Mass spectrometry in glycomics research: Application to IgA nephropathy

Part I

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IgA Nephropathy

The most common primary glomerulonephritis in the world

- Hematuria and proteinuria
  - episodic gross hematuria x mucosal (upper respiratory tract) infections - viruses, bacteria
- Afflicts preferentially children and young adults
- Male to female ratio is about 2 : 1
- Sporadic or familial (hereditary) forms
- Henoch-Schönlein purpura
  - –may include renal involvement - nephritis (similar to IgAN; in young children)

IgA nephropathy

Immune complex glomerulonephritis

Diagnosis of glomerulonephritis - one of the following:

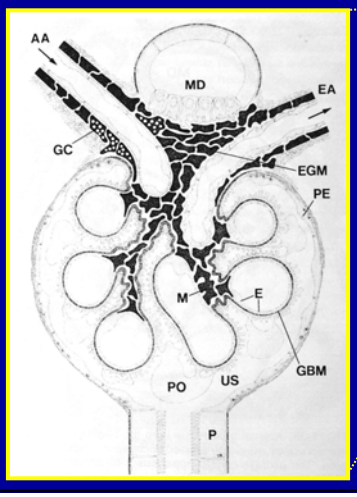
- History of macroscopic hematuria
- Microscopic hematuria: >5 RBC/high-power field
- Proteinuria: dipstick ≥1+ or UP/Cr ratio ≥0.2

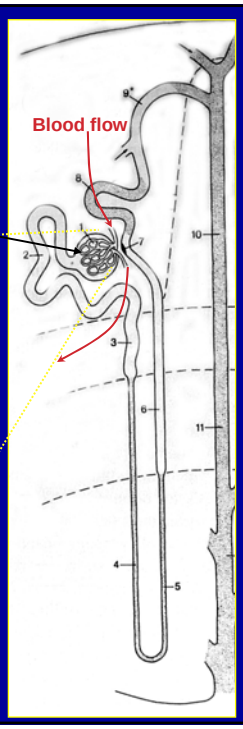
In the absence of menstrual bleeding, known urologic source or nephrolithiasis

Nephron

Number of glomeruli and podocytes given

Glomerulus





Cortex

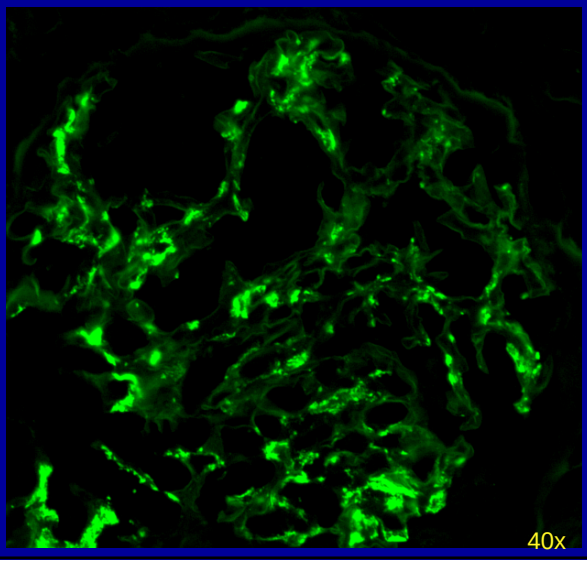
Outer medulla

Inner medulla

From Heptinstall's Pathology of the Kidney, 1998

IgA nephropathy: Diagnosis

IgA1 mesangial deposits by immunofluorescence



IgA1 (but not IgA2)

Mostly polymeric IgA1 (but not secretory IgA1)

C3 (but not C1q) co-deposits

Often IgG (IgM) co-deposits

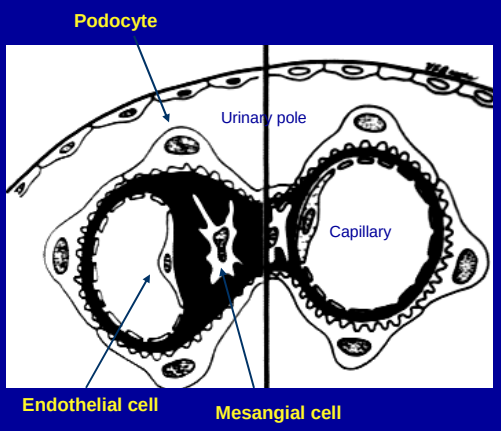
Expansion of extracellular matrix

Proliferation of mesangial cells

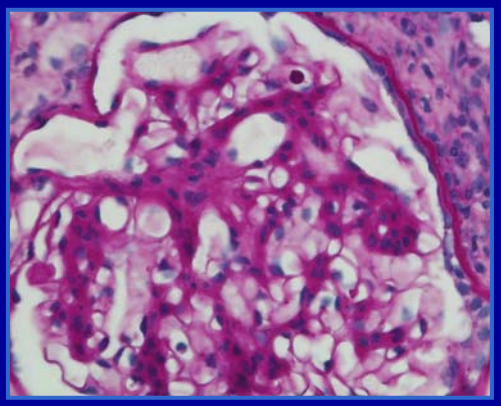
IgAN: Histology

Expansion of Extracellular Matrix

Proliferation of Mesangial Cells



IgAN **Normal**



IgAN (Periodic acid-Schiff stain)

Prognosis

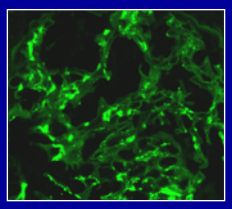
- Usually slow progression towards glomerular and interstitial sclerosis (no disease-specific treatment of IgAN)
- 30-40% patients develop end-stage renal disease within 20 years
- Dialysis, transplantation
- IgN cause is extrarenal:
  - IgAN recurrent >50% after transplantation
  - IgAN kidney transplanted to non-IgAN recipient cleared IC

Circulating Immune Complexes (CIC) in IgA nephropathy

- IgA1-containing CIC present in most IgAN patients
- IgA1-CIC levels correlate with the disease activity

IgA1 deposits originate from CIC

Immune complex glomerulonephritis (GN)

