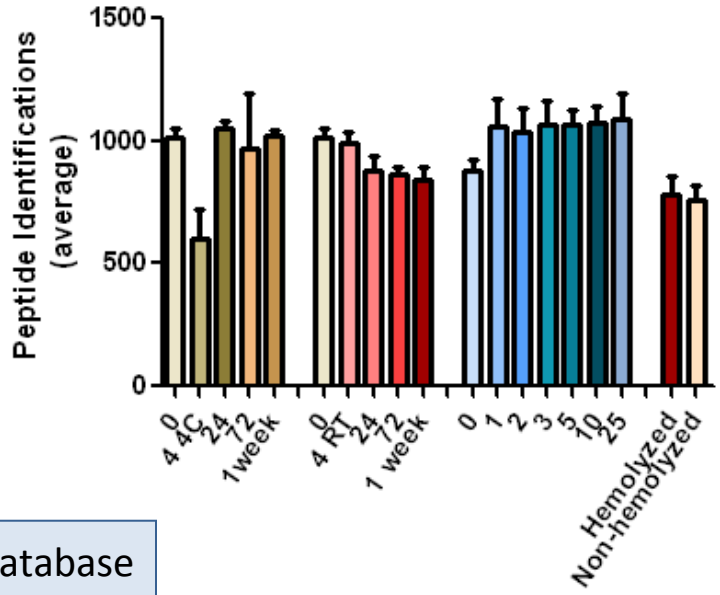
Statisical Analysis using QuasiTel
And
Targeted MRM Analysis

C. Hemolyzed verses Non-hemolyzed

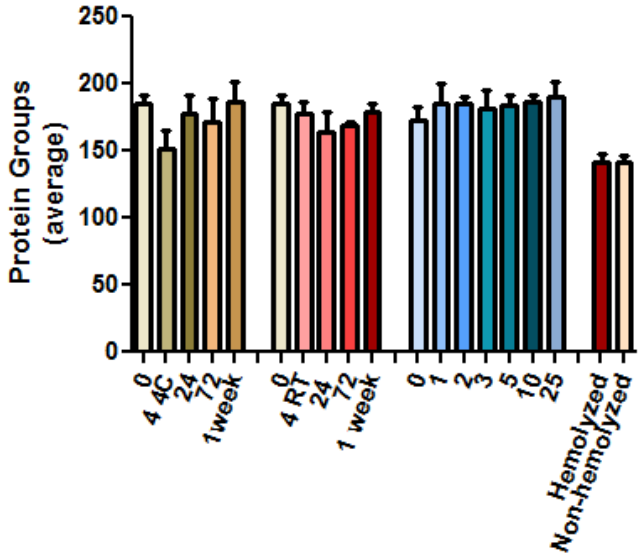
-10 each of hemolyzed and non-hemolyzed

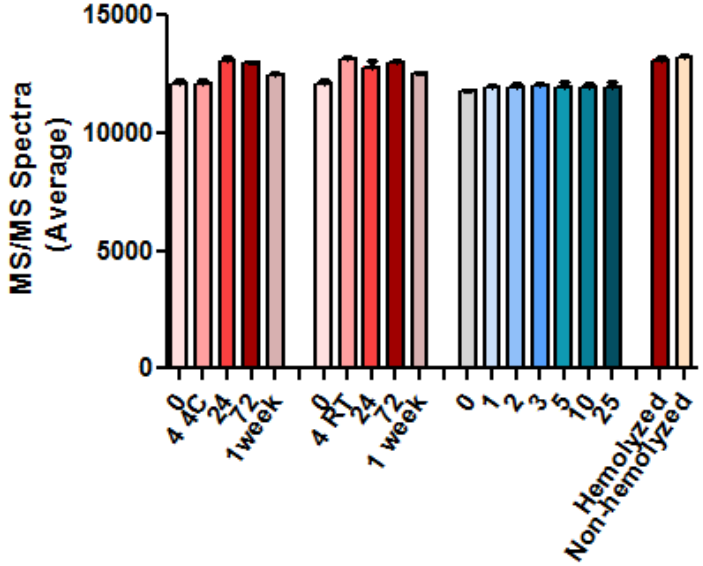
Results of Shotgun Proteomic Analysis on Protein Identificatons



