THE FLYING IONS

A UAB Mass Spec Interest Group

“I know my protein is modified…why couldn’t you find it?”

Matt Renfrow, PhD

Wednesday, March 25th 2009

I Know My Protein is Modified- Why Can’t You Find It?

Matthew B. Renfrow

Assistant Professor
Department of Biochemistry & Molecular Genetics

The Flying Ions MSIG
March 25, 2009

UAB
Biomedical FT-ICR MS
LABORATORY

High Resolution Mass Spectrometry

Ernie PTM
Success Stories

Glycosylation
- *O*-glyc osylation IgA1
- *N*-glycosylation IgA1
- *N*-glycosylation HIV gp120
- Oral bacterial protein
- O-GlcNAc signaling

Ubiquitination
- Histone ___ (in vitro reaction)

Phosphorylation
- Drosophila protein

Oxidative Modifications
- hCKB (in vitro / in vivo)
- hBAT (in vitro)

UAB
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High Resolution Mass Spectrometry

Major Points for locating sites of modification

Assumptions: This is NOT protein discovery.
Requires some level of familiarity.

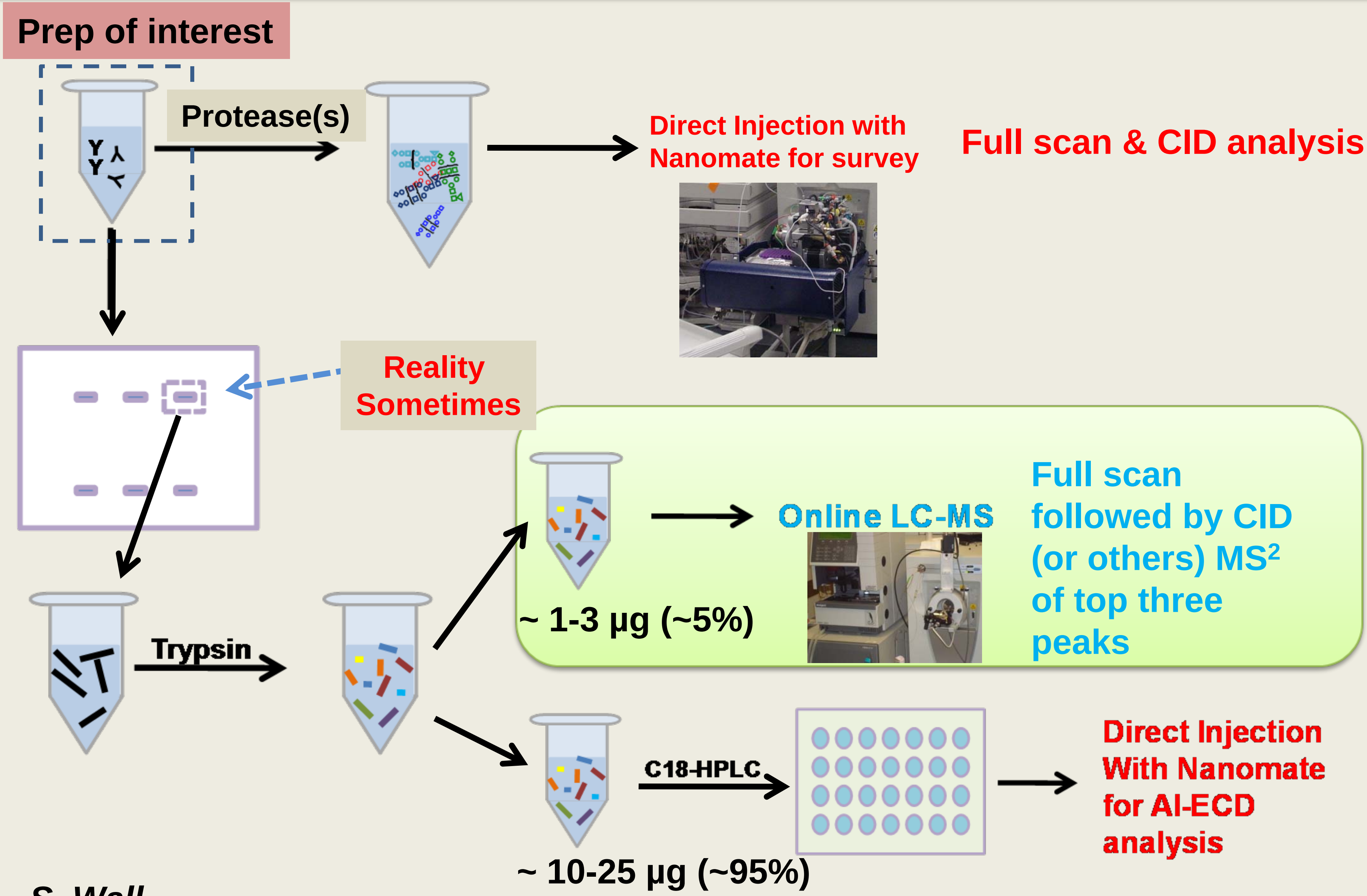
#1 Requirement for locating a site of MODIFICATION:
You must see the fragment!

#2 Major Point: Scale doesn’t matter if you are not
Sampling the MODIFIED FORM

#3 MAJOR POINT: What can potentially mask your modification and how can you get around that?

#4 Major Point: How do you visualize the results?
Qualitative alone may not work?

Analysis of *PTM's*



Prep of interest

Protease(s)

Direct Injection with Nanomate for survey

Full scan & CID analysis

Reality Sometimes

Trypsin

Online LC-MS

Full scan followed by CID (or others) MS^2 of top three peaks

~ 1-3 µg (~5%)

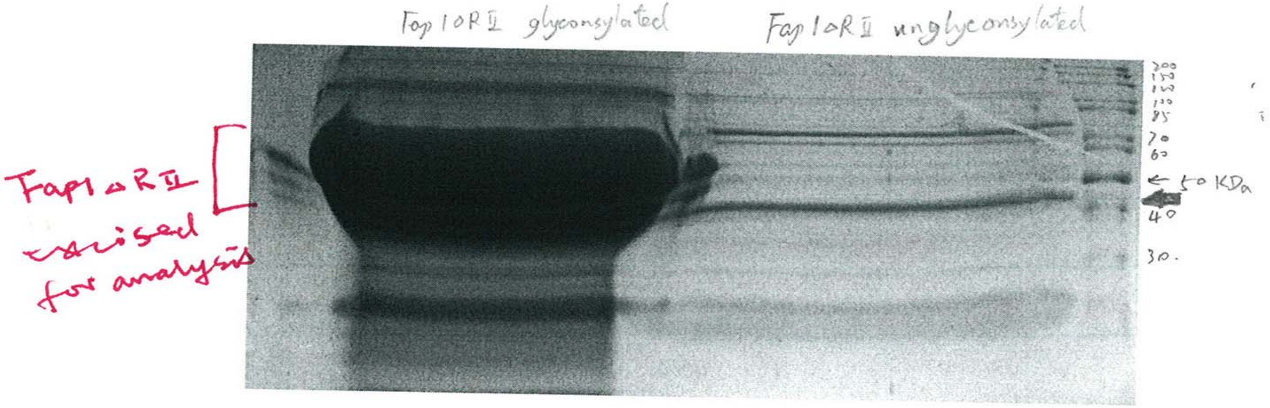
C18-HPLC

Direct Injection With Nanomate for AI-ECD analysis

~ 10-25 µg (~95%)

S. Wall

Committed to the analysis



H. Wu,
Pediatric Dentistry

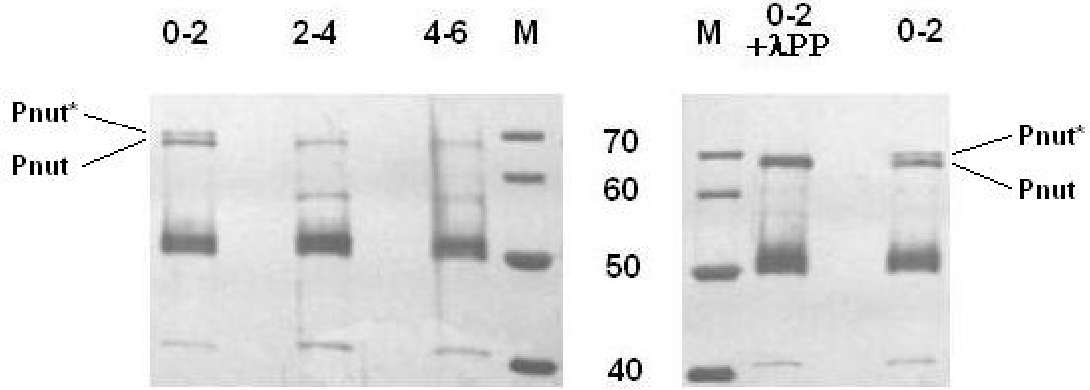
Phosphorylation analysis

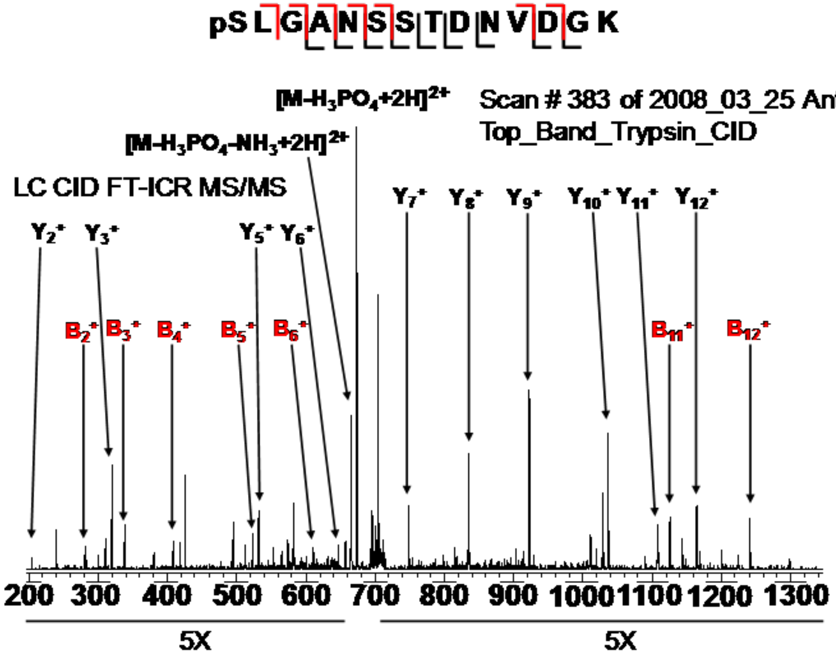
1. Their characterized complex

2. Previously worked on identifying components

3. They knew what it took to isolate this complex before we ever saw the sample.

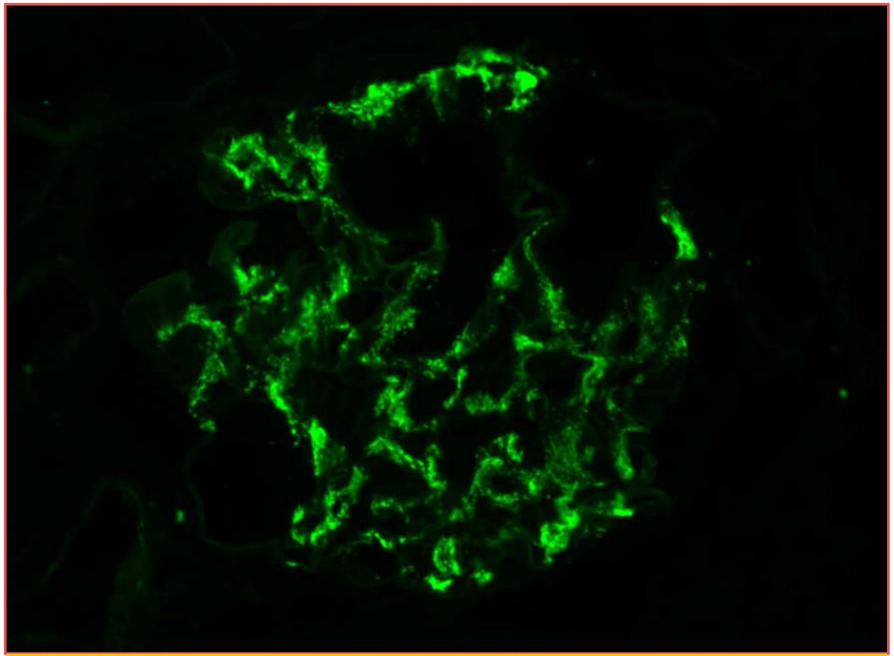
4. You can do discovery of Phosporylation sites.





A. Svitin
I. Chesnokov, Biochemistry

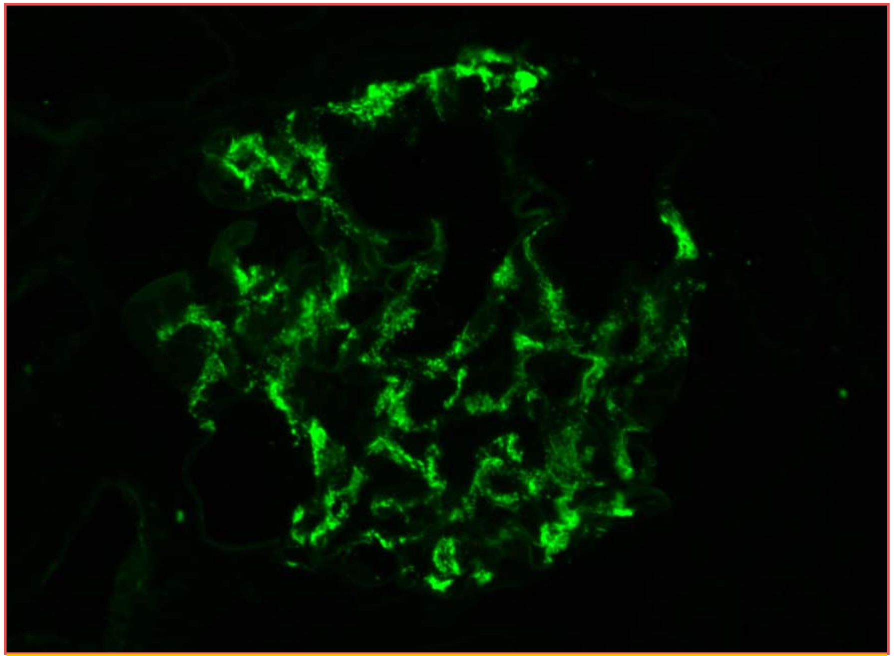
IgA Nephropathy (IgAN)



Immunoflouresence stained kidney biopsy

- Most predominant form of glomerulonephritis
- Children & young adults
- Male:female ~ 2 : 1
- 20-40% of IgAN patients will require dialysis or kidney transplant within 25 – 30 years after onset
- Caused by deposition of IgA induced circulating immune complexes (CIC’s) in the kidney and in serum of IgAN patients

IgA1 *O*-glycosylation



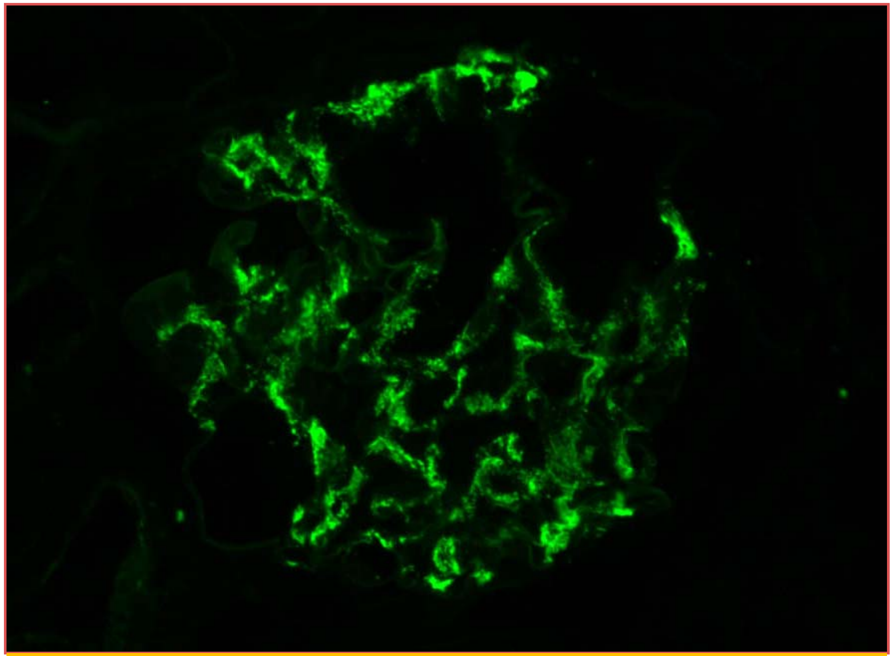
Immunoflouresence stained kidney biopsy

- Modified protein involved in disease pathogenesis.
- Biomarker
- MS Analytical Challenge

KEEP IN MIND THE FOLLOWING

- Source of the sample
- Orders of Magnitude (modified vs. unmodified)
- What assumptions are being made about the sample?
- What assumptions are being made about the sample prep?
- Microheterogeneity (total population)

Why MS needed?