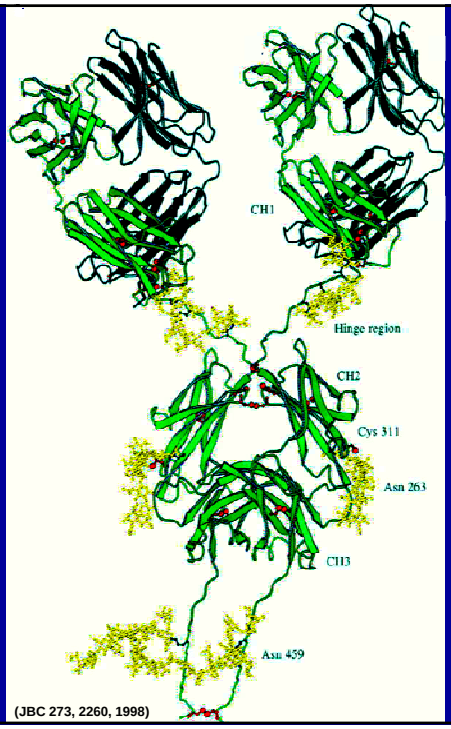


Initial events in immune deposit formation:

- deposition of CIC
  - **pre-formation of CIC**
    only certain complexes are "nephritogenic"
  - **host factors promoting glomerular IC deposition**
    reduced clearance or complement-mediated solubilization
- in-situ formation
  - **Ab recognize glomerular antigens**
  - **Ab bind to planted Ag (models vs. naturally-occurring diseases)**

Secondary events:

- formation of aggregates detectable by IF and EM
  (redistribution of IC; addition of Ab, IC, other reactants)



(JBC 273, 2260, 1998)

Human IgA1

Structure and glycosylation

Monomeric or polymeric (with J-chain) forms

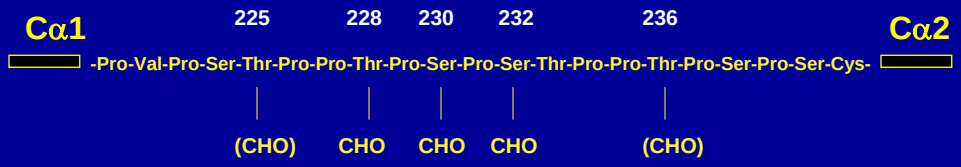
O-linked glycans

N-linked glycans

Hinge regions of human IgA subclasses: IgA1 and IgA2

IgA1

hinge sequence similar to mucins, recognized by IgA proteases of bacterial pathogens

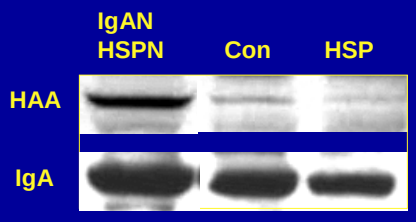


IgA2m(1)

IgA1 glycosylation in IgAN: Initial analyses

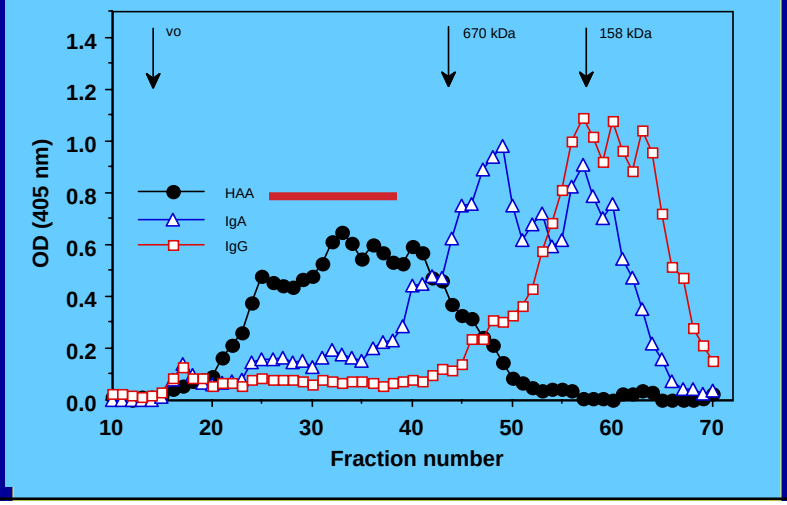
Monosaccharide composition: Gas-liquid chromatography
Terminal saccharides: Lectin ELISA, Western blot

Gal-deficient *O*-linked glycans in the hinge-region



Gal-deficient IgA1 forms CIC with IgG

Size-exclusion chromatography of serum



Pooled CIC

↓

Dissociated (pH 3)

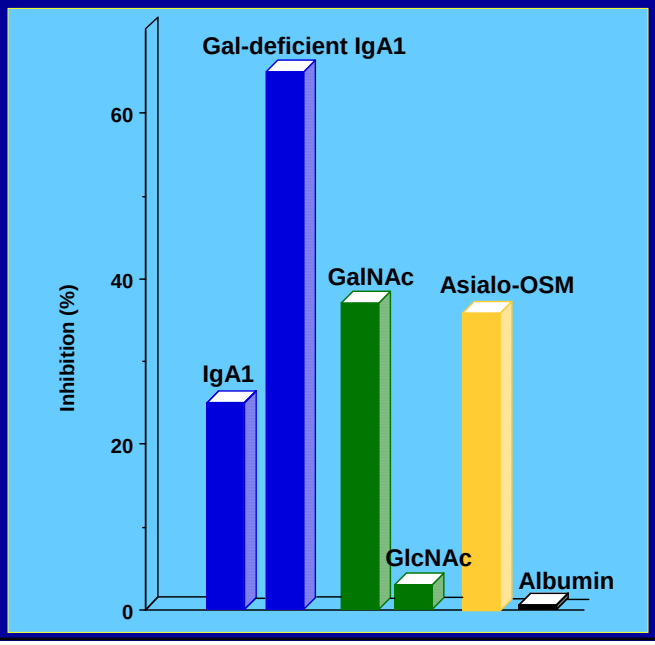
↓

Fractionated (HPLC)

↓

pIgA and IgG detected by ELISA

Inhibition of reformation of IgAN IgG-IgA1 CIC



IgA1-CIC isolated

↓

Dissociated (pH 3)

↓

Immobilized inhibitors added

↓

Sample neutralized

↓

After o/n incubation free IgG measured

Localization of glycan-dependent antigenic determinants of Gal-deficient IgA1

- **Gal-deficient IgA1 is present in sera in IgG-IgA1 immune complexes (IC)**
- **Free and IC-bound IgG and IgA1 anti-IgA1 antibodies are specific for the hinge region *O*-linked glycans**

(cross-reactive antibodies specific for mucosal pathogens or viruses?)