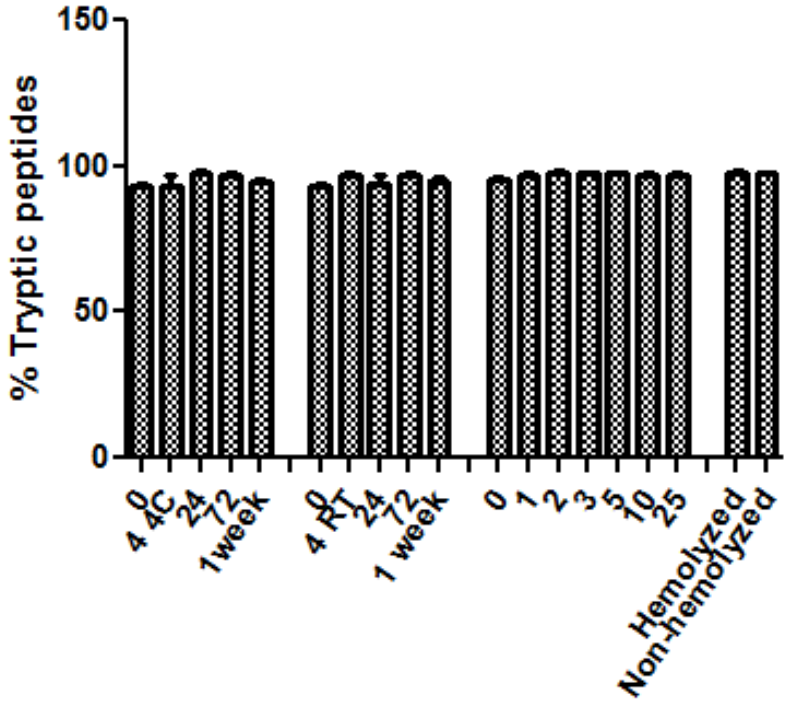


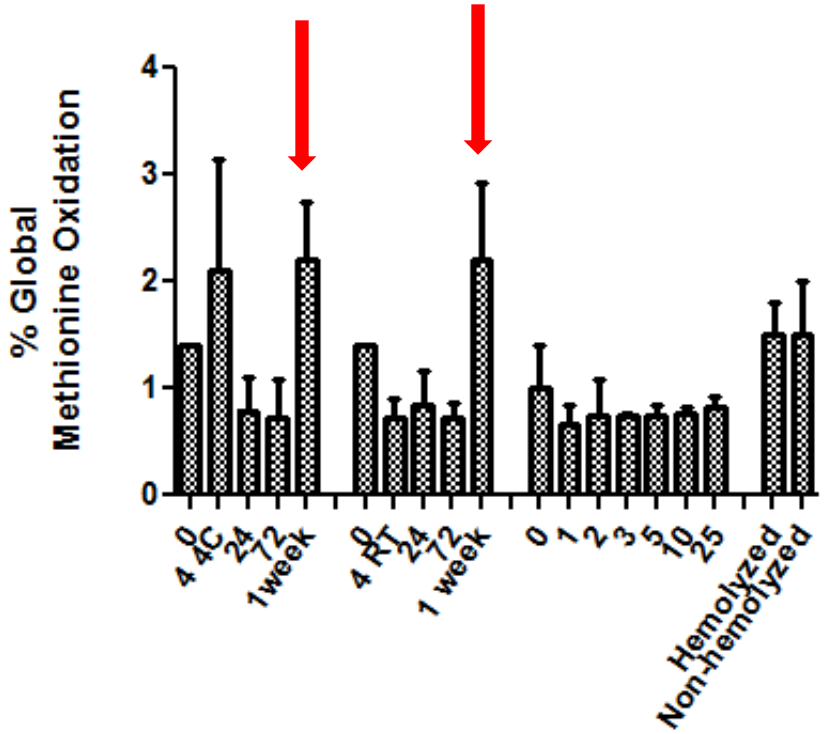
-Searched against human database
-Filtered to achieve a 5% FDR





Additional Qualitative Parameters Characterizing Global Plasma Proteome





Statistical Analysis of Shotgun Proteomic Datasets Using QuasiTel

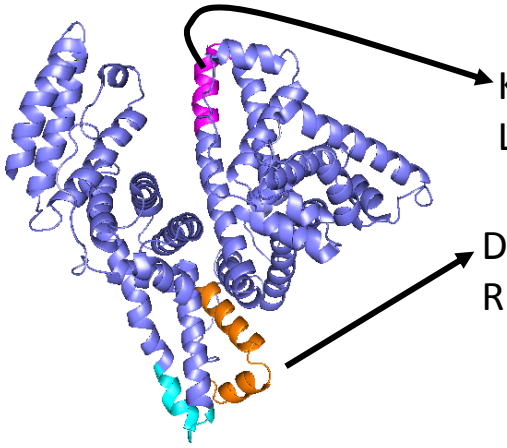
-Fold changes in spectral count data of peptides identified were compared using quasi-likelihood modeling while correcting for false discovery rate FDR.