Immunoflouresence stained kidney biopsy

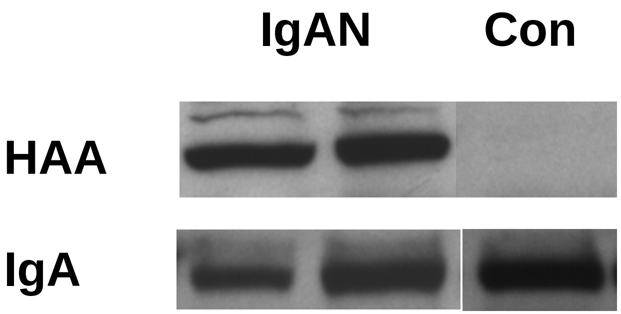
- **Locate sites of modification?**
- **Changes in *O*-glycans linked to the disease.**

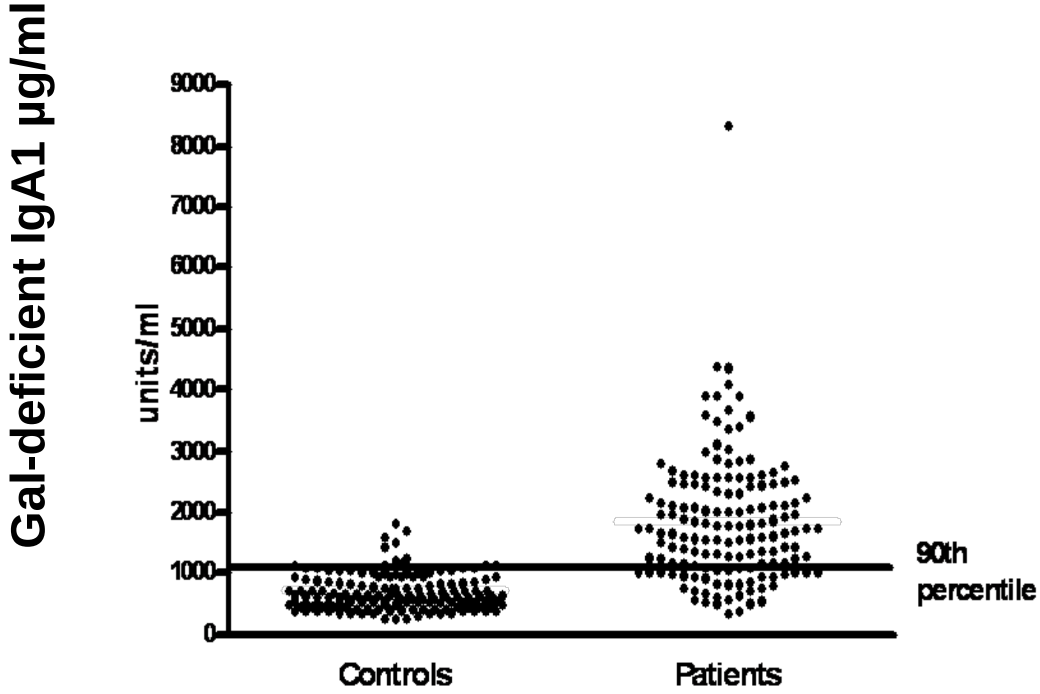
IgA1 HR lectin analysis

Total Serum IgA1

HA ELISA assay

Glycan Specific Lectin Western Blots

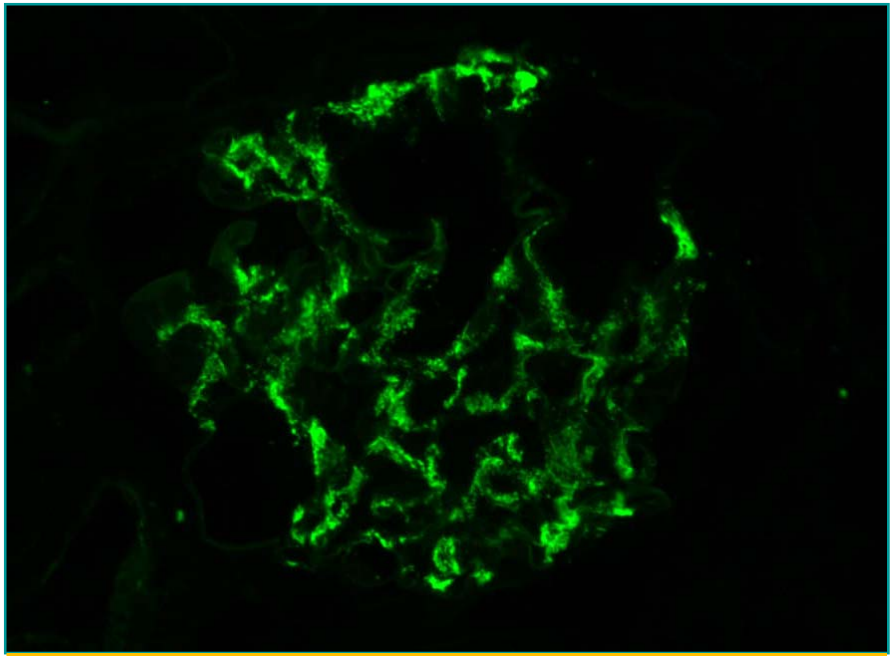




Moldeveanu et al., KI (2007)

Moore et al., MI (2007)

Why MS needed?



Immunoflouresence stained kidney biopsy

- **Locate sites of modification?**
- **Changes in *O*-glycans linked to the disease.**
- **Where do these changes occur? (specific or random sites)**