- **The antigenic determinant(s) comprises GalNAc and/or GalNAc -α2,6 SA glycans**

In vitro model to study IgA1-CIC biological activity

• IgA1-CIC fractionated -> added to cultured MC

-> Binding, proliferation, activation markers,…

• **Proteomics** (ID proteins up- or down-regulated or with altered post-translational modifications)

• **High-density DNA arrays** (ID genes up- or down-regulated)

IgA1 binding to mesangial cells *in vitro*

- **Putative receptor (R) binds the Fc portion of IgA1**
- **Asialo-agalacto-IgA1 > normally glycosylated IgA1**
- **CIC from IgAN patients >>>> asialo-agalacto-IgA1**
- **CIC from IgAN patients >> CIC from healthy controls**
- **Binding of CIC inhibited by IgA1 but not by IgG**
- Fcα R (CD71, Fcα/μ possible candidates but not CD89)

Novak et al., Kidney Int. 2002

In-vitro assay of biological activity of CIC: proliferation of MC

CIC from serum fractionated by size-exclusion chromatography
(Superose 6 column; calibrated)

↓

Collect CIC V_0 - ~700 kDa

↓

Filter-sterilize and add to quiescent MC
Incubate for 20 h

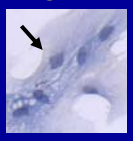
↓

Add ^{3}H-thymidine for 4 h

↓

Harvest, measure ^{3}H-thymidine incorporation

PCNA -



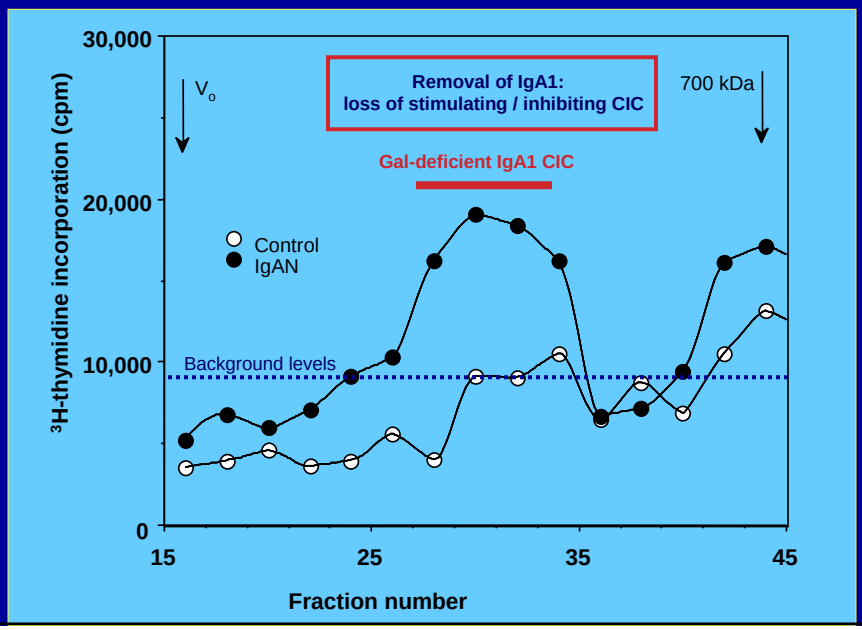
PCNA +

Additional experiments: depletion of IgA, IgG
supplementation of IgA

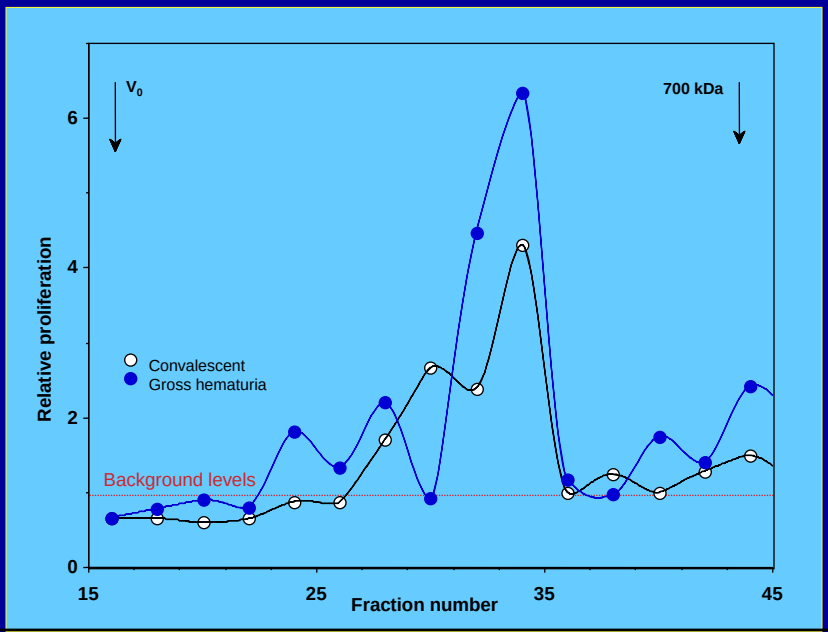
Controls: PDGF, negative control (no CIC)