- Peptides found to be significantly different were monitored using multiple reaction monitoring (MRM) analysis.

Peptides from 0-1 week RT	Parent Protein	Total	zero	1wkRT	zero_rate	1wkRT_rate	RateRatio	Quasi_FDR
EFNAETFTFHADIC(57.0215)TLSEK	Albumin	214	16	198	0.0036	0.0381	-3.42	0.00120
DVFLGMFLYEYAR	Albumin	177	11	166	0.0025	0.0319	-3.70	0.00735
ALVLIAFAQYLQQC(57.0215)PFEDHVK	Albumin	92	14	78	0.0031	0.0150	-2.27	0.01255
ADDKETC(57.0215)FAEEGKK/ADDKETC(57.0215)FAEEGQK	Albumin	30	10	20	0.0022	0.0038	-0.79	0.03287
Q(-17.0265)EPERNEC(57.0215)FLQHK	Albumin	27	9	18	0.0020	0.0035	-0.79	0.04085
VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK	IgG	15	0	15	0.0000	0.0029	-32.84	0.02634
Peptides from 0-25 freeze thaw cycles	**Parent Protein**	**Total**	**zero**	**25 FT Cycles**	**zero_rate**	**25_rate**	**RateRatio**	**Quasi_FDR**
EFNAETFTFHADIC(57.0215)TLSEK	Albumin	209	16	193	0.0038	0.0335	3.1316	0.0178
SHC(57.0215)IAEVENDEMPADLPSLAADFVESK	Albumin	31	3	28	0.0007	0.0049	2.7615	0.0064
LESDVSAQMEYC(57.0215)R	Fibrinogen Beta	15	3	12	0.0007	0.0021	1.5391	0.0386
WQQGNVFSC(57.0215)SVMHEALHNHYTQK	IgG	13	3	10	0.0007	0.0017	1.2761	0.0317
VDGALC(57.0215)MEK	Hemopexin	9	3	6	0.0007	0.0010	0.5391	0.0396
VRVELLHNPAFC(57.0215)SLATTK	Complement C3	9	3	6	0.0007	0.0010	0.5391	0.0396

-Additional peptides that had similar spectra counts between 0 and 1 week time points were also monitored.