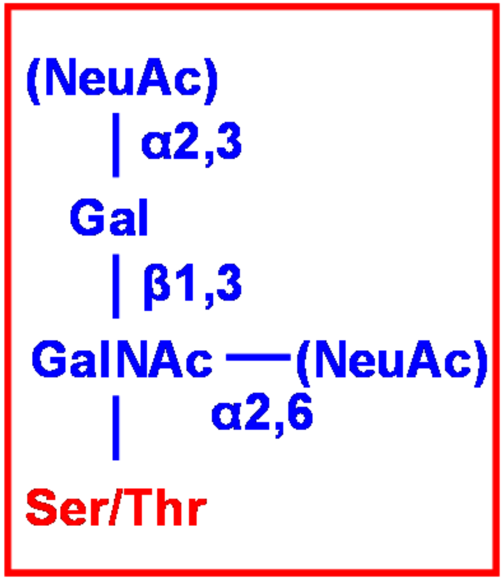
IgA1 Hinge Region

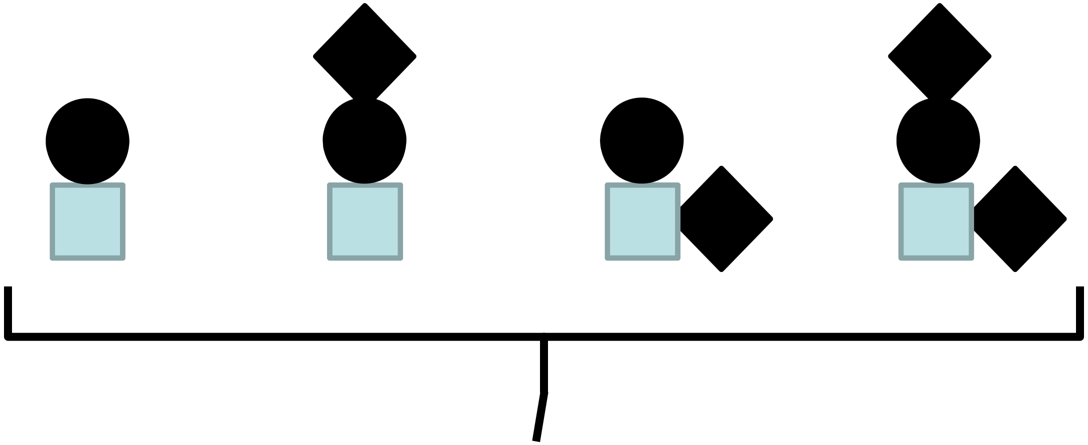
🟩 = GalNac

◆ = NeuAc

● = Gal

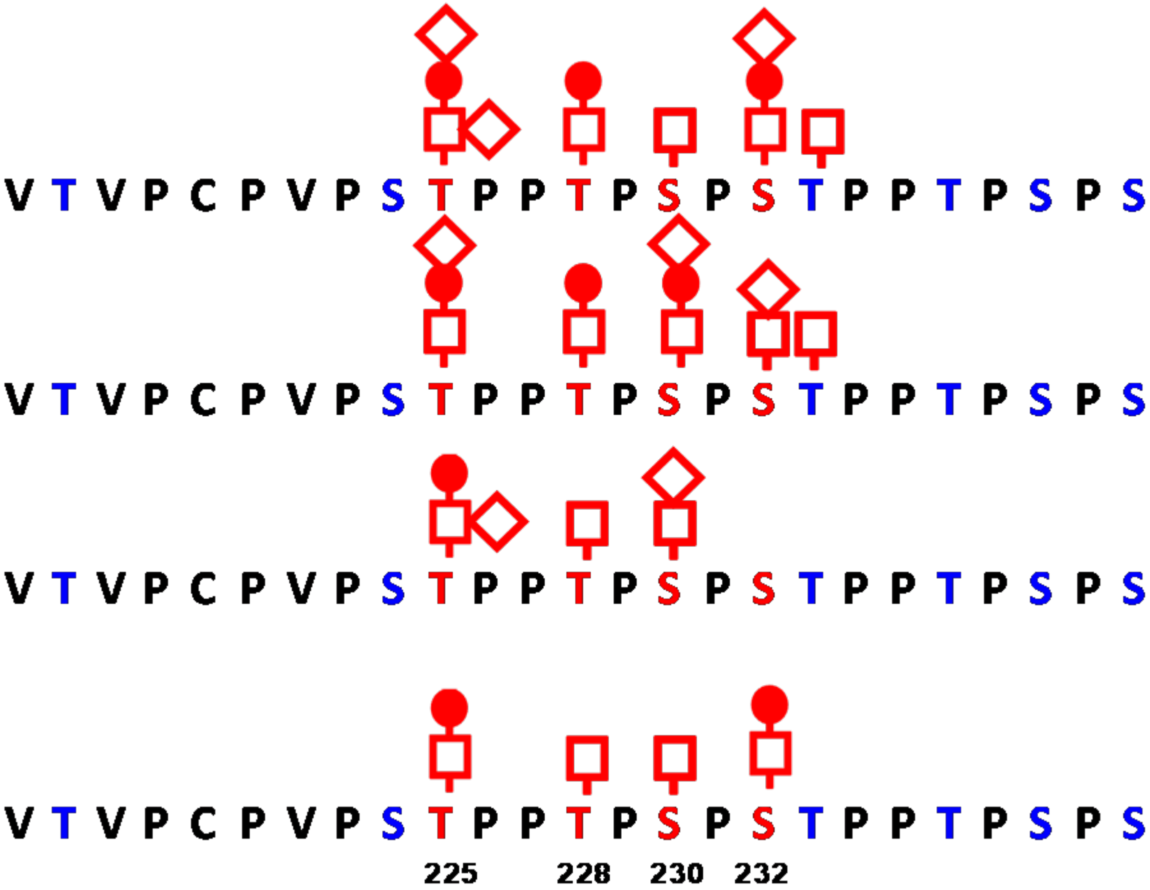
O-glycosylation


(NeuAc)
α2,3
Gal
β1,3
GalNAc —(NeuAc)
α2,6
Ser/Thr

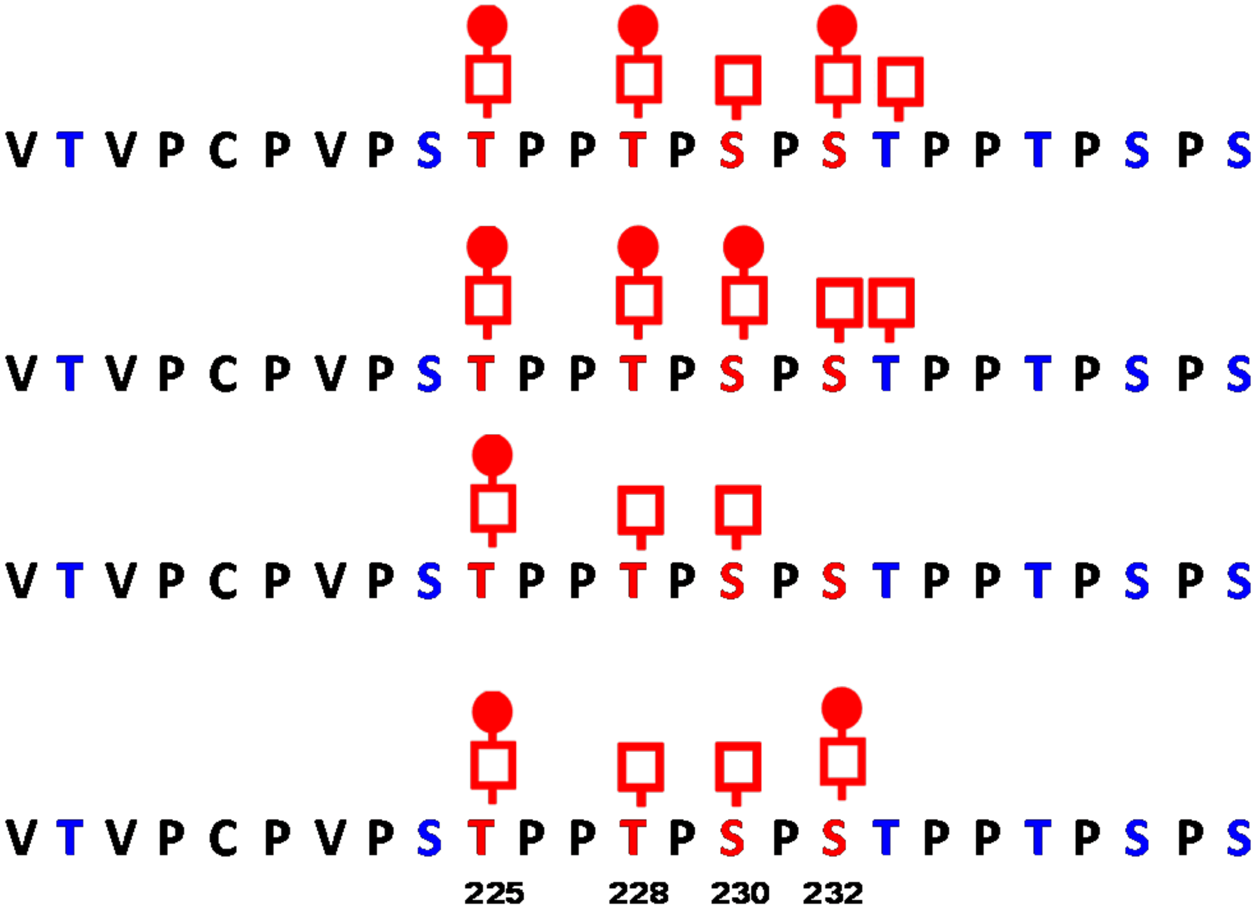


V T V P C P V P S T P P T P S P S T P P T P S P S C C H P

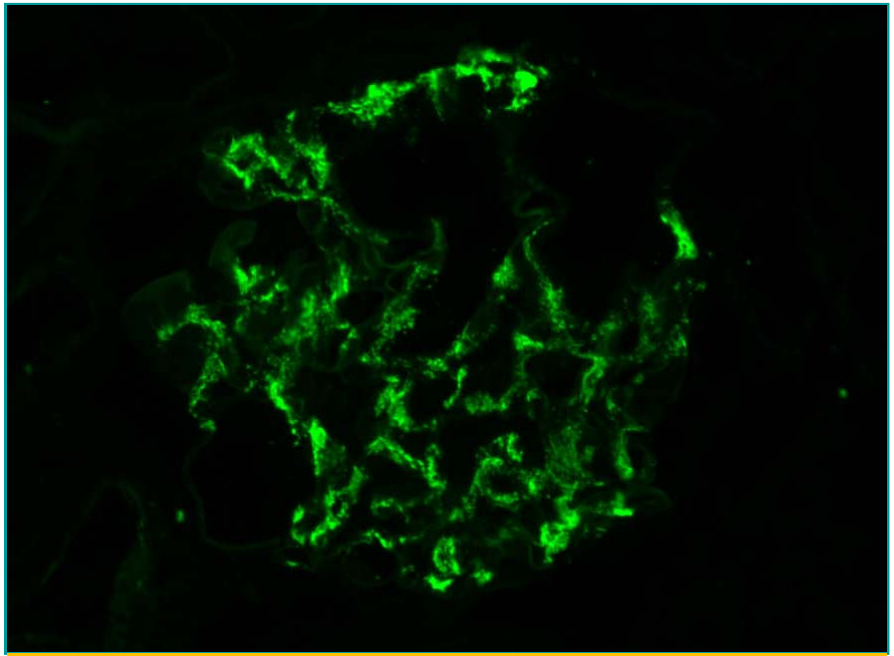
IgA1 Population
Many possible combinations



IgA1 Population Microheterogeneity



Why MS needed?



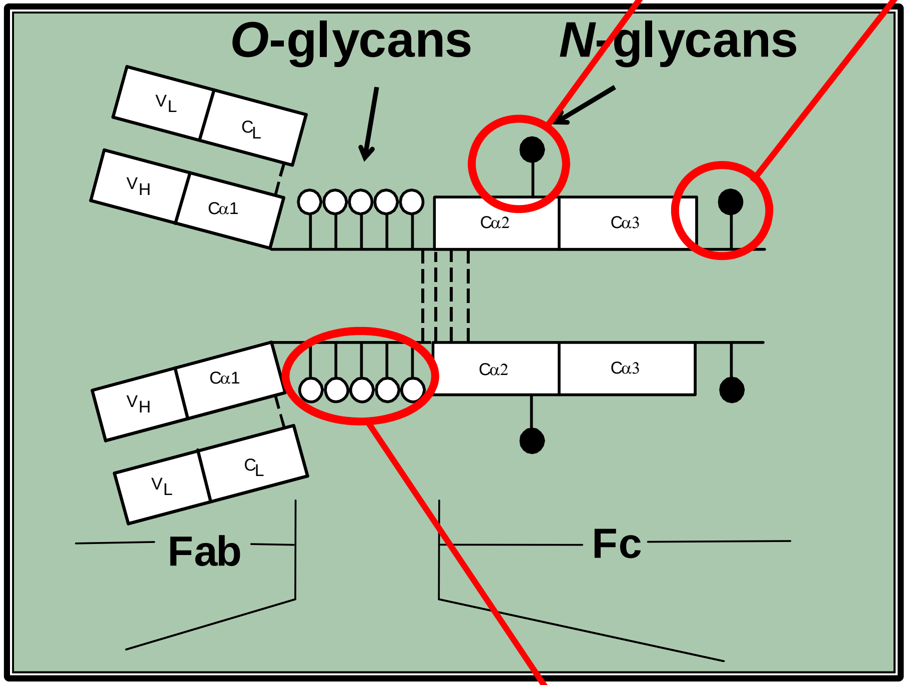
Immunoflouresence stained kidney biopsy

- **Locate sites of modification?**
- **Changes in *O*-glycans linked to the disease.**
- **Where do these changes occur? (specific or random sites)**
- **What is the pathogenic form of IgA1?**
- **What does the normal POPULATION of IgA1 look like?**

IgA1

LSLHRPALEDLLLGSEANLTCTLTGLR

LAGKPTHVNVSVVMAEVDGTCY



HYTNPSQDVTVPCPVPSTPPTPSPSTPPTPSPSCCHPR

P V P ———————————————— P P P P C IgA2

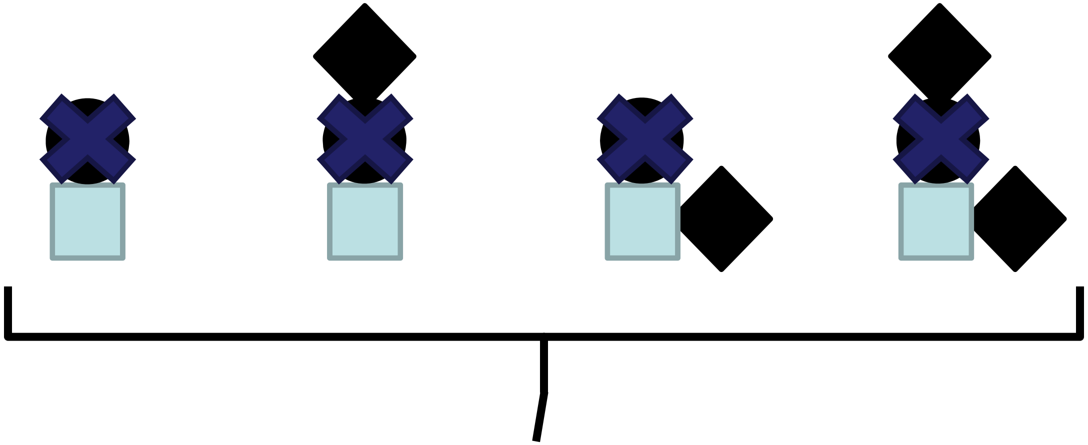
Hinge Region

Gal-deficient IgA1 *O*-glycosylation

🟩 = GalNac
◆ = NeuAc
● = Gal

***O*-glycosylation**

(NeuAc)
| α2,3
Gal
| β1,3
GalNAc —(NeuAc)
| α2,6
Ser/Thr



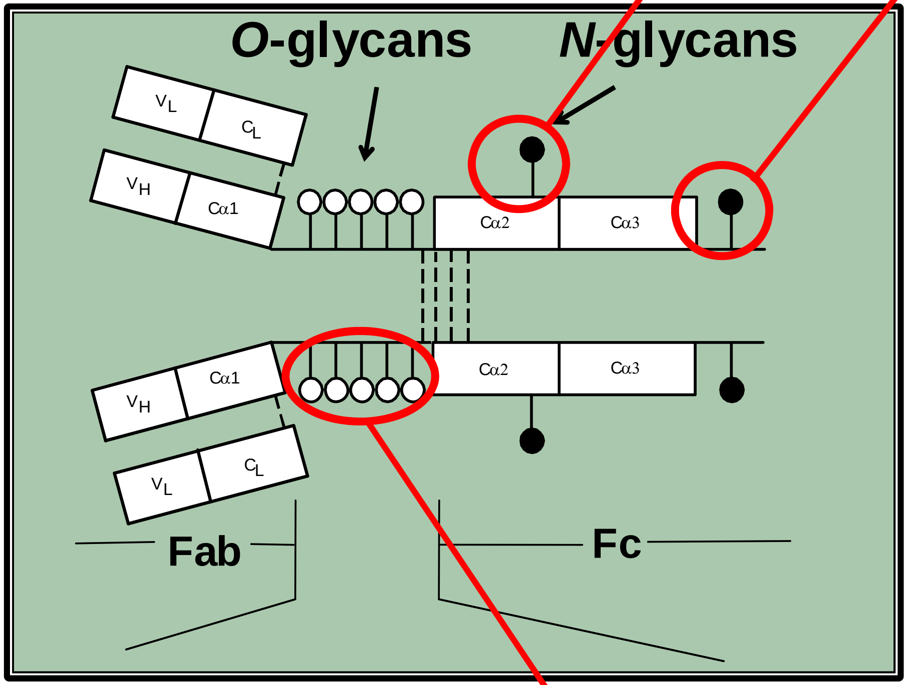
VTVPCPVPSTPPTPSPSTPPTPSPSCCHP

#1 Requirement for locating a site of MODIFICATION:
You must see the fragment!