IgAN-CIC differentially stimulate or inhibit MC proliferation

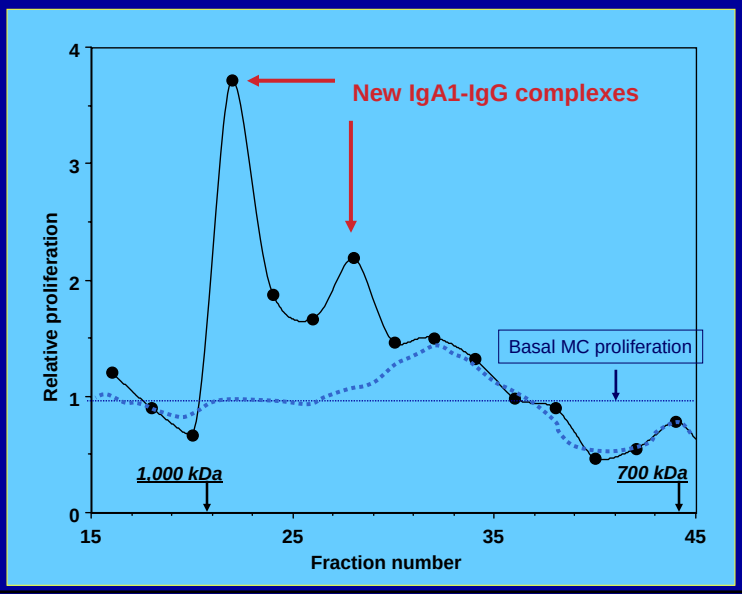
Size-exclusion chromatography



Primary IgAN: IgA1-CIC activate MC

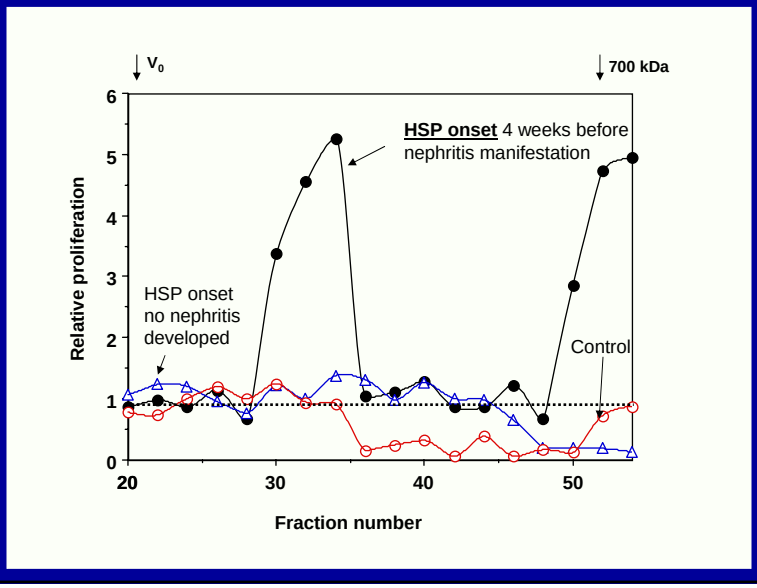


IgA1-supplementation of serum increased stimulating activity

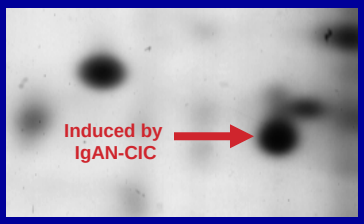


MC proliferation after stimulation with CIC from patients with HSP

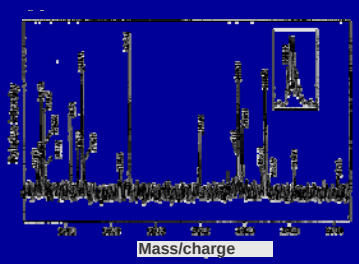
Pre-existing "nephritogenic" CIC at onset



Proteome analysis of IgAN-CIC-stimulated MC



Pick a spot on 2-D gel, digest with trypsin



MALDI peptide mass map "fingerprint"

