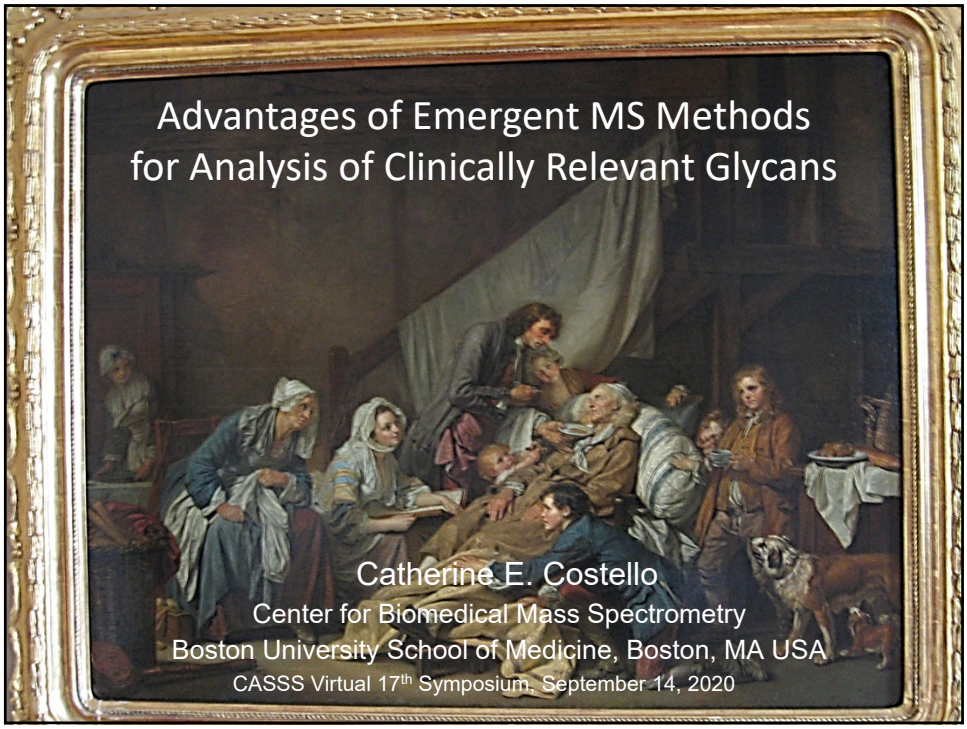
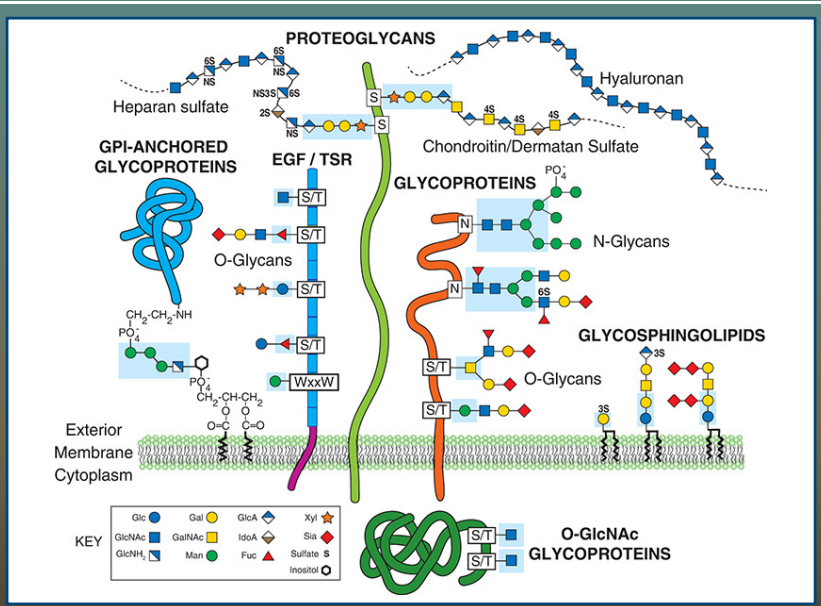
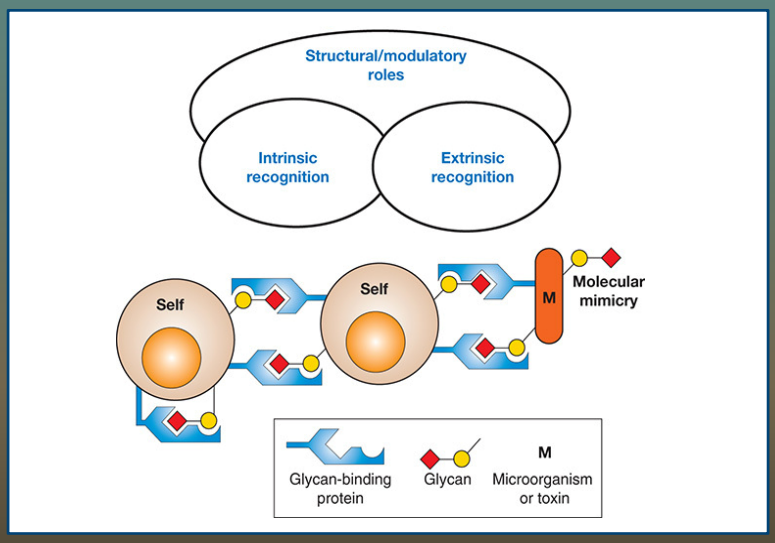



## Representative mammalian glycans and glycoconjugates



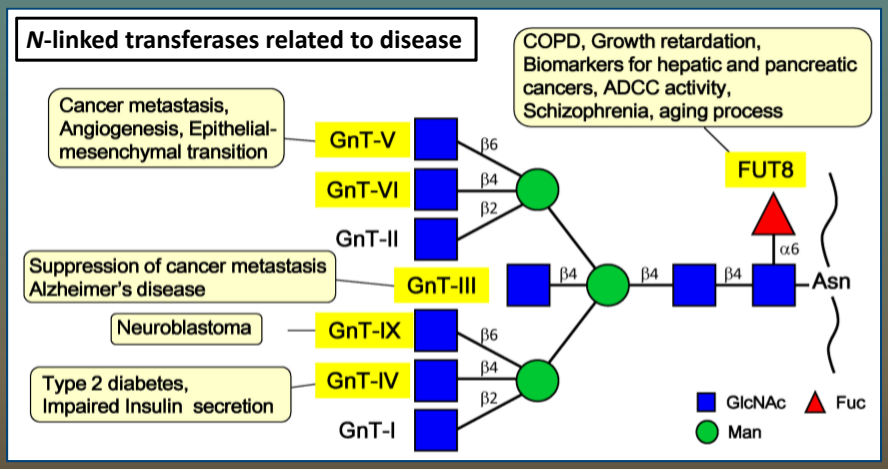

A Varki, S Kornfeld, *Essentials of Glycobiology*, 3rd ed, 2017

## Self- and non-self glycan interactions




A Varki, P Gagneux., *Essentials of Glycobiology*, 3rd ed, 2017

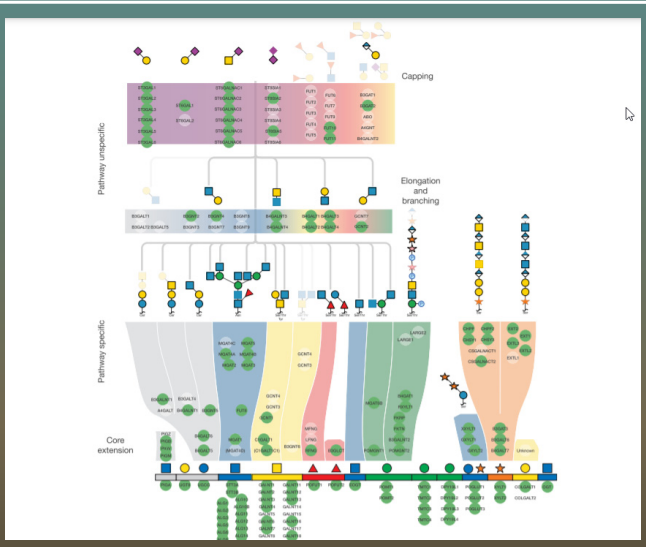
## Glycosyltransferases affect disease glycoform distributions




N Taniguchi, H Korekane, BMB Reports, 2011, 44, 772-781

→→→ *O*-linked glycoform distributions are also disease-dependent.

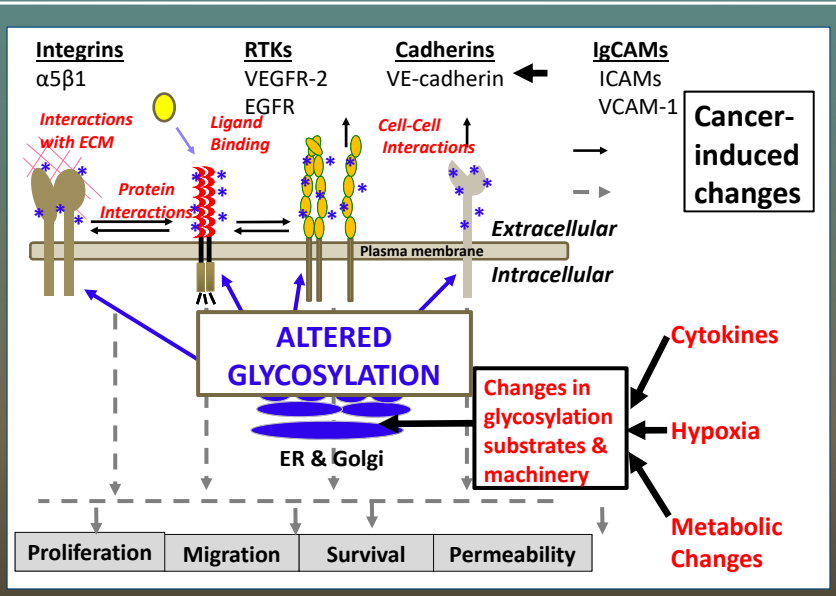
## Human glycosylation pathway maps reveal native complexity




~170 pathways are open to congenital disruption

Y Narimatsu, HJ Joshi, R Nason, J Van Coillie, R Karlsson, L Sun, Z Ye, YH Chen, KT Schjoldager, C Steentoft, S Furukawa, BA Bensing, PM Sullam, AJ Thompson, JC Paulson, C Büll, GJ Adema, U Mandel, L Hansen, EP Bennett, A Varki, SY Vakhrushev, Z Yang, H Clausen. *Mol Cell,* 2019, *75*, 394-407

## Infection & disease change *N*-glycosylation machinery




KB Chandler, CE Costello, N Rahimi, *Cells* 2019, *8*, 544