IgA1

LSLHRPALEDLLLGSEANLTCTLTGLR

LAGKPTHVNVSVVMAEVDGTCY



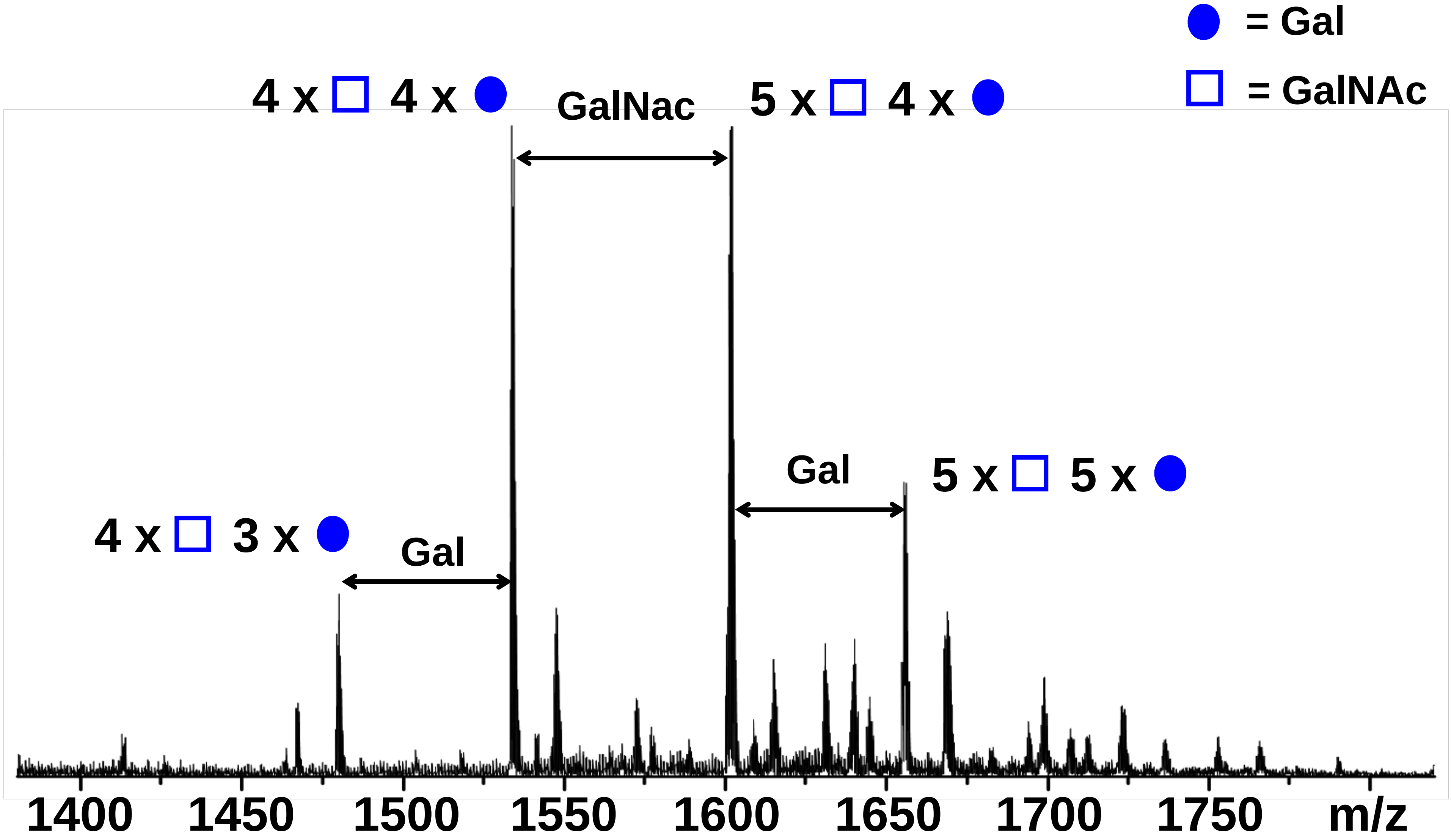
HYTNPSQDVTVPCPVPSTPPTPSPSTPPTPSPSCCHPR

P V P ———————————————— P P P P C IgA2

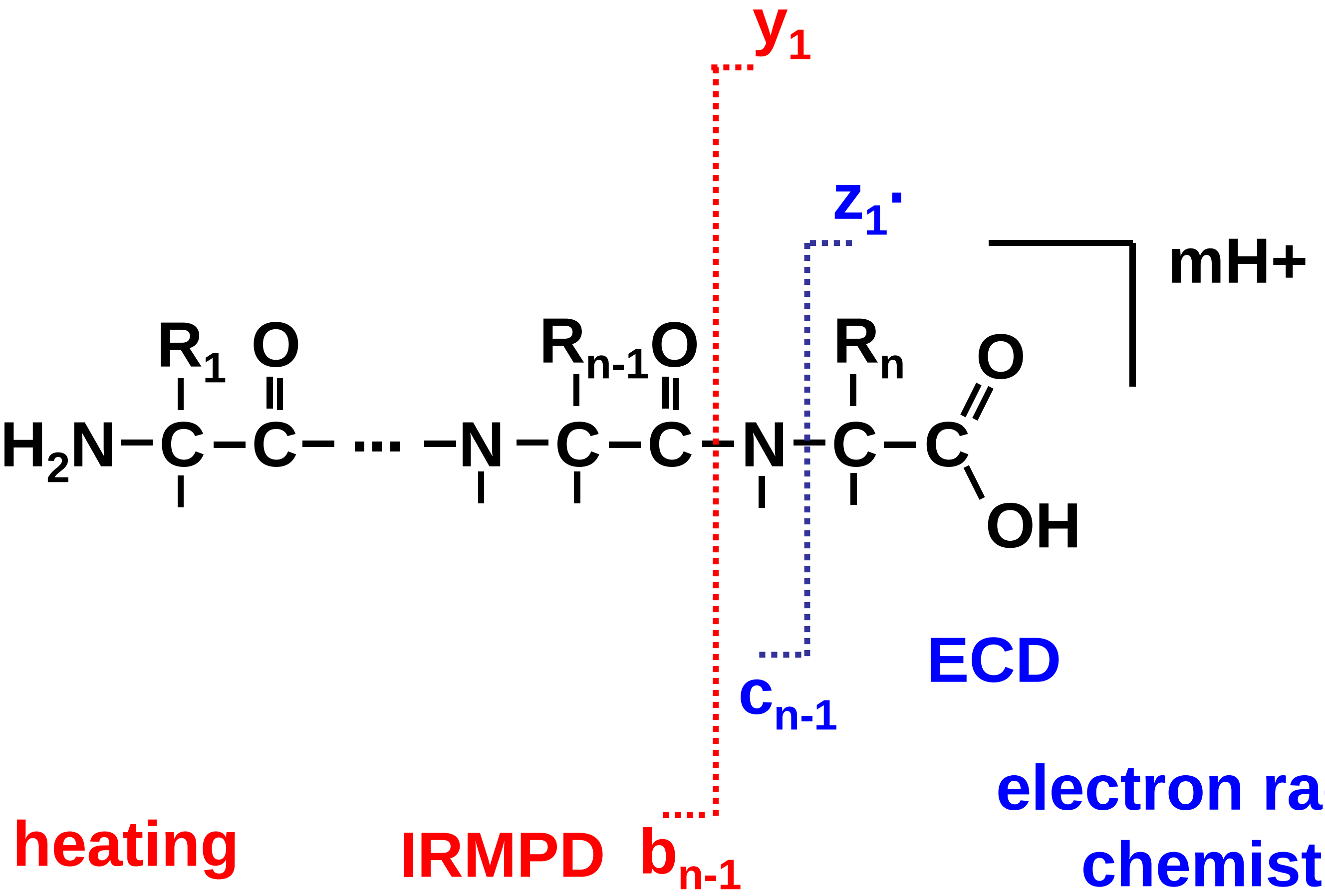
Hinge Region

2003, starting with 500 mg of IgA1

VTVPCPVPSTPPTPSPSTPPTPSPSCCHPRL



How do Peptides Cleave?



ECD

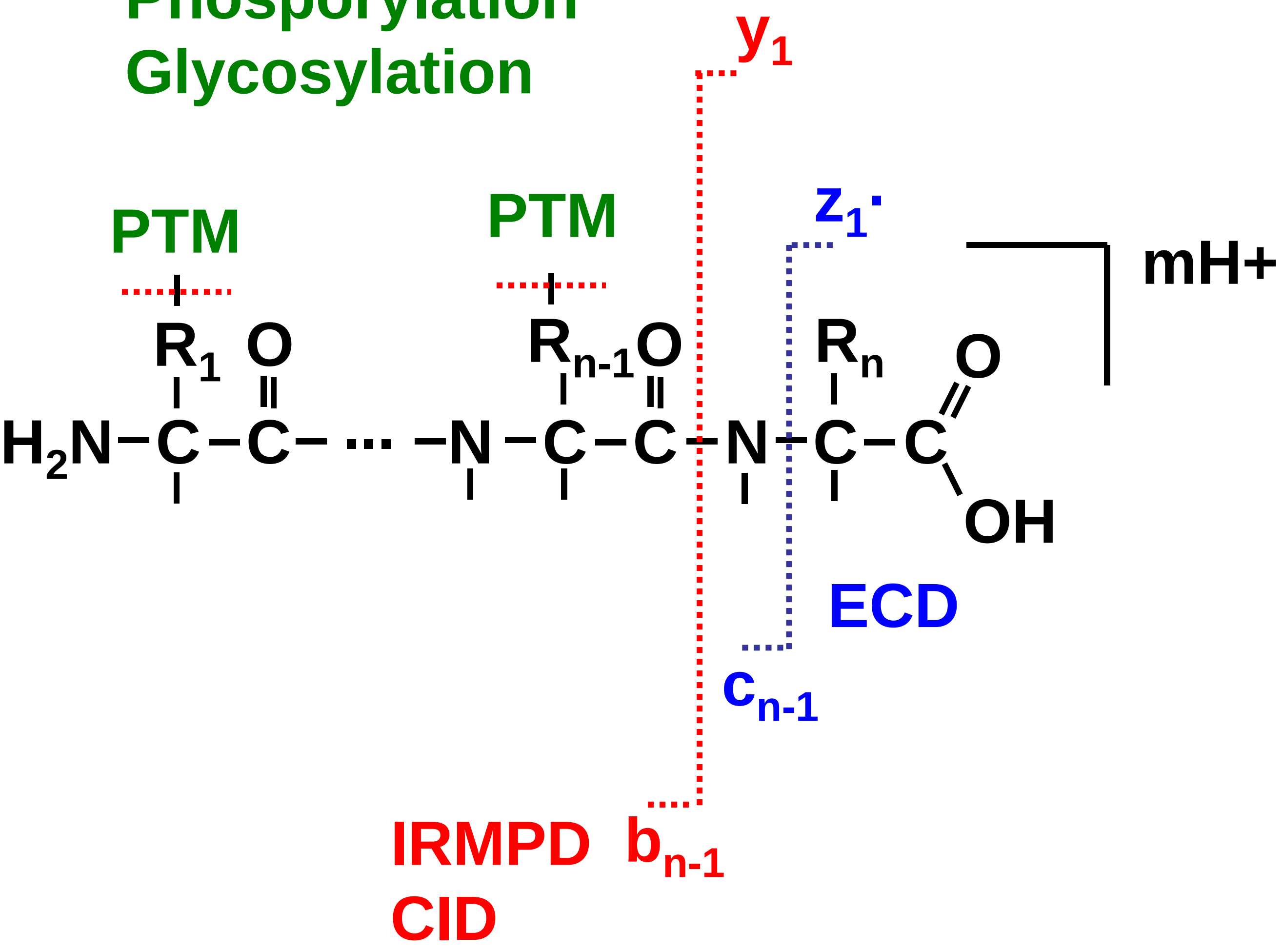
electron radical chemistry

slow heating methods

IRMPD
CID

How do Peptides With PTMs Cleave?

Phosporylation
Glycosylation



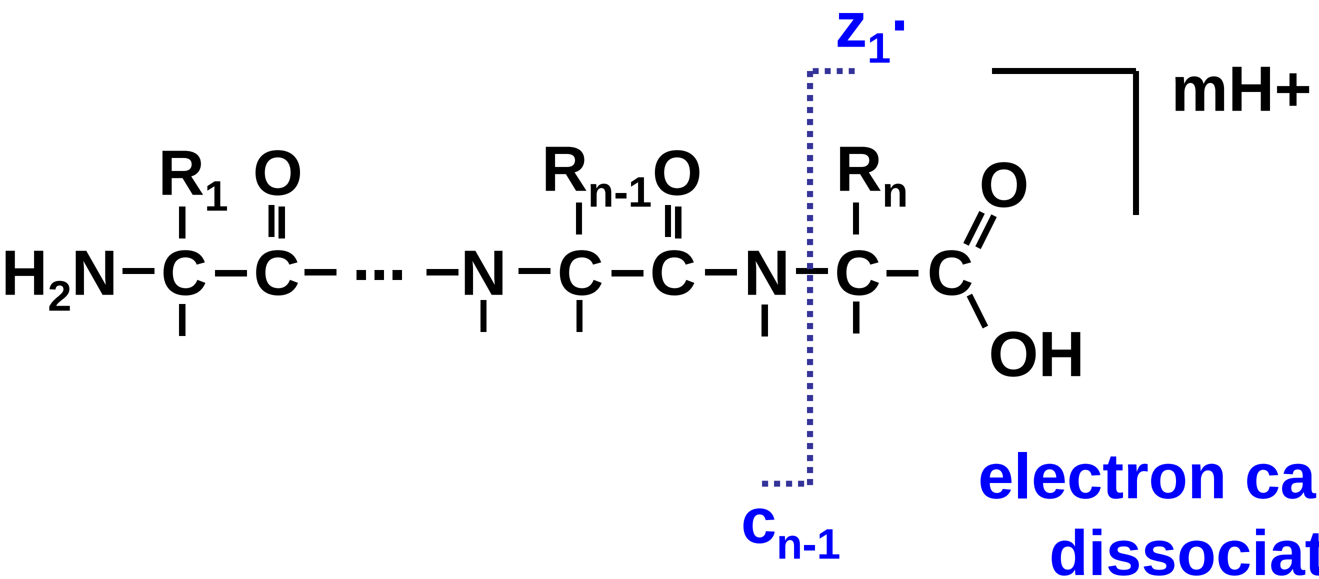
Can We Locate *O*-glycosylation Sites?