Protein identification: **vimentin** --> Q-TOF (AA sequence)

```
    | 10   | 20   | 30   | 40   | 50   | 60   | 70   | 80   | 90   | 100
  1 MSTRSVSSSS YRRMFGGPGT ASRPSSSRSY VTTSTRTYSL GSALRPSTSR  SLYASSPGGV YATRSSAVRL RSSVPGVRLL QDSVDFSLAD AINTEFKNTR 100
101 TNEKVELQEL NDRFANYIDK VRFLEQQNKI LLAELEQLKG QGKSRLGDLY  EEEMRELRRQ VDQLTNDKAR VEVERDNLAE DIMRLREKLQ EEMLQREEAE 200
201 NTLQSFRQDV DNASLARLDL ERKVESLQEE IAFLKKLHEE EIQELQAQIQ  EQHVQIDVDV SKPDLTAALR DVRQQYESVA AKNLQEAEEW YKSKFADLSE 300
301 AANRNNDALR QAKQESTEYR RQVQSLTCEV DALKGTNESL ERQMREMEEN  FAVEAANYQD TIGRLQDEIQ NMKEEMARHL REYQDLLNVK MALDIEIATY 400
401 RKLLEGEESR ISLPLPNFSS LNLRETNLDS LPLVDTHSKR TLLIKTVETR  DGQVINETSQ HHDDLE                                    466
    | 10   | 20   | 30   | 40   | 50   | 60   | 70   | 80   | 90   | 100
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Vimentin expression in mesangial cells

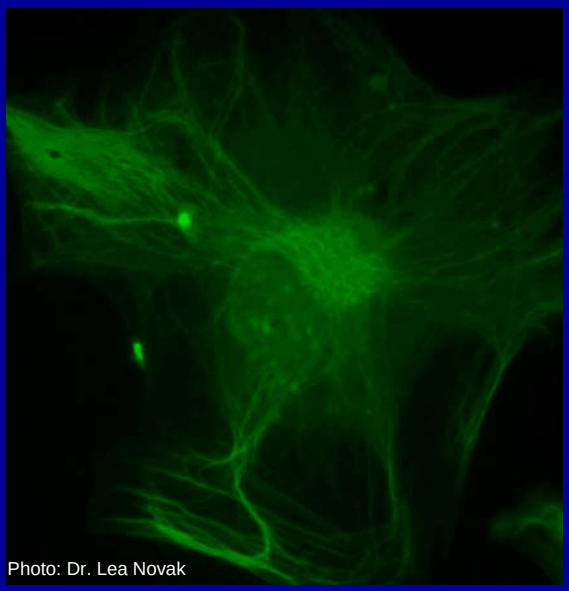
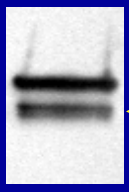
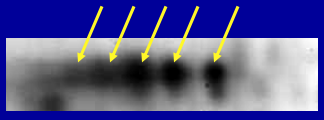


Photo: Dr. Lea Novak



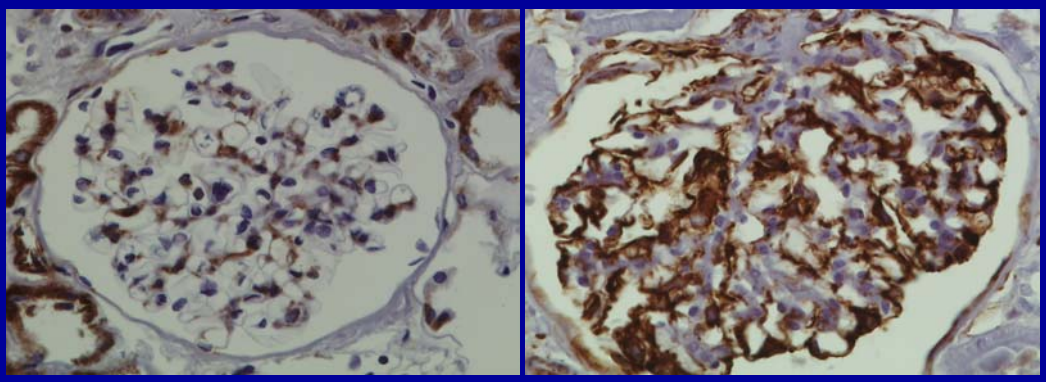
Western blot

Phosphorylation variants



2-D gel

Vimentin over-expressed in IgAN renal biopsies



Normal glomerulus

IgAN glomerulus

Overproduction of extracellular matrix proteins

IgAN-CIC induced laminin expression in MC *in vitro*

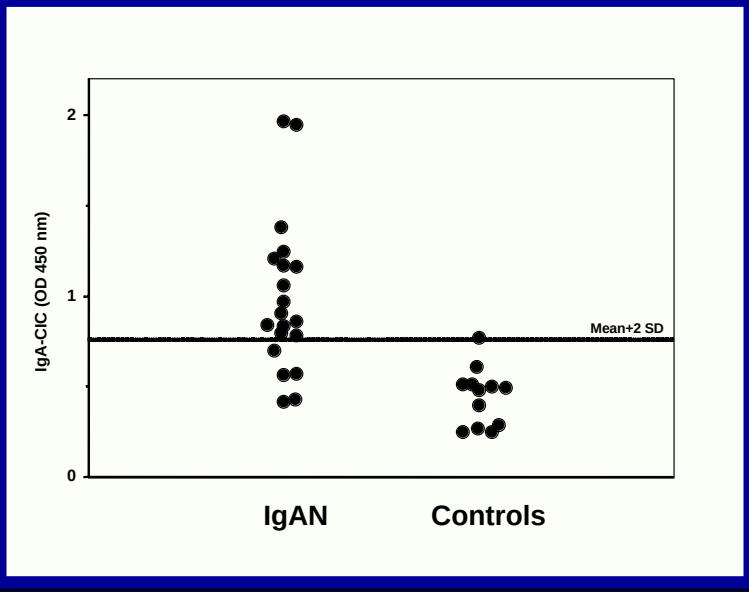
Cell lysates

Medium supernatants

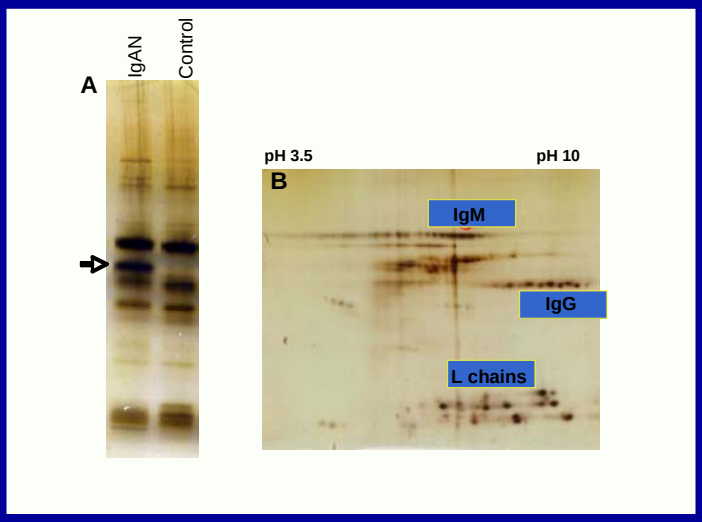
IgAN-CIC induce cytokine/chemokine mRNA in MC

	IL-6	IL-8	MCP-1	PDGF B/ PDGF ßR
Control (No CIC added)	+	±	+	+
Large CIC (800-900 kDa)	⇑	⇑	⇑	⇑
Small CIC (<800 kDa)	⇓	⇑⇑	⇓	⇑

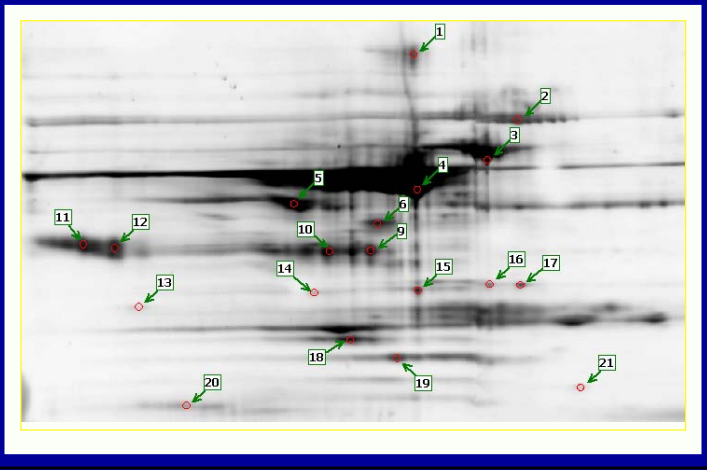
Elevated IgA-CIC in IgAN patients