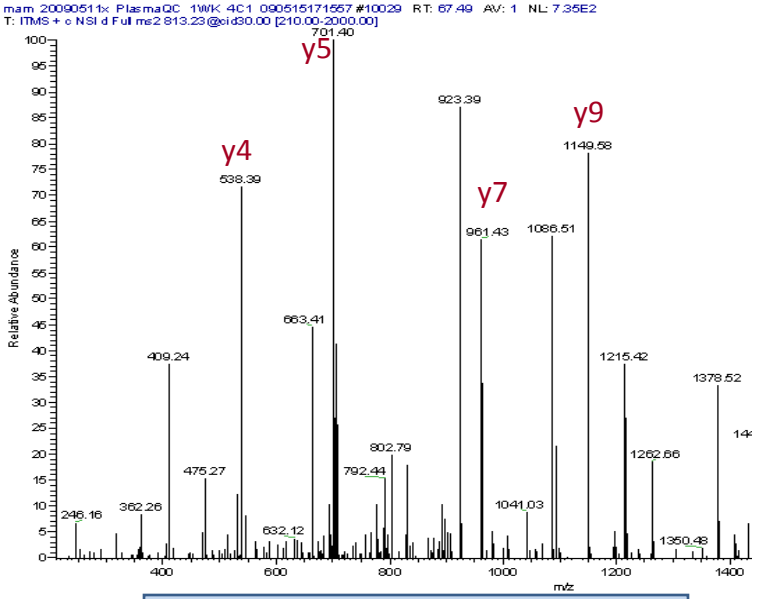
Targeted MRM Analysis



KLVAASQAALGL

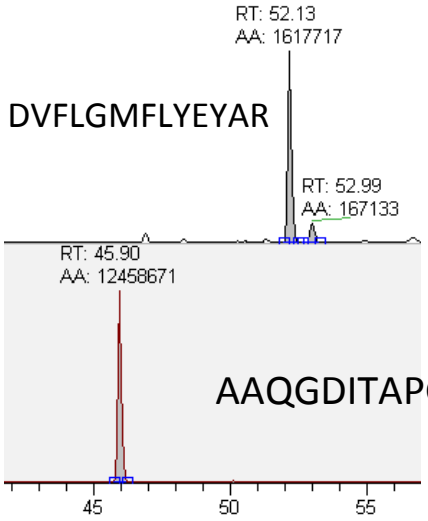
DVFLGMFLYEYAR

Select peptide-specific transitions



D-V-F-L-G-M-F-L-Y-E-Y-A-R

y9 y7 y5 y4

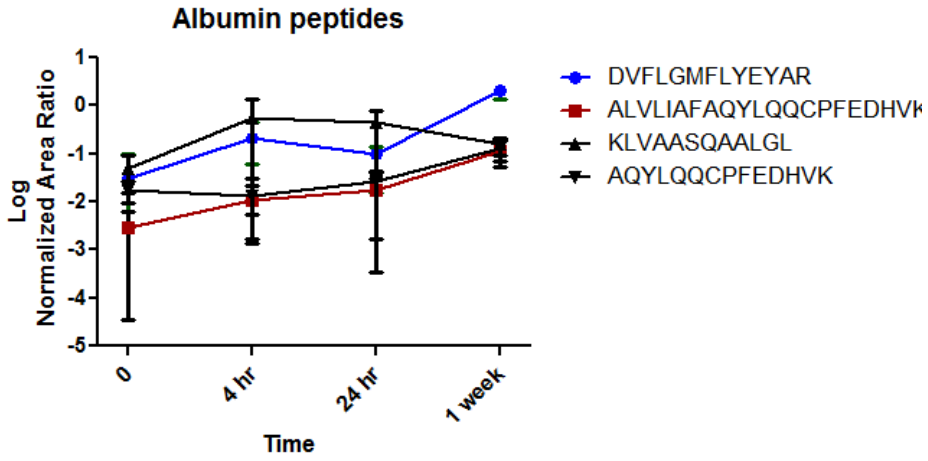


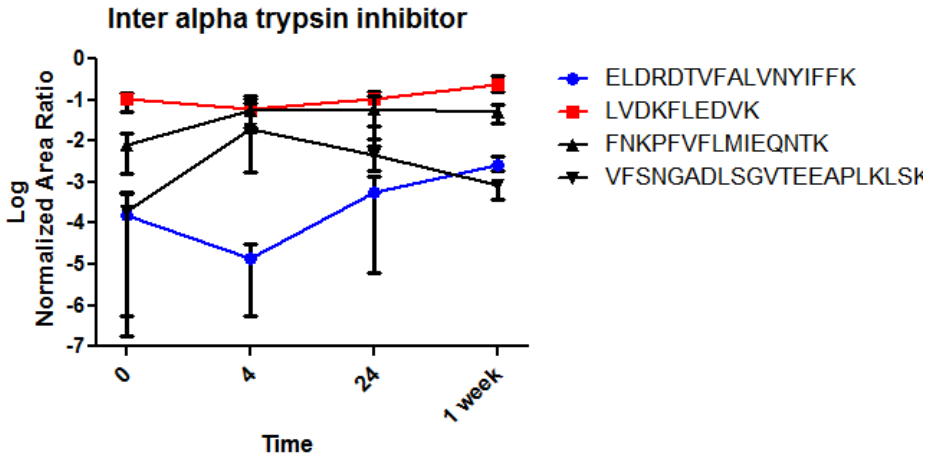
AAQGDITAPGGA*R[$^{13}C^{15}N$]*

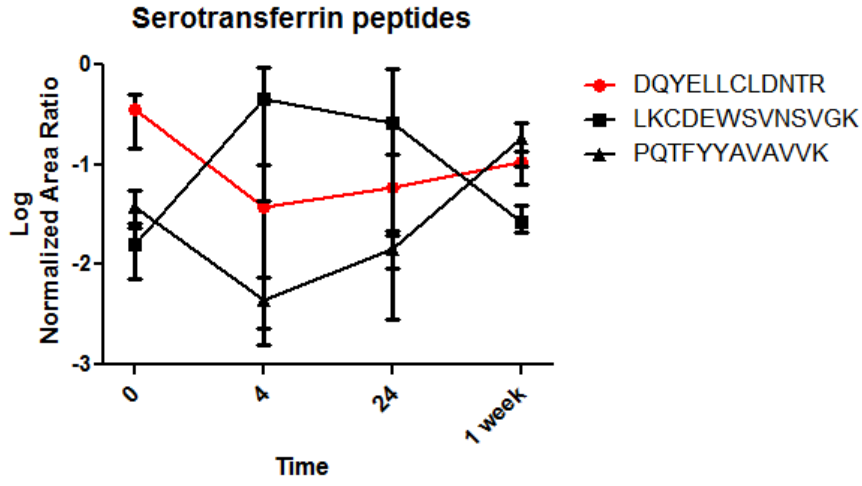
Spike in labeled peptide from *E. Coli* bacterial protein [alkaline phosphatase] at a known concentration for normalization.