V T V P C P V P S T P P T P S P S T P P T P S P S C C H P R L

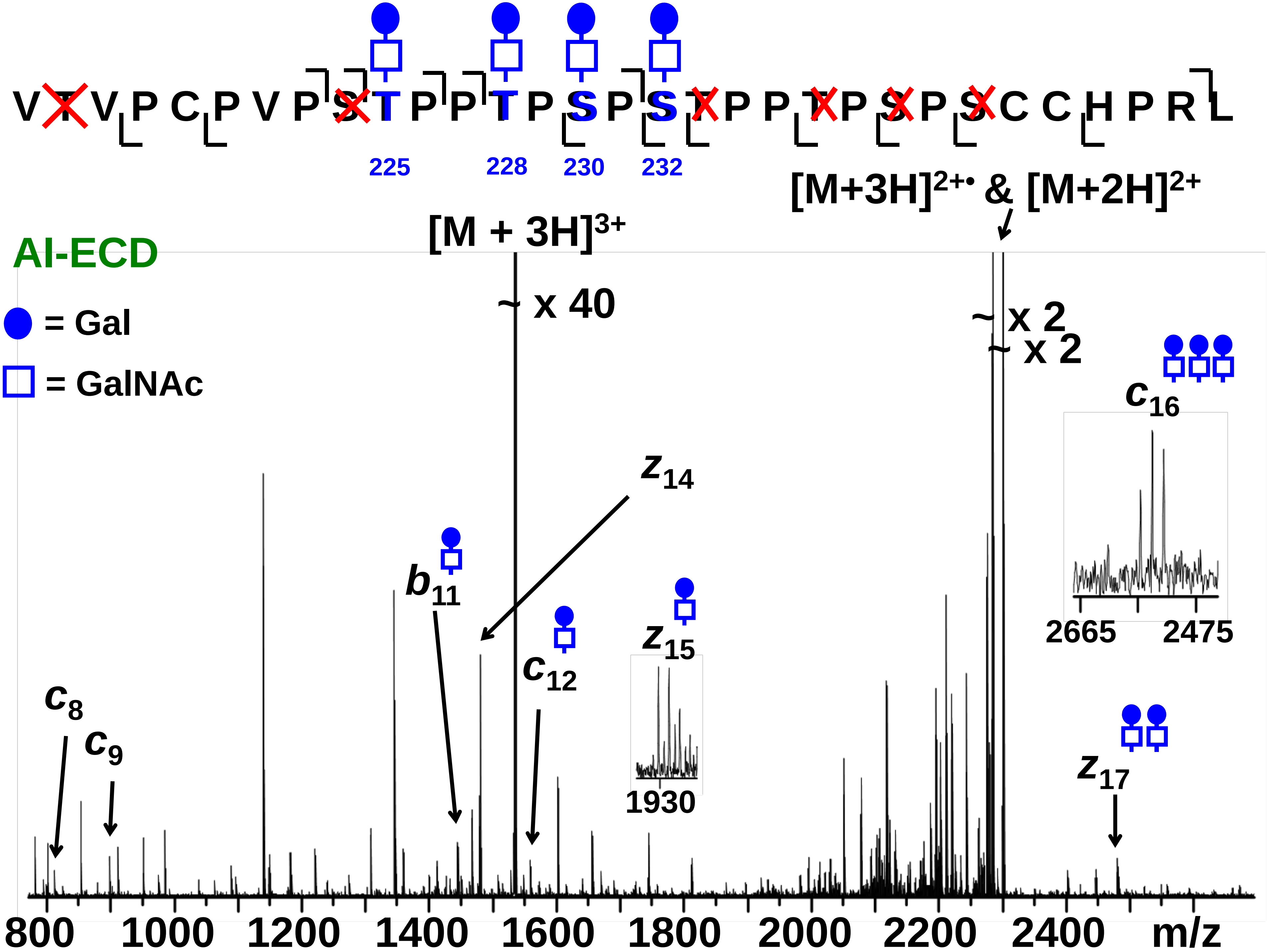
T S T T S S T T S S

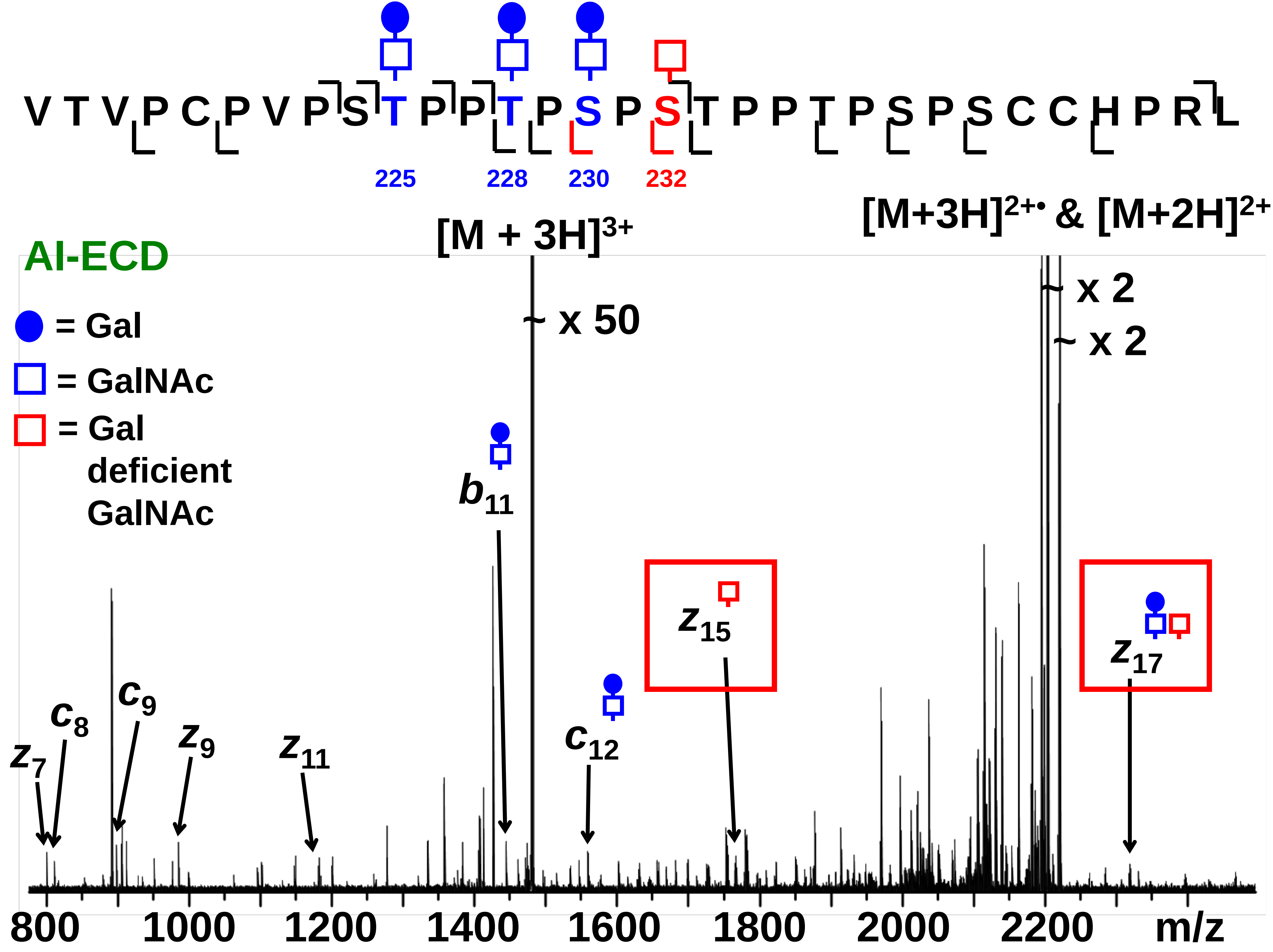
P V P S T P P T P S P S T P P T P S P S

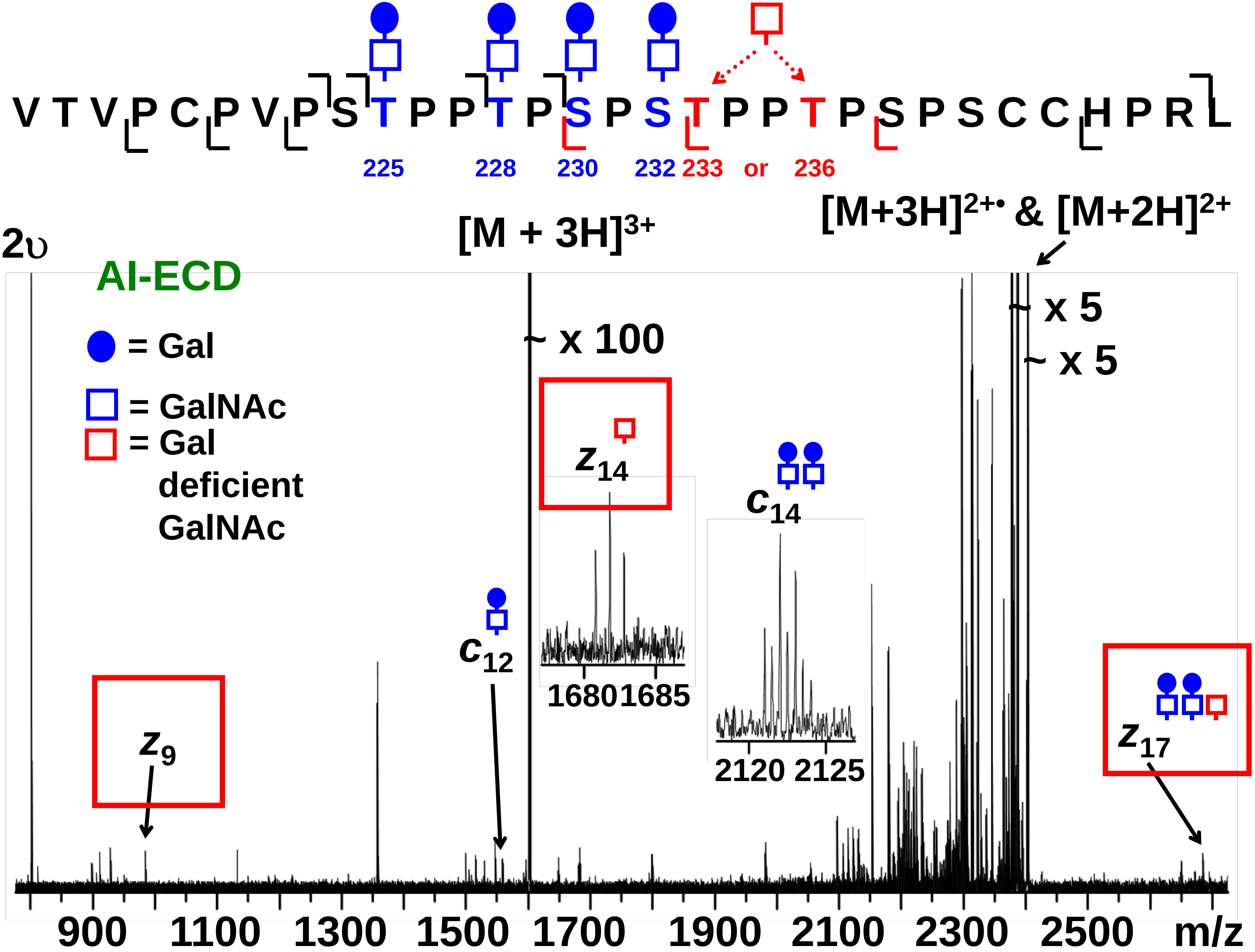
P P P P P P P P P P P P



electron capture dissociation


V T V P C P V P S T P P T P S P S T P P T P S P S C C H P R L
225
228
230
232
AI-ECD
● = Gal
☐ = GalNAc
[M + 3H]3+
~ x 40
[M+3H]2+• & [M+2H]2+
~ x 2
~ x 2
z14
b11
c12
z15
1930
c16
2665
2475
z17
c8
c9
800
1000
1200
1400
1600
1800
2000
2200
2400
m/z


V T V P C P V P S T P P T P S P S T P P T P S P S C C H P R L
225
228
230
232
[M + 3H]3+
[M+3H]2+• & [M+2H]2+
AI-ECD
● = Gal
□ = GalNAc
□ = Gal deficient GalNAc
~ x 50
~ x 2
~ x 2
b11
c12
z15
z17
z7
c8
c9
z9
z11
800
1000
1200
1400
1600
1800
2000
2200
m/z



V T V P C P V P S T P P T P S P S T P P T P S P S C C H P R L
225
228
230
232
233
or
236
[M + 3H]$^{3+}$
[M+3H]$^{2+\bullet}$ & [M+2H]$^{2+}$
2υ
AI-ECD
● = Gal
□ = GalNAc
□ = Gal deficient GalNAc
~ x 100
~ x 5
~ x 5
z_{14}
1680
1685
c_{14}
2120
2125
c_{12}
z_9
z_{17}
900
1100
1300
1500
1700
1900
2100
2300
2500
m/z

Why MS needed?

Immunoflouresence stained kidney biopsy

- **Locate sites of modification?**
- **Changes in *O*-glycans linked to**

MAJOR SCALE ISSUES!

500 mg is too much.

es occur?
tes)

- **What is the pathogenic form of IgA1?**
- **What does the normal POPULATION of IgA1 look like?**

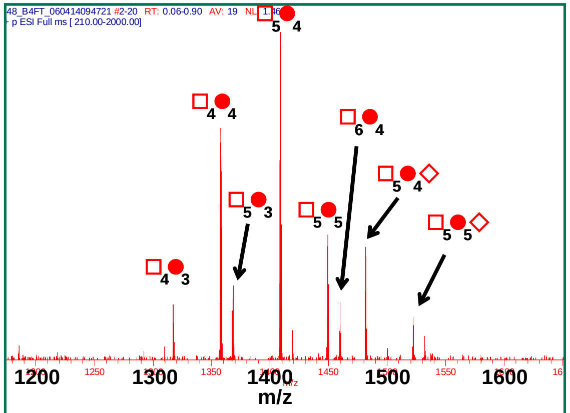
Scaling Down
MS profiles

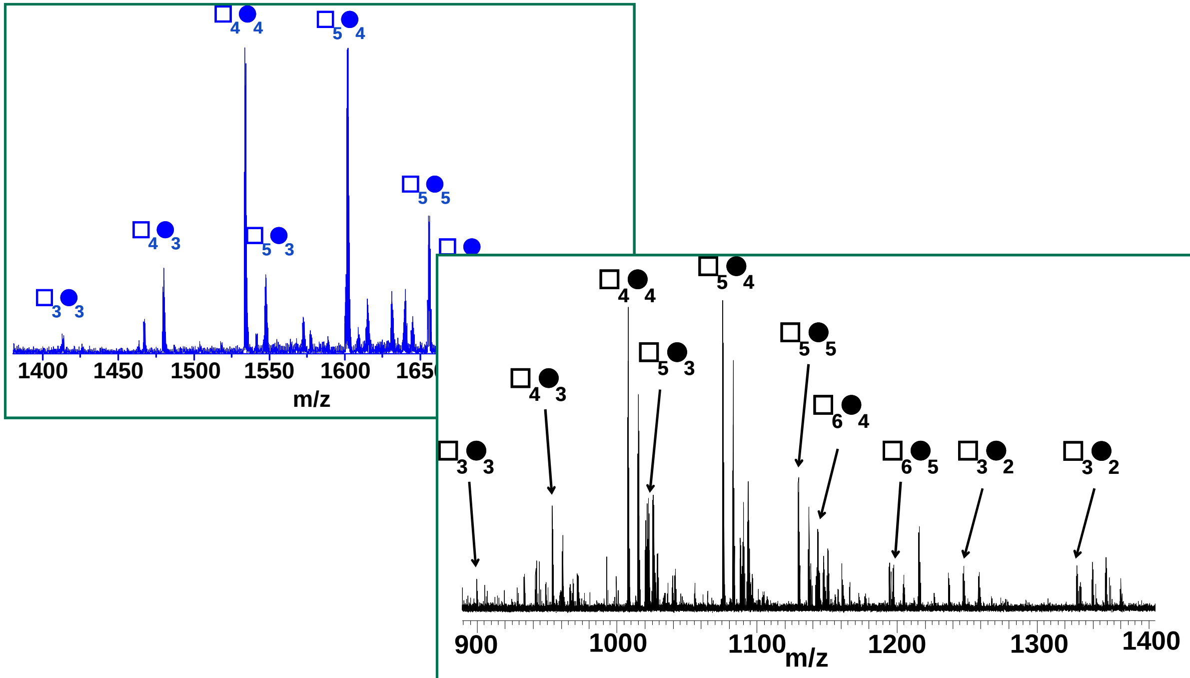
HYTNPSQDVTVPCPVPSTPPTPSPSTPPTPSPSCCHPR

VTVPCPVPSTPPTPSPSTPPTPSPSCCHPRL

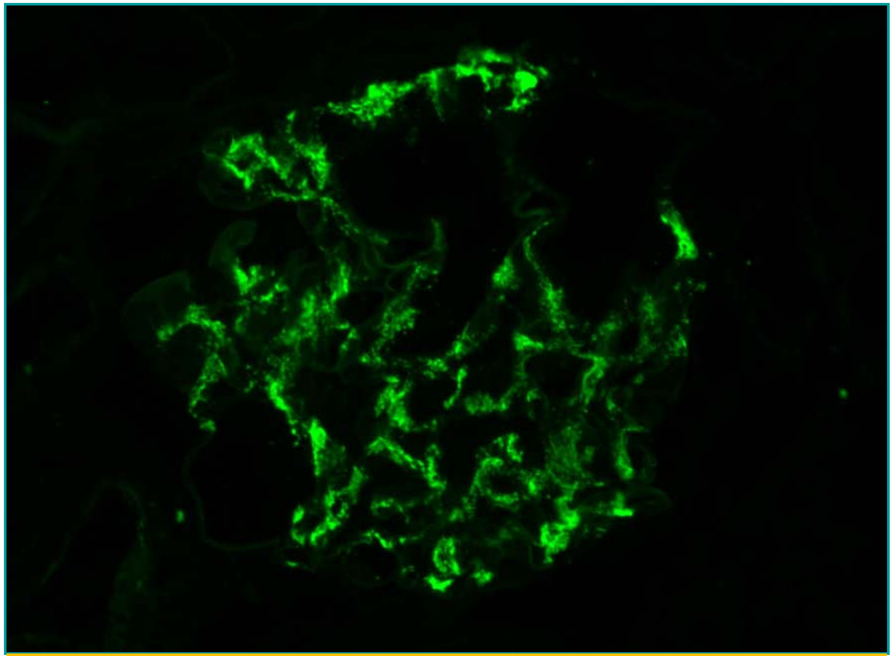
VPSTPPTPSPSTPPTP

2003 – 500 mg
2004 – 500 µg
2005 – 100 µg – 25 µg
2006 – 10 µg – 250 ng





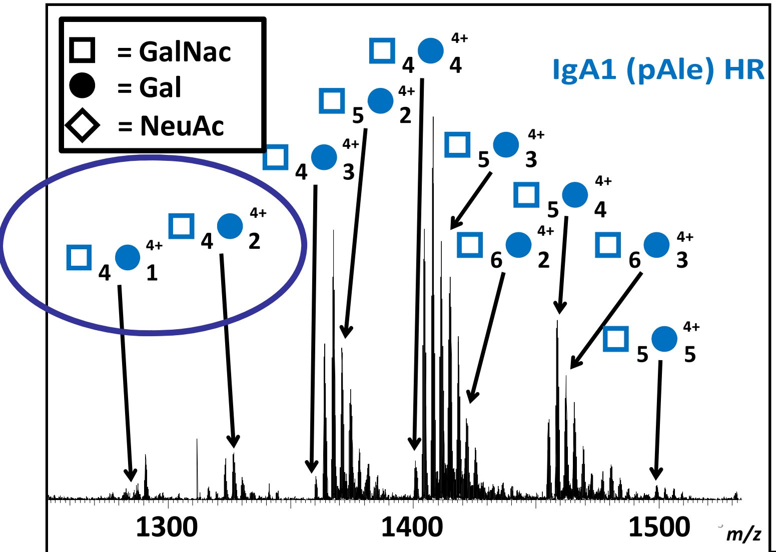
Why MS needed?