Total CIC isolated with IC-specific affinity matrix

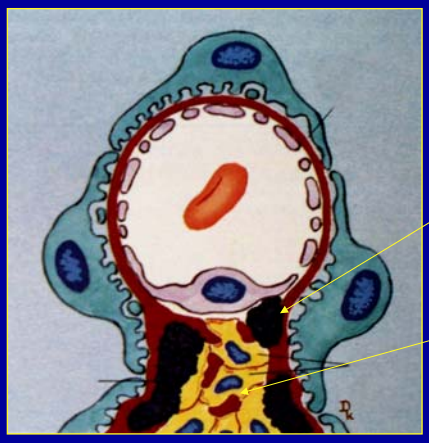


Analysis of IgAN CIC by proteomic approaches



2- plasmin precursor
3- transferrin precursor
5- alpha-1-antitrypsin
9,10- haptoglobin (acute phase prt)
11,12- Ig H chain VHDJ region
18- apolipoprotein A lipid binding protein
19- chromosome 14 ORF transcript variant 1

Hypothesis for pathogenesis of IgAN



Formation of IgA1-CIC

Gal-deficient IgA1 bound by anti-glycan Ab (IgG, IgA1)

↓

Mesangial deposition

↓

Activation of MC

(Proliferation, ECM expansion)

IgAN is an autoimmune disease

Antigen - galactose-deficient *O*-glycan-containing pIgA1

possibly induced by mucosal pathogens or their products

Antibody - glycan-specific IgG, IgA1

possibly induced by mucosal pathogens bearing *O*-glycans (viruses, bacteria)

Ratio of Ag:Ab determines **size** (and thus **biological activity**)

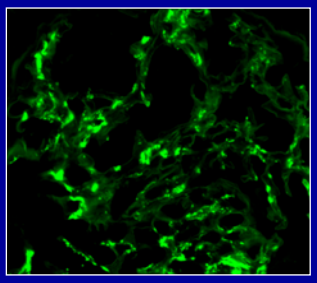
(Serum sickness may be a prototype of this kind of IC-disease)

Mesangial cells have **IgA receptor**(s) bind **IgA1-CIC** with high affinity

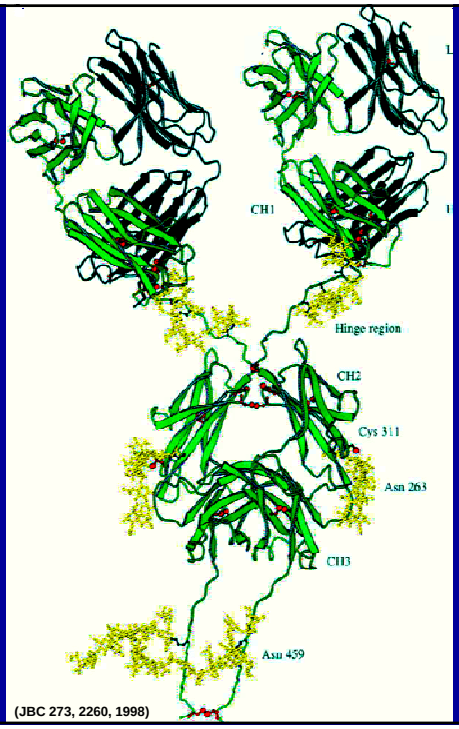
-> differential cellular activation by IgA1-CIC of different sizes

IgA nephropathy: a disease of abnormal post-translational modification?

- Abnormal *O*-glycosylation of IgA1 as etiopathogenic factor in IgAN (Mestecky 1993)
- Gal-deficient IgA1 complexed in CIC with anti-glycan IgG/IgA1 (Tomana 1997, 1999)
- Gal-deficient IgA1 in mesangial deposits (Allen 2001, Hiki 2001)



- What is the heterogeneity of *O*-glycosylation of IgA1, and what are the sites of *O*-glycan attachment?
- Does Gal deficiency in IgAN occur randomly or preferentially at specific sites?



(JBC 273, 2260, 1998)

Human IgA1

Structure and glycosylation

O-linked glycans

IgA glycosylation: Analytical approaches

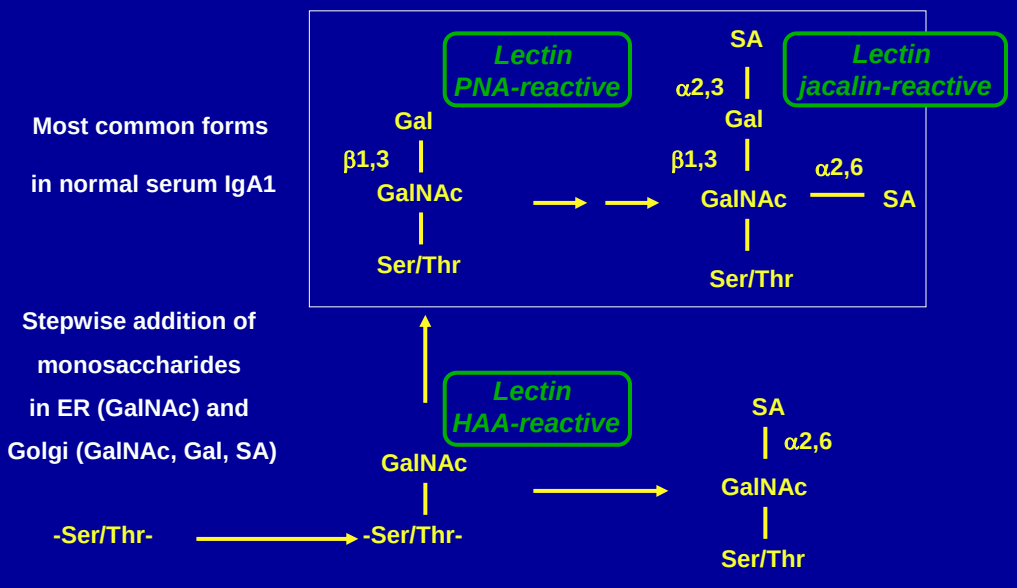
Monosaccharide composition (Gas-liquid chromatography)

Terminal saccharides (Lectin analyses: ELISA, Western blots)

N-linked glycans profile -> Composition & heterogeneity
(*N*-glycanase release -> MALDI-TOF MS)
-> Localization: predicted (Asn-X-Ser/Thr)
verification (NMR, MS)

O-linked glycans: Monosaccharide composition (GalNAc)
Terminal saccharides (lectin analyses)
Heterogeneity & Localization (NMR, FT-MS?)

O-glycan biosynthesis of circulatory IgA1

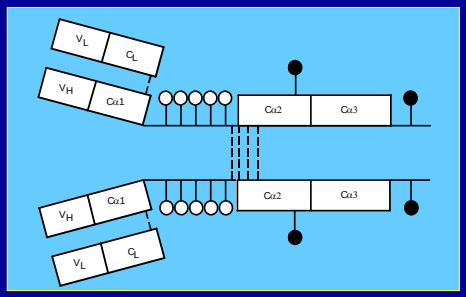


Methods

- Naturally Gal-deficient pIgA1 myeloma protein mimicking IgA1 from IgAN patients (Tomana 1999) analyzed after enzymatic removal of sialic acid
- Isolated trypsin-pepsin-thermolysin fragments
- IgA1 protease-generated fragments
  (single and double digests: Fc and Fd or released hinge region)
- Analyses: Gas-liquid chromatography
  Mass spectrometry
  Western blots with lectins

IgA1

Generation of fragments



Trypsin + pepsin

CHVKHYTNPSQD **VTVPCPVPSTPPTPSPSTPPTPSPSCCHPRL** SLHR

CH1 (CHO) CHO CHO CHO (CHO) CH2

-Pro-Val-Pro-Ser-Thr-Pro-Pro-Thr-Pro-Ser-Pro-Ser-Thr-Pro-Pro-Thr-Pro-Ser-Pro-Ser-Cys-

225 228 230 232 236

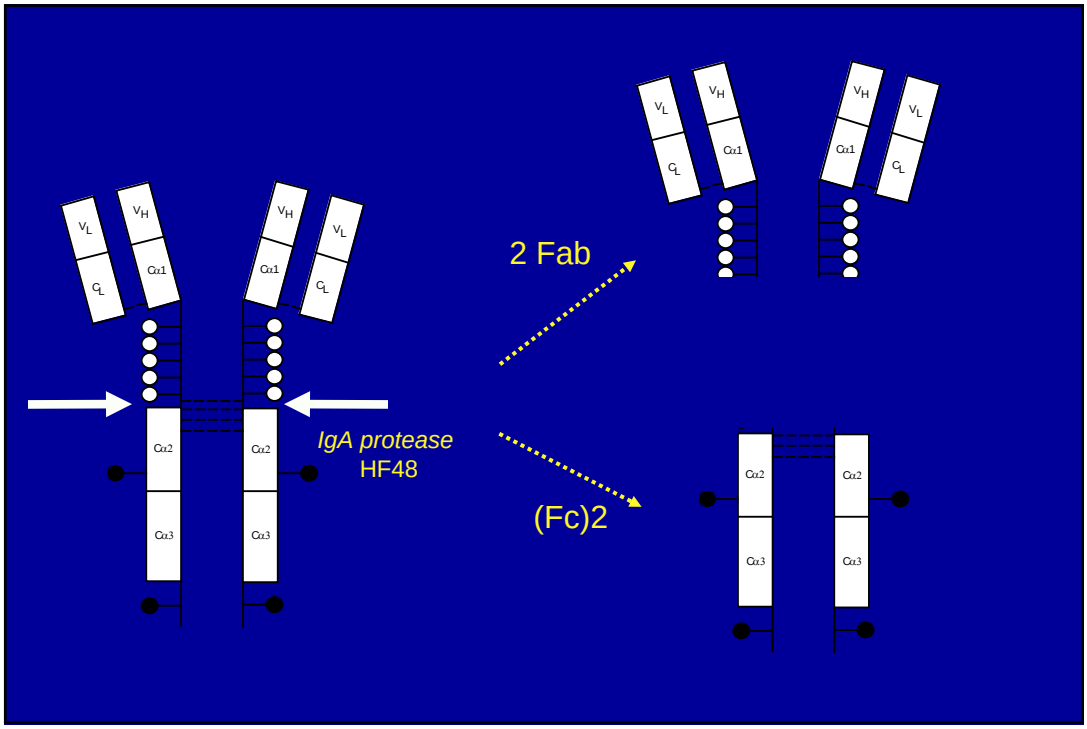
IgA proteases

1 *Clostridium*

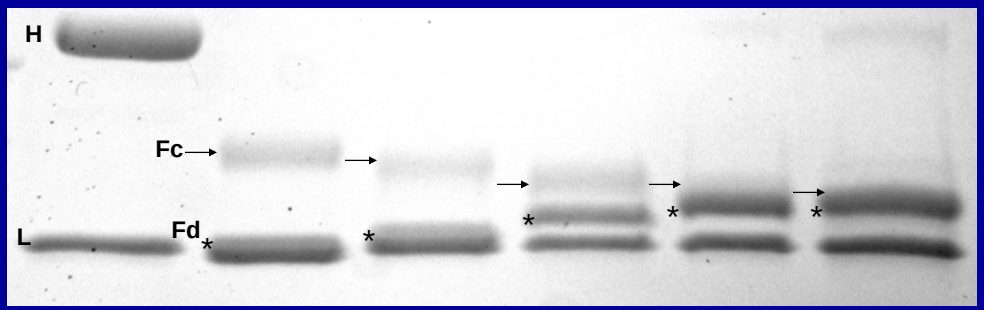
2 *Streptococcus*

3 *Haemophilus*

4 5 *Neisseria (HF 13 , 48)*

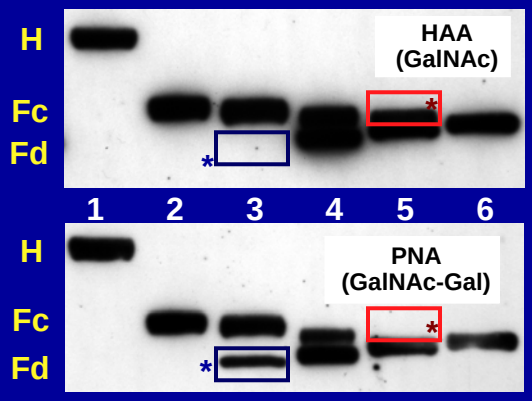


IgA1 Mce fragments in SDS PAGE (reducing 4-20% gradient)



1 - no protease
2 - IgA1 protease from *C. ramosum* AK183
3 - IgA1 protease from *S. pneumoniae TIGR4*
3 - IgA1 protease from *H. influenzae*
4 - IgA1 protease from *N. gonorrhoeae* HF13
5 - IgA1 protease from *N. gonorrhoeae* HF48

Western blots with *O*-glycan-specific lectins of Gal-deficient IgA1 myeloma protein digested with IgA proteases

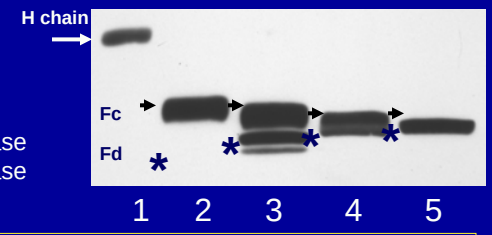


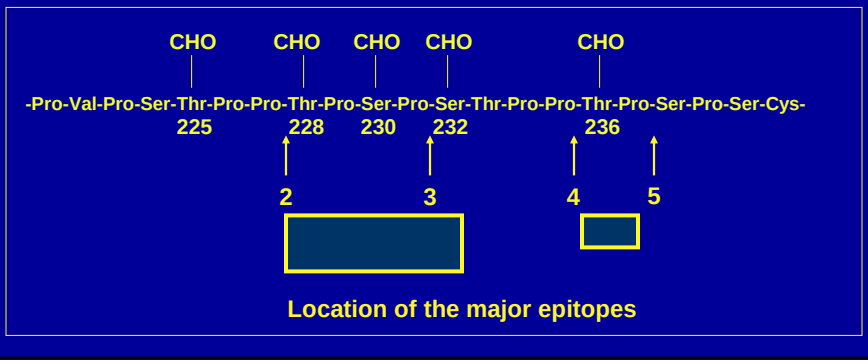
1 - no protease
2 - *C. ramosum*
3 - *S. pneumoniae*
4 - *H. influenzae*
5 - *N. gonorrhoeae* HF13
6 - *N. gonorrhoeae* HF48

(neuraminidase-treated samples)

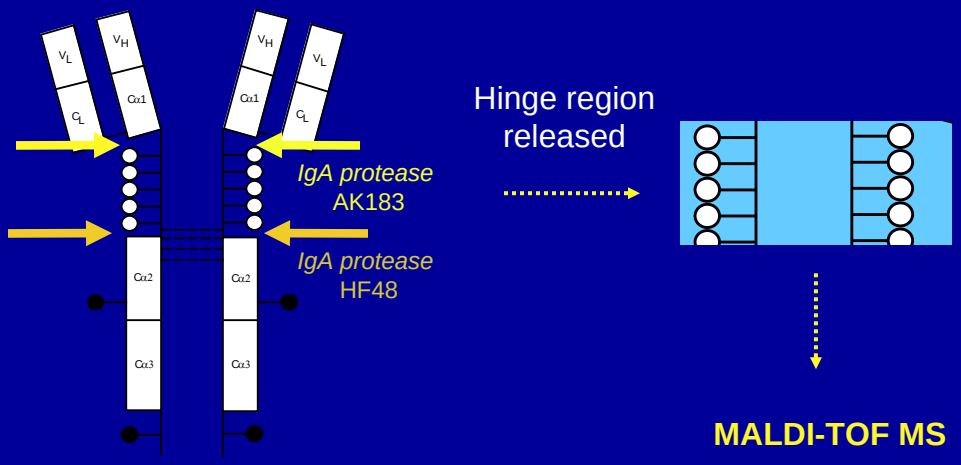
Reactivity of IgG antibodies with desialylated IgA1 and its fragments generated by IgA proteases

1 - uncleaved
2 - *S. pneumoniae* IgA1 protease
3 - *H. influenzae* IgA1 protease
4 - *N. gonorrhoeae* HF13 IgA1 protease
5 - *N. gonorrhoeae* HF48 IgA1 protease



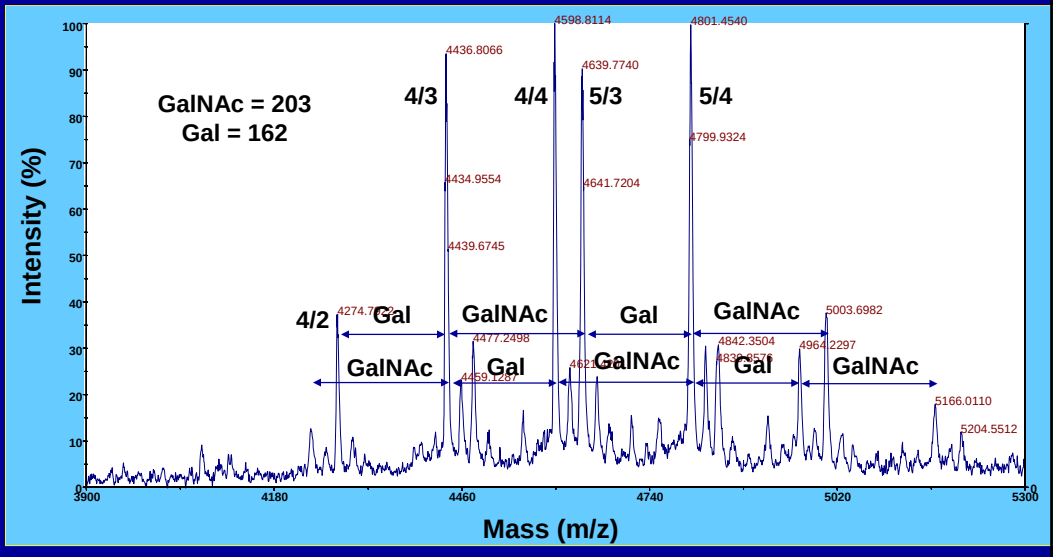


Hinge region can be released using two IgA proteases



MALDI-TOF MS of IgA1 HR glycopeptides (trypsin-pepsin)

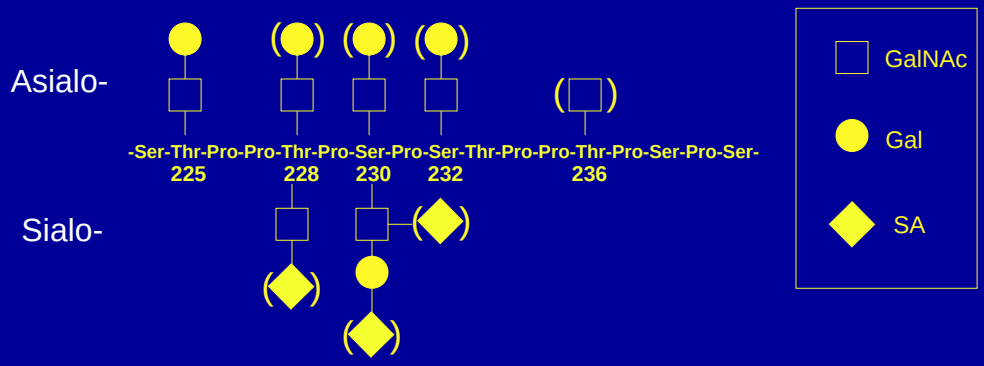
(multiple *O*-glycans of variable composition and number)



Summary

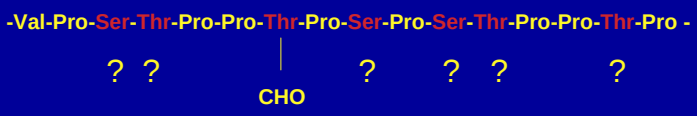
- **IgA1 HR showed heterogeneity in number and composition of glycans**
- **Sites of glycan attachment were determined by MS and WB with lectins using proteolytically generated HR fragments**
- **Sites that are aberrantly glycosylated may be specific and not random and may thus serve as biomarkers**

Major glycosylation forms:



Analysis of glycan attachment sites by mass spectrometry

Example of hinge variant with one glycan:



? One specific site of attachment or mixture of variants ?

Fourier transform-ion cyclotron resonance (FT-ICR) mass spectrometry

Fragmentation of peptide
Electron capture dissociation (ECD)

Fragmentation of glycosidic bond
Infrared multiphoton dissociation (IRMPD)