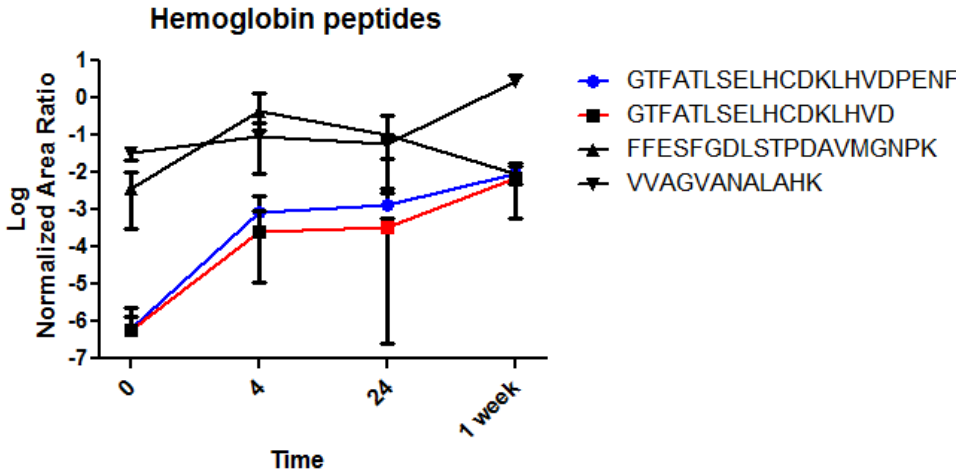
Relative quantitation based on peak areas for target peptide and labeled standard/s