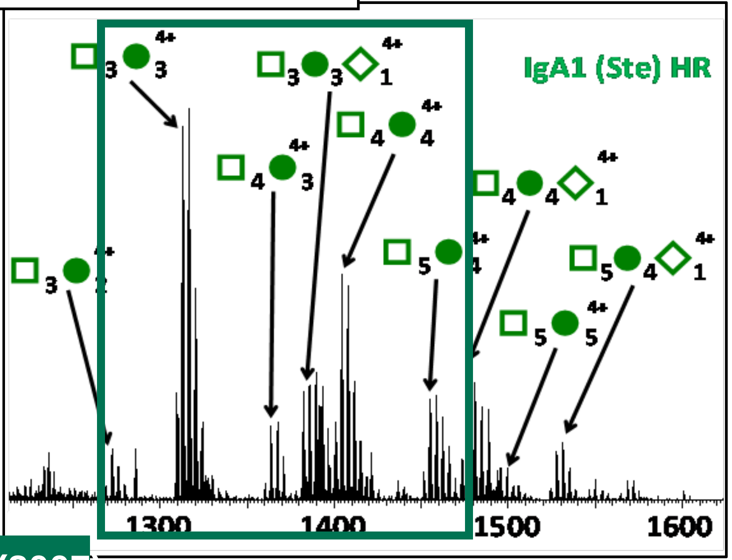
Immunoflouresence stained kidney biopsy

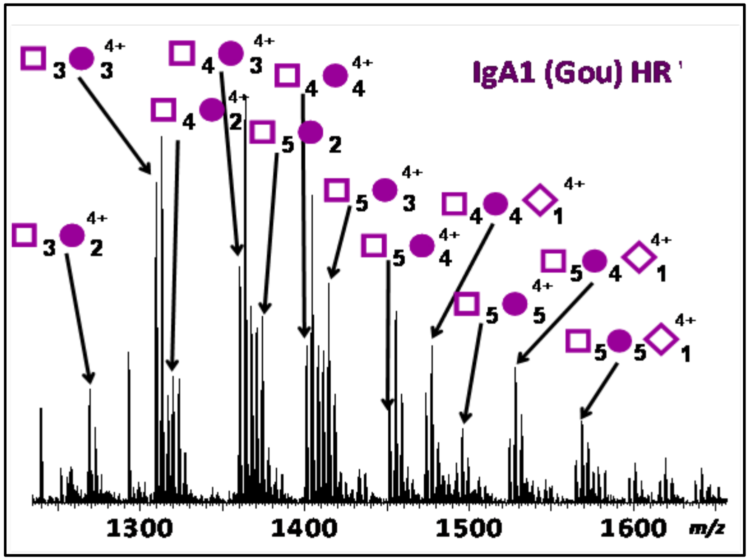
- **Locate sites of modification?**
- **Changes in *O*-glycans linked to the disease.**
- **Where do these changes occur? (specific or random sites)**
- **What is the pathogenic form of IgA1?**
- **What does the normal POPULATION of IgA1 look like?**

myeloma IgA1 HR
LC FT-ICR MS profiles



We can see clear differences in profiles



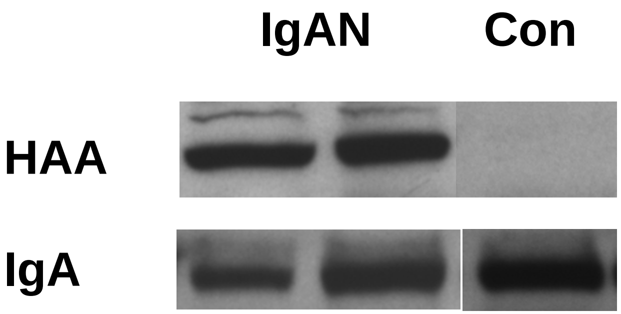


Renfrow et al., *ABC* (2007

IgA1 HR lectin analysis

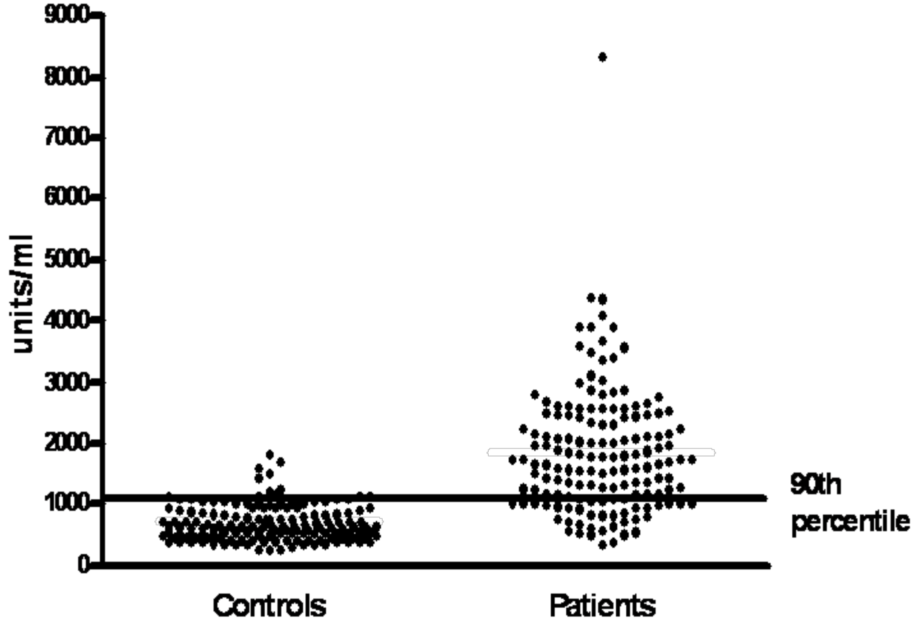
Total Serum IgA1

Glycan Specific
Lectin Western Blots



HA ELISA assay

Gal-deficient IgA1 µg/ml

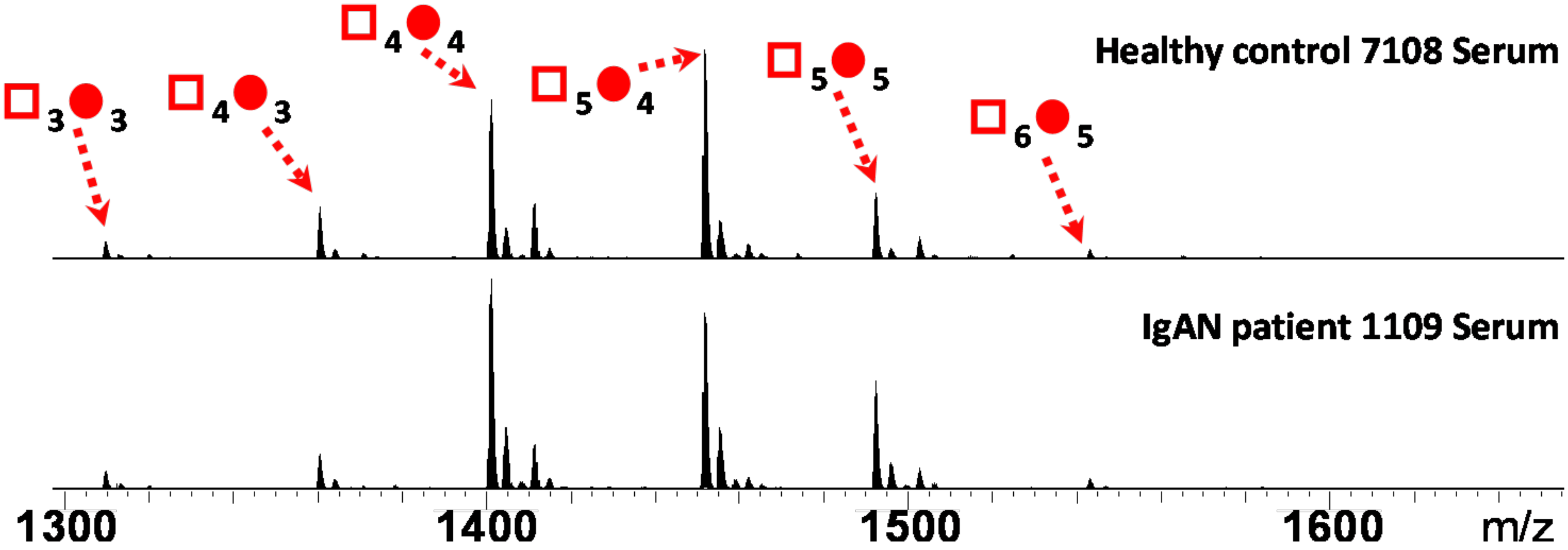


Moldeveanu et al., KI (2007)

Moore et al., MI (2007)

IgAN Patient vs Healthy control Serum IgA1

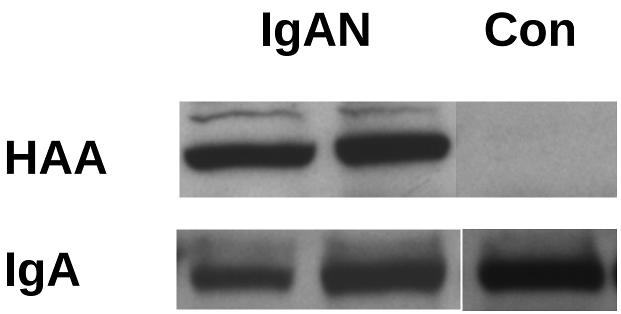
LC-FT-ICR MS



#2 Major Point: Scale doesn’t matter if you are not Sampling the PATHOGENIC (or modified) FORM

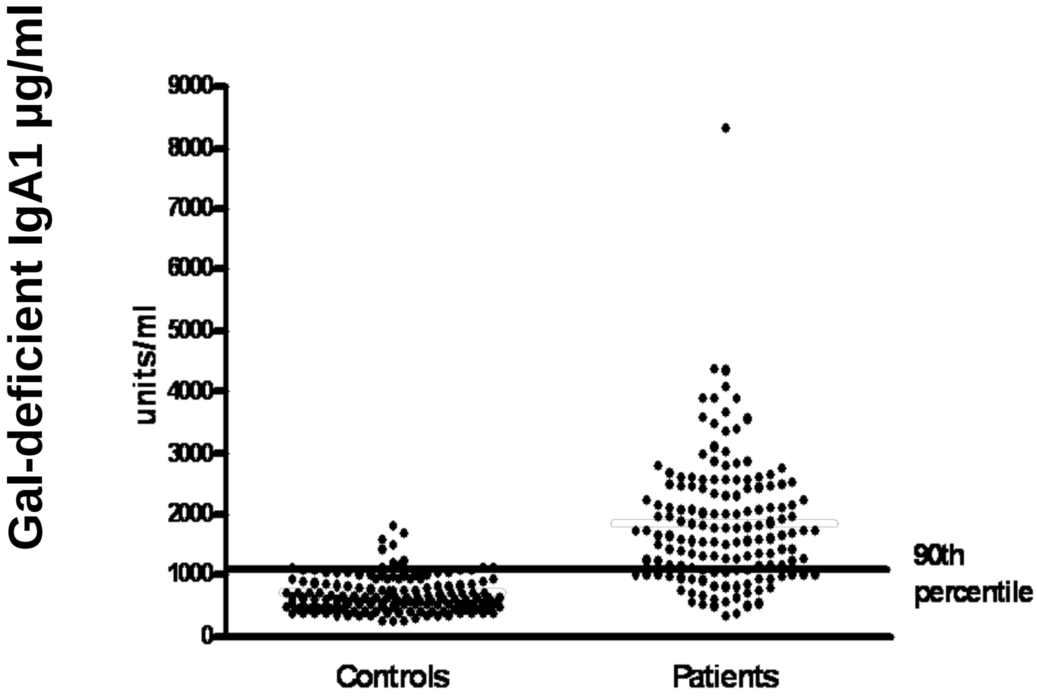
IgA1 HR lectin analysis

Glycan Specific Lectin Western Blots



Total Serum IgA1

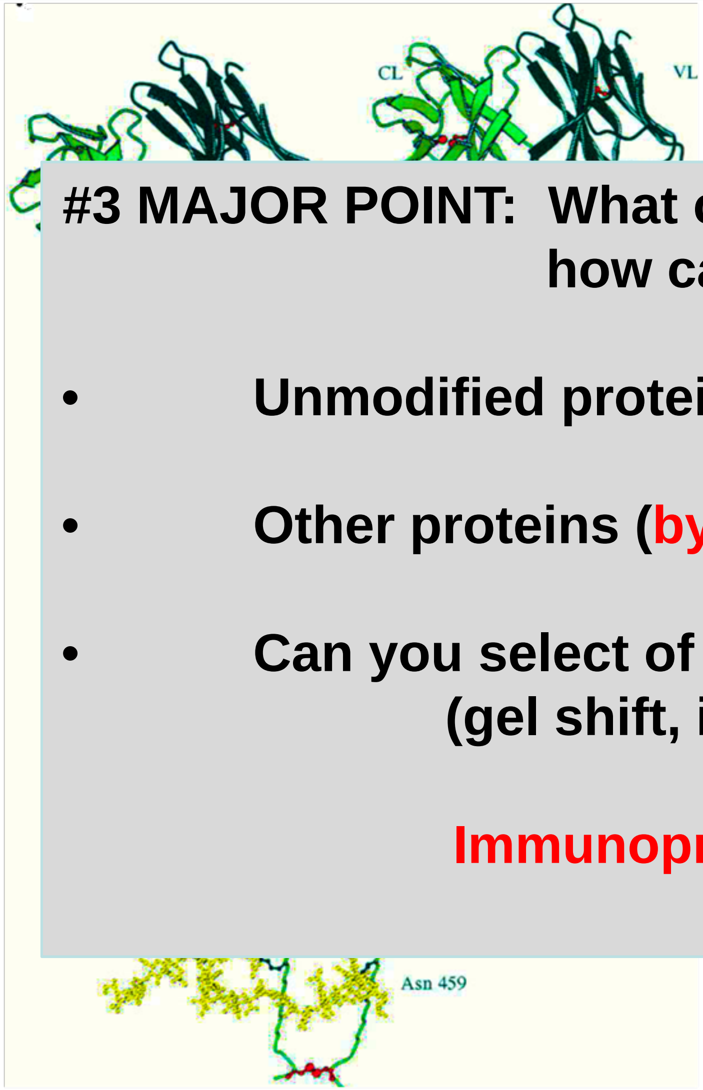
HA ELISA assay



Moldeveanu et al., KI (2007)

Moore et al., MI (2007)

Let’s think about our analytical sample



IgA1

- Monomeric, multimeric, polymeric

#3 MAJOR POINT: What can potentially mask your modification and how can you get around that?