Results of Targeted MRM Analysis of Time Points

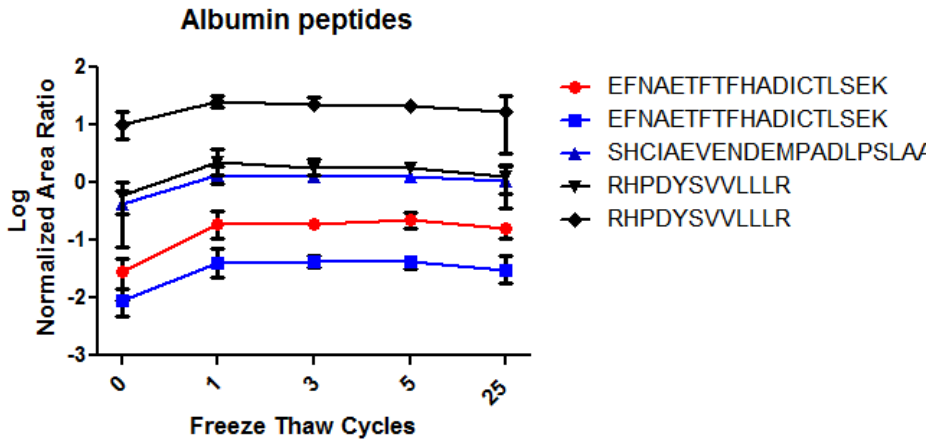


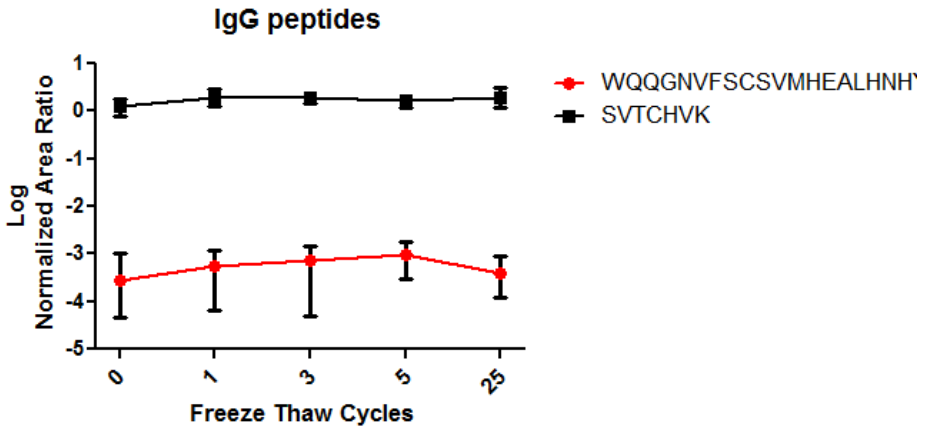


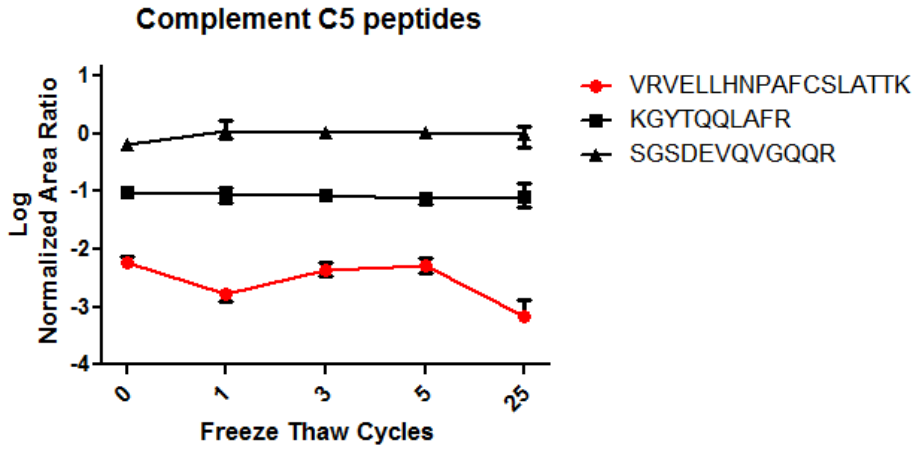


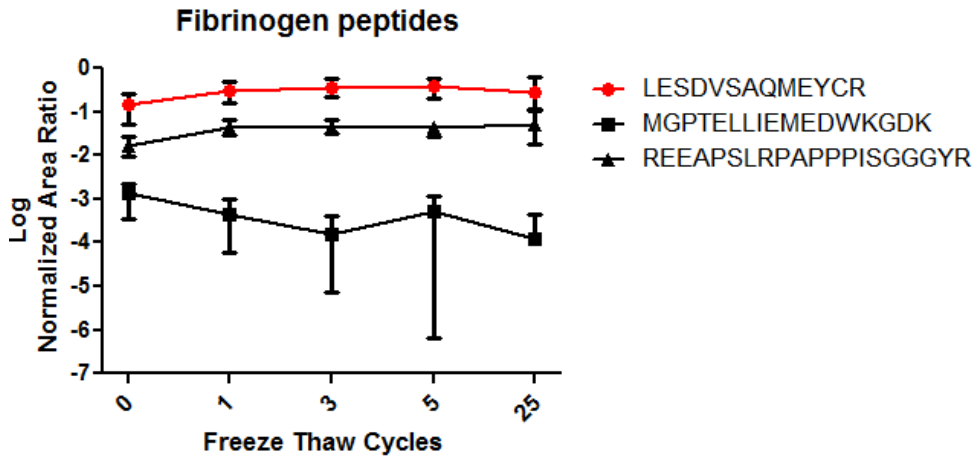


Results of Targeted MRM Analysis of Freeze-Thaw Cycles









MRM Analysis of Truncated Fragments of Fibrinogen

ADSGEGDFLAEGGGVR
ADSGEGDFLAEGGGVR
ADSGEGDFLAEGGGVR
ADSGEGDFLAEGGGVR
ADSGEGDFLAEGGGVR
ADSGEGDFLAEGGGVR
DSGEGDFLAEGGGVR
DSGEGDFLAEGGGVR
DSGEGDFLAEGGGVR
DSGEGDFLAEGGGVR
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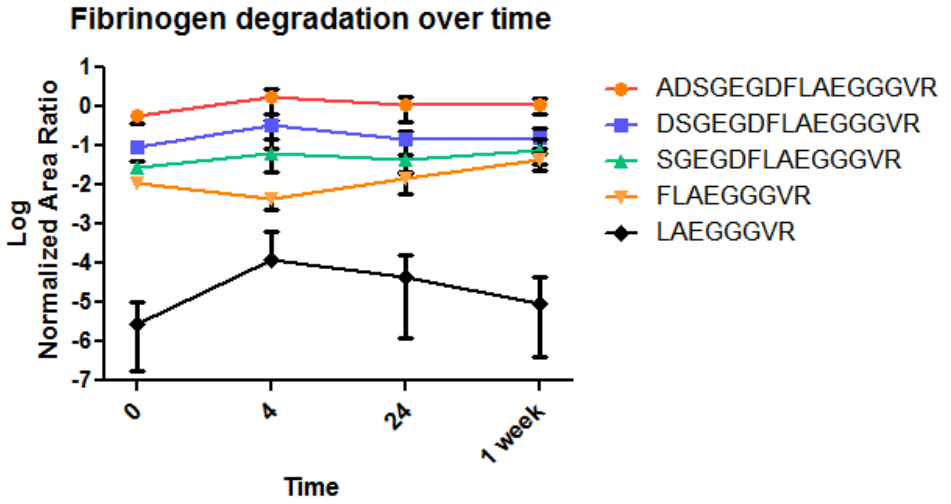
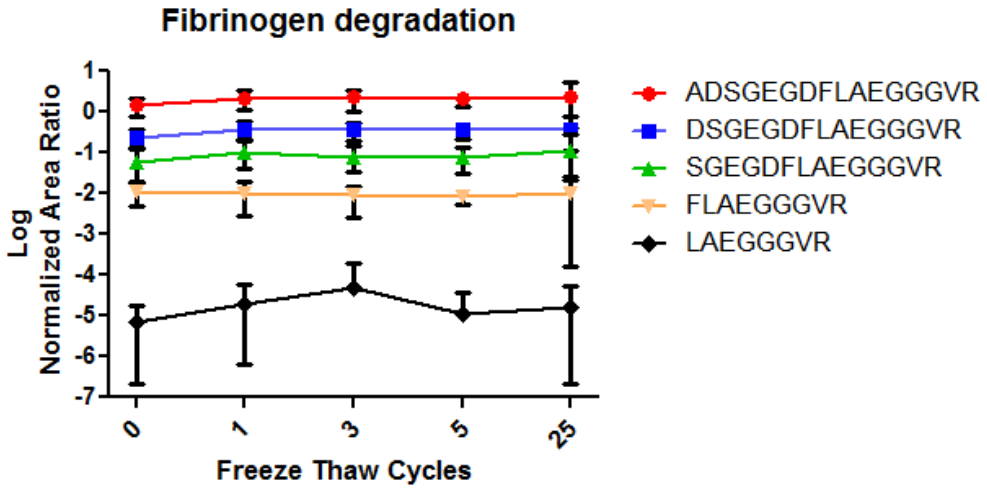
LAEGGGVR