- **Unmodified protein (by an order of magnitude)**
- **Other proteins (by two or more orders of magnitude)**
- **Can you select of the modified form**
  (gel shift, isoelectric focusing, enrichment)

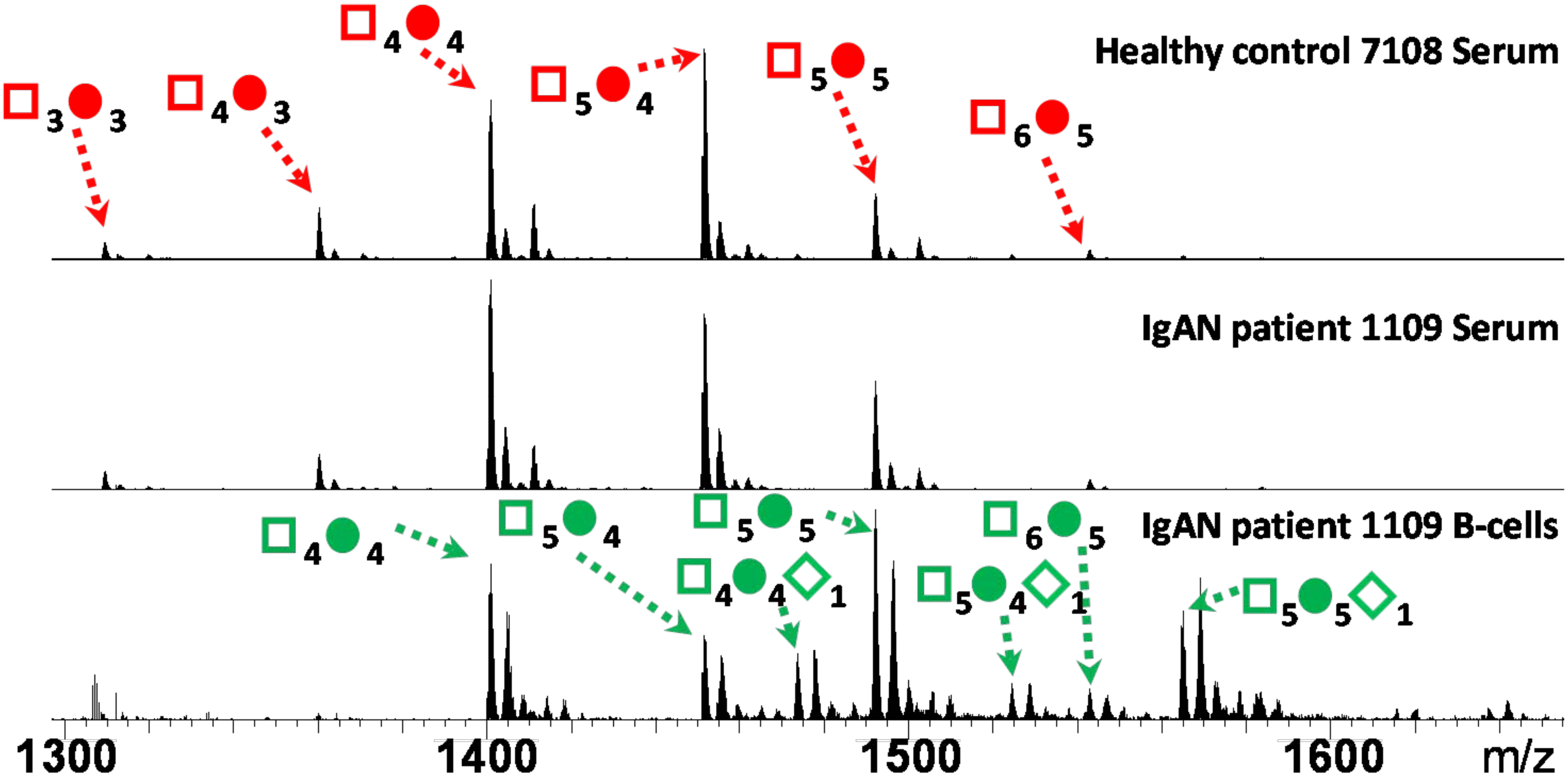
Immunoprecipitation is not a cure all.

- Lectins react with primaryly polymeric
- CIC’s represent <1% if Serum IgA1
  (<1 µg/ml)

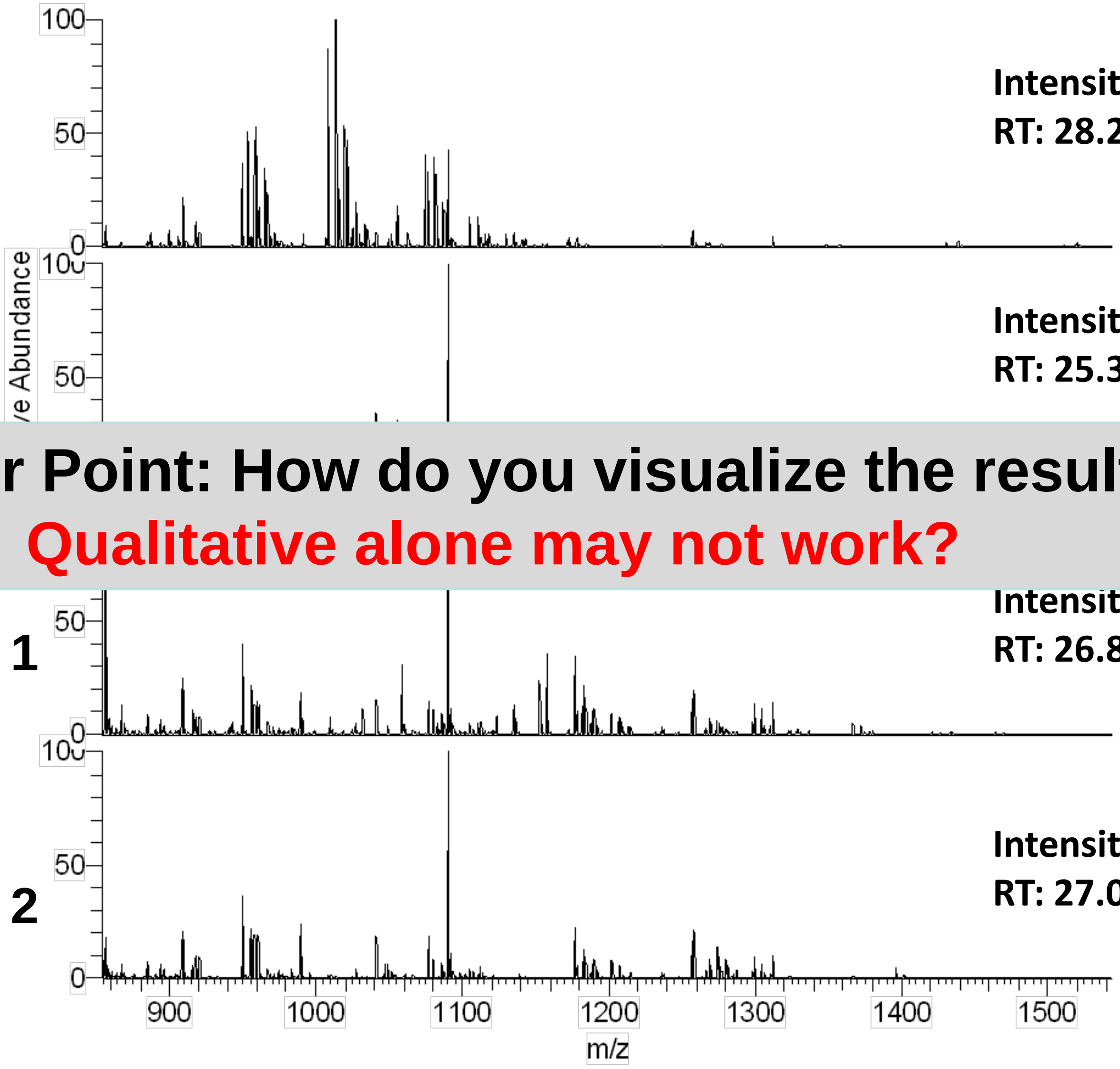
(JBC 273, 2260, 1998)

Cultured B-cells from IgAN patients

LC-FT-ICR MS



LC-MS of Cultured B-cells from IgAN patients vs. healthy controls



IgAN 1

Intensity: 2.38E5
RT: 28.20-32.63

IgAN 2

Intensity: 6.24E4
RT: 25.35-33.66

#4 Major Point: How do you visualize the results?
Qualitative alone may not work?

Control 1

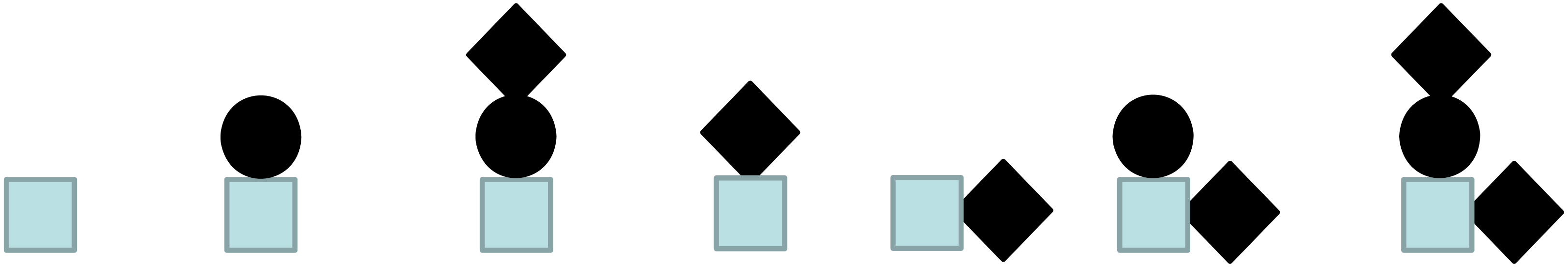
Intensity: 6.21E4
RT: 26.80-34.91

Control 2

Intensity: 9.41E4
RT: 27.03-31.52

Cultured B-cells from IgAN patients vs. H Controls

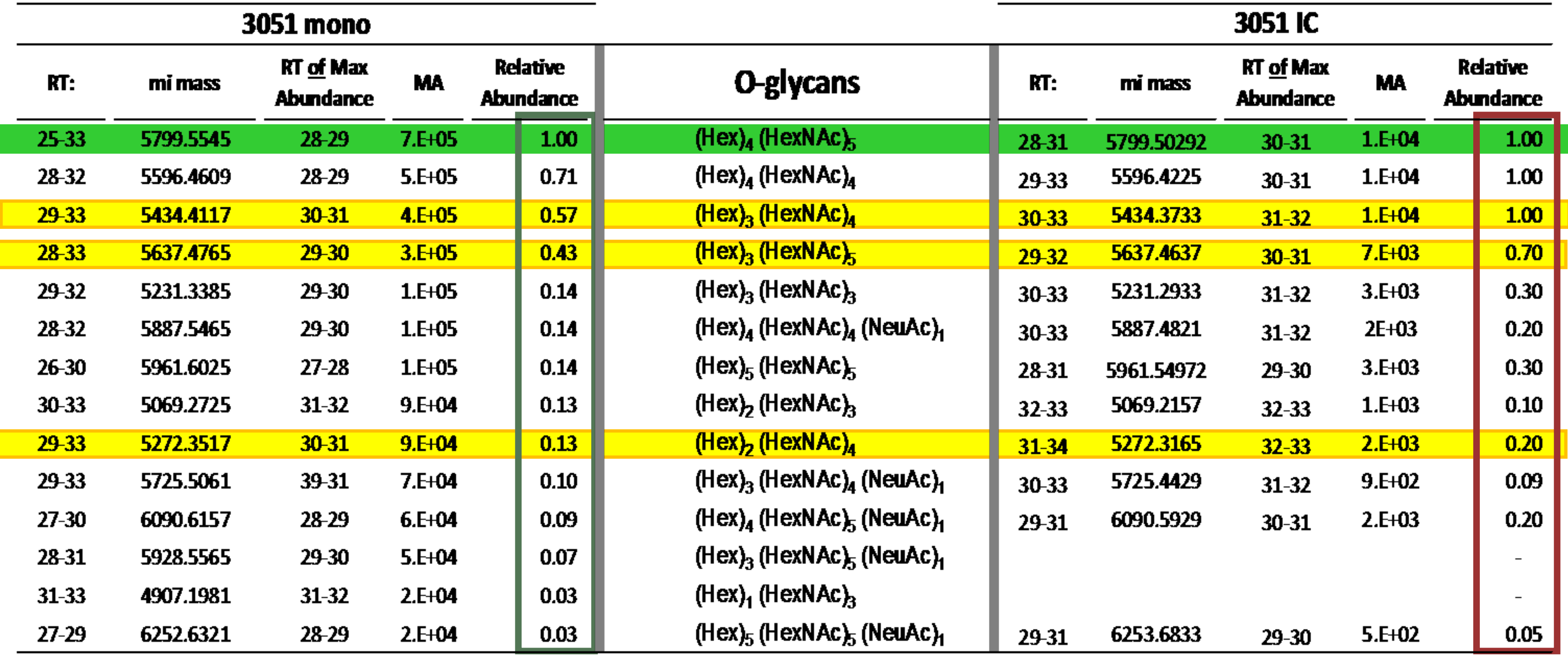
	IgAN1				IgAN2				Control1				Control2			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Gal deficient sp.	54.4	51.6	50.0	47.3	10.2	2.6	10.9	2.9	1.4	0.1	2.6	0.2	0	0	0	0
SA Gal deficient sp.	3.0	1.8	5.6	3.6	17.5	15.1	18.1	16.6	20.7	9.0	23.4	10.7	14.9	1.6	20.0	2.2
Full Gal Sp	38.0	41.6	38.6	42.6	10.6	5.7	10.5	5.7	5.4	0.4	5.0	0.5	4.0	0.8	5.8	1.4
SA Full Gal sp.	4.7	5.0	5.7	6.4	62.1	76.7	60.6	74.7	72.6	90.8	68.9	88.5	81.2	97.6	74.1	96.3



	IgAN1		IgAN2		Control1		Control2	
Neuraminidase treatment	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
HAA-IgA reactivity (ELISA)	1.717	0.772	1.268	0.379	0.509	0.359	0.404	0.241

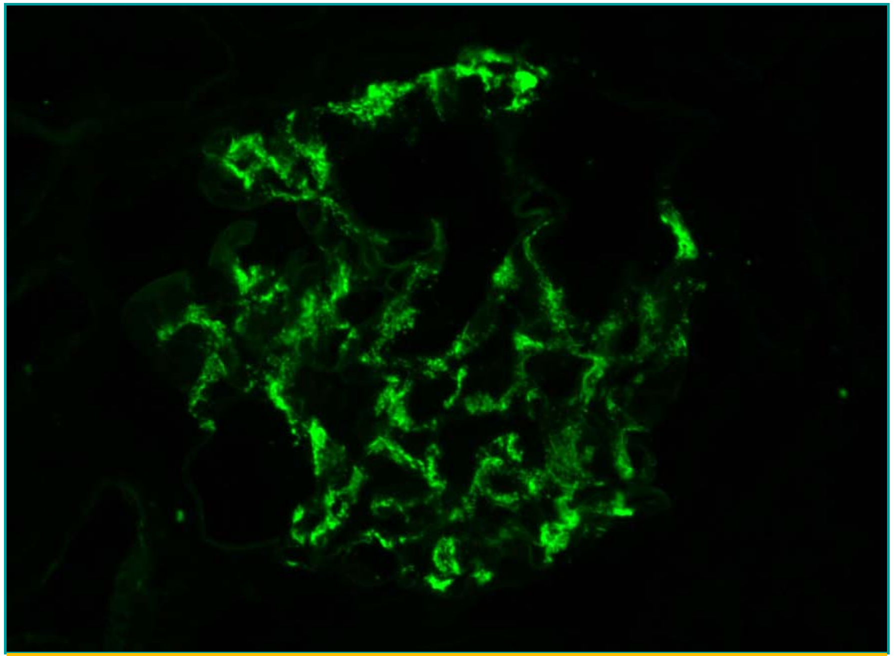
Serum IgA1 vs CIC IgA1

Gal-deficient glycoforms more abundant in CICs



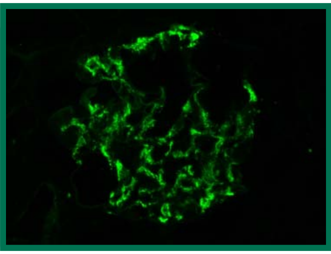
3051 mono						3051 IC				
RT:	mi mass	RT of Max Abundance	MA	Relative Abundance	O-glycans	RT:	mi mass	RT of Max Abundance	MA	Relative Abundance
25-33	5799.5545	28-29	7.E+05	1.00	(Hex)$_4$ (HexNAc)$_5$	28-31	5799.50292	30-31	1.E+04	1.00
28-32	5596.4609	28-29	5.E+05	0.71	(Hex)$_4$ (HexNAc)$_4$	29-33	5596.4225	30-31	1.E+04	1.00
29-33	5434.4117	30-31	4.E+05	0.57	(Hex)$_3$ (HexNAc)$_4$	30-33	5434.3733	31-32	1.E+04	1.00
28-33	5637.4765	29-30	3.E+05	0.43	(Hex)$_3$ (HexNAc)$_5$	29-32	5637.4637	30-31	7.E+03	0.70
29-32	5231.3385	29-30	1.E+05	0.14	(Hex)$_3$ (HexNAc)$_3$	30-33	5231.2933	31-32	3.E+03	0.30
28-32	5887.5465	29-30	1.E+05	0.14	(Hex)$_4$ (HexNAc)$_4$ (NeuAc)$_1$	30-33	5887.4821	31-32	2E+03	0.20
26-30	5961.6025	27-28	1.E+05	0.14	(Hex)$_5$ (HexNAc)$_5$	28-31	5961.54972	29-30	3.E+03	0.30
30-33	5069.2725	31-32	9.E+04	0.13	(Hex)$_2$ (HexNAc)$_3$	32-33	5069.2157	32-33	1.E+03	0.10
29-33	5272.3517	30-31	9.E+04	0.13	(Hex)$_2$ (HexNAc)$_4$	31-34	5272.3165	32-33	2.E+03	0.20
29-33	5725.5061	39-31	7.E+04	0.10	(Hex)$_3$ (HexNAc)$_4$ (NeuAc)$_1$	30-33	5725.4429	31-32	9.E+02	0.09
27-30	6090.6157	28-29	6.E+04	0.09	(Hex)$_4$ (HexNAc)$_5$ (NeuAc)$_1$	29-31	6090.5929	30-31	2.E+03	0.20
28-31	5928.5565	29-30	5.E+04	0.07	(Hex)$_3$ (HexNAc)$_5$ (NeuAc)$_1$					-
31-33	4907.1981	31-32	2.E+04	0.03	(Hex)$_1$ (HexNAc)$_3$					-
27-29	6252.6321	28-29	2.E+04	0.03	(Hex)$_5$ (HexNAc)$_5$ (NeuAc)$_1$	29-31	6253.6833	29-30	5.E+02	0.05

MS answers our questions?

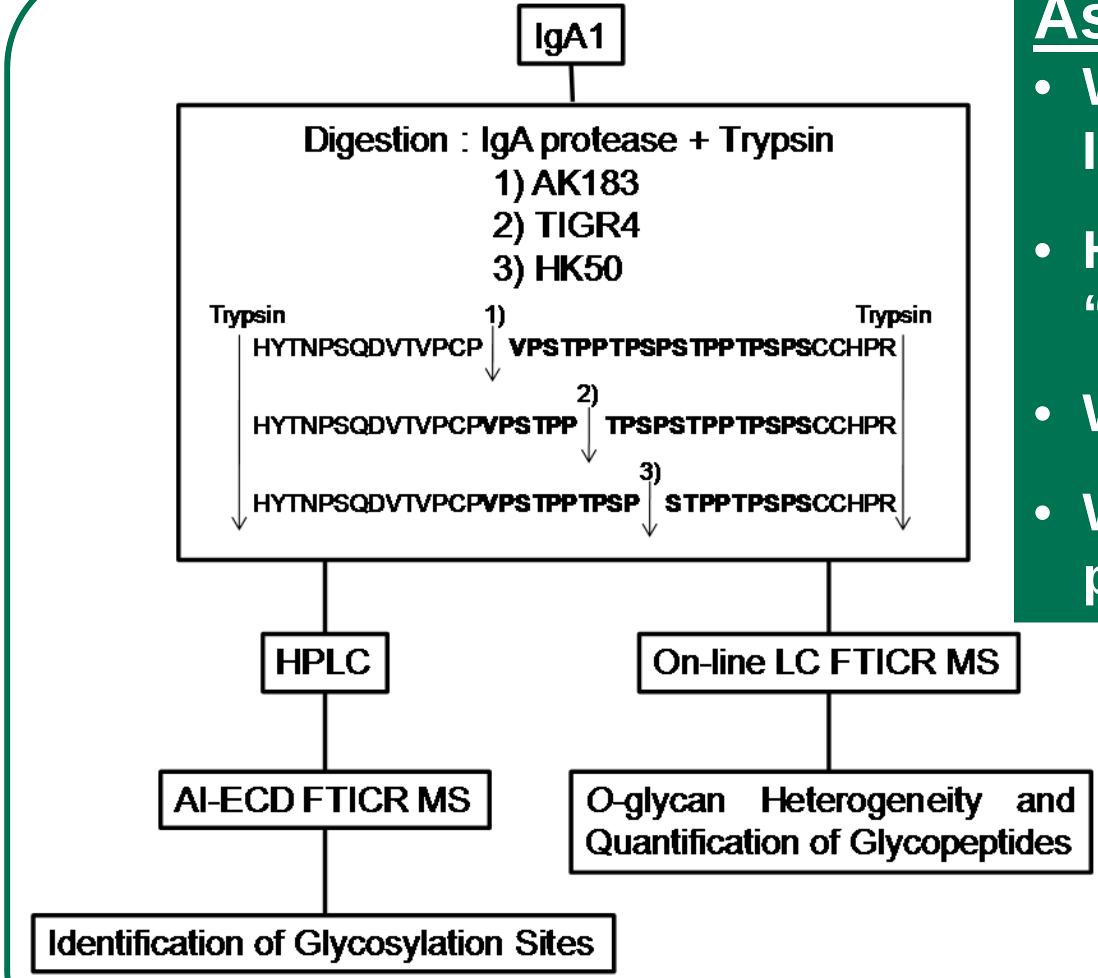


Immunoflouresence stained kidney biopsy

- Locate sites of modification?
- Changes in *O*-glycans linked to the disease.
- Where do these changes occur? (specific or random sites)
- What is the pathogenic form of IgA1?
- What does the normal POPULATION of IgA1 look like?



Conclusions (WORKFLOW)



IgA1

Digestion : IgA protease + Trypsin
1) AK183
2) TIGR4
3) HK50

Trypsin 1) Trypsin

HYTNPSQDVTVPCP **VPSTPPTPSPSTPPTPSPS**CCHPR

2)

HYTNPSQDVTVPCP**VPSTPP** **TPSPSTPPTPSPS**CCHPR

3)

HYTNPSQDVTVPCP**VPSTPPTPSP** **STPPTPSPS**CCHPR

HPLC

On-line LC FTICR MS

AI-ECD FTICR MS

O-glycan Heterogeneity and Quantification of Glycopeptides

Identification of Glycosylation Sites

Ask the right questions?

- What is the source of the IgA1?
- How do we avoid the “healthy” IgA1?
- What is normal?
- What glycoforms are pathogenic?

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Working through your PTM analysis

#1 Requirement for locating a site of MODIFICATION:
You must see the fragment!

#2 Major Point: Scale doesn’t matter if you are not
Sampling the MODIFIED FORM.

#3 MAJOR POINT: What can potentially mask your modification and how can you get around that?

#4 Major Point: How do you visualize the results?
Qualitative alone may not work?

Next Meeting:

THE FLYING IONS

A UAB Mass Spec Interest Group

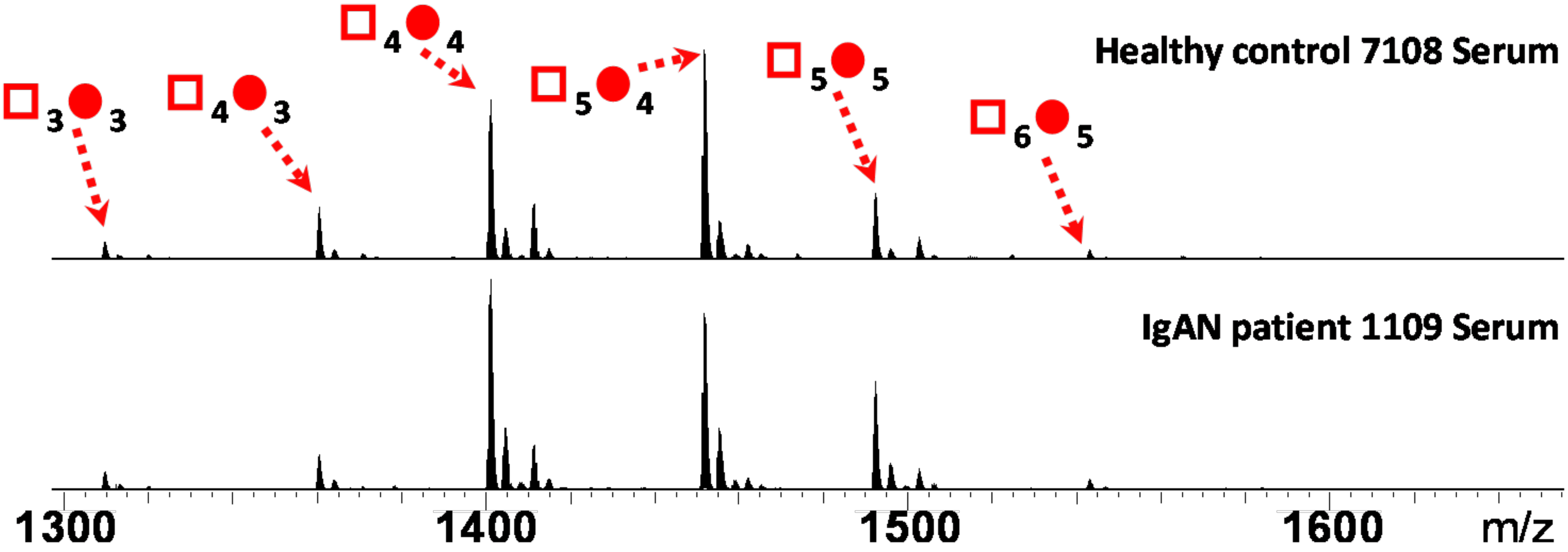
"Pretty pictures and more, turning a mass spec into a (chemical) microscope.

Eric Monroe, PhD

Wednesday, April 15th 2009

Normal distribution of *O*-glycans is disrupted

LC-FT-ICR MS



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FT-ICR MS instrumentation

Ernie (Electron Capture Dissociation)

- **Protein ID**
  - **IP's and Column Fractions**
  - **RP C18 Chromatography (LC-MS)**
- **Protein PTM's**
  - **LC-MS**
  - **Offline preparation**
  - **4 Fragmentation techniques**
    - **CID / PQD**
    - **ECD**
    - **IRMPD**
  - **Direct infusio**
- **Lipids**
- **Organics**
- **Anything**

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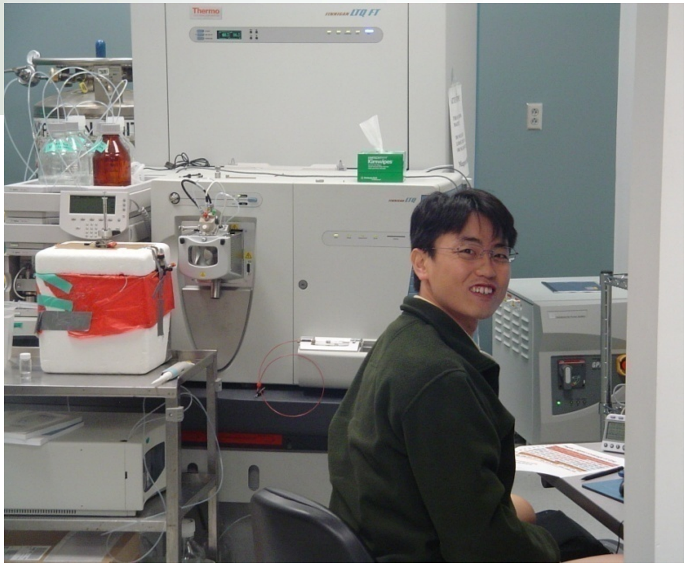
High Resolution Mass Spectrometry

FT-ICR MS instrumentation



Heidi (Hydrogen/Deuterium Exchange)

Dynamic Structural Analysis
- **Protein-Protein**
- **Protein-Ligand**
- **Protein-Nucleic Acid**
- **Protein-Ligand-Coactivator**

Peter Prevelige Group
- **HIV/Bacteriophage**
  - **Capsid Structures**

Steve Barnes Group
- **HBat active site**

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High Resolution Mass Spectrometry