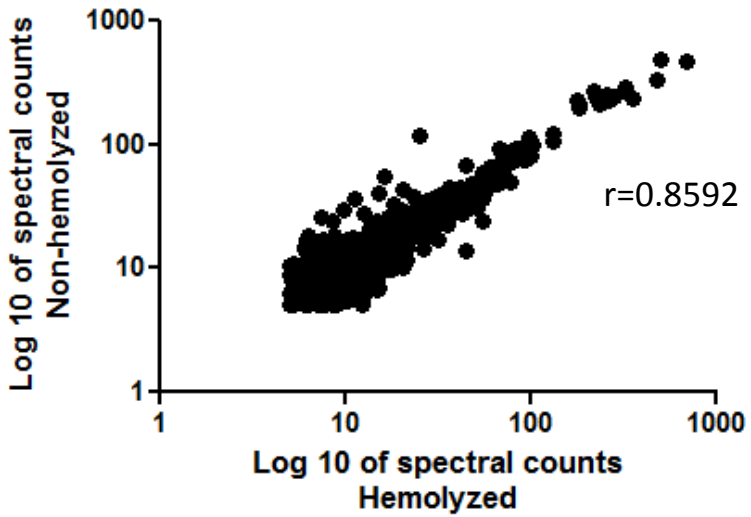
Fibrinogen

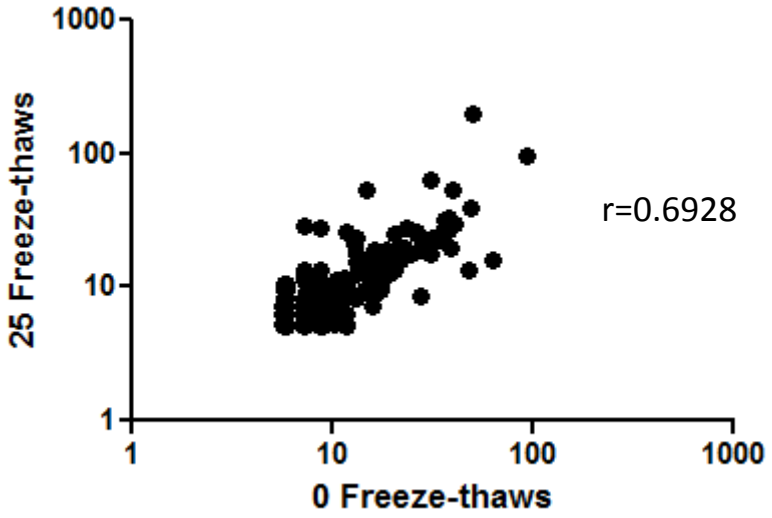


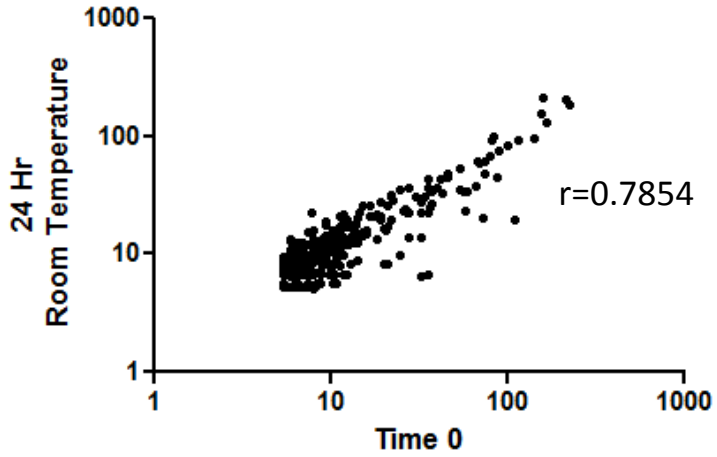


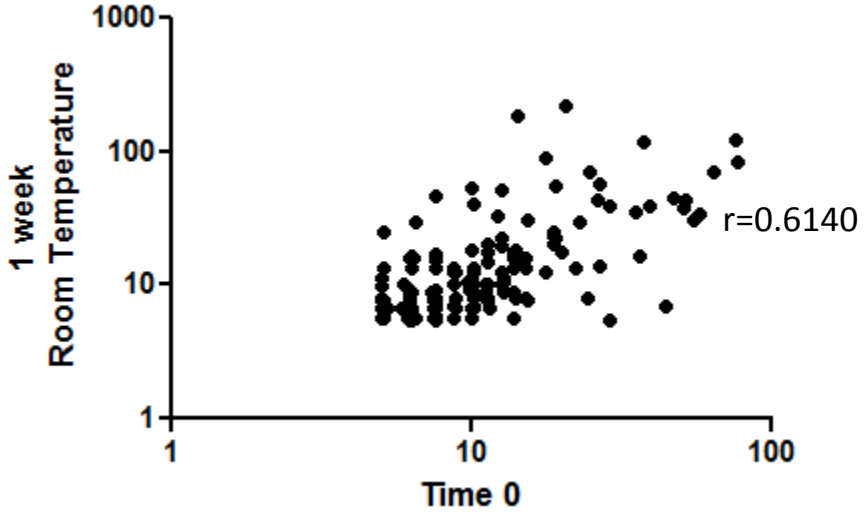
Koomen, J. M., et al. *J. Proteome Res*. **4**, 3, 2005, 972-981.

Correlation Scatter Plots









Conclusions and Future Directions

- Many of the recommendations for plasma processing are based upon MALDI-MS based results (Protein level).

-However, changes in the global distribution at the peptide level, both tryptic and semi-tryptic, in stored plasma are not detected upon digestion and LC-MS/MS analysis.
-

-Observed minimal changes in proteins of plasma collected in EDTA tubes with only modest degradation of those proteins previously shown to be highly susceptible to cleavage (i.e. fibrinogen, complement C3).

- Our results indicate that degradation occurring during storage is randomly distributed, and while some proteins may display higher instability, most proteins are relatively unaffected under such conditions.

-These results suggest that plasma samples previously thought to be unusable may be viable for biomarker discovery and follow-up verification studies.

-Similar experiments planned with *serum and tissue samples.*

Acknowledgements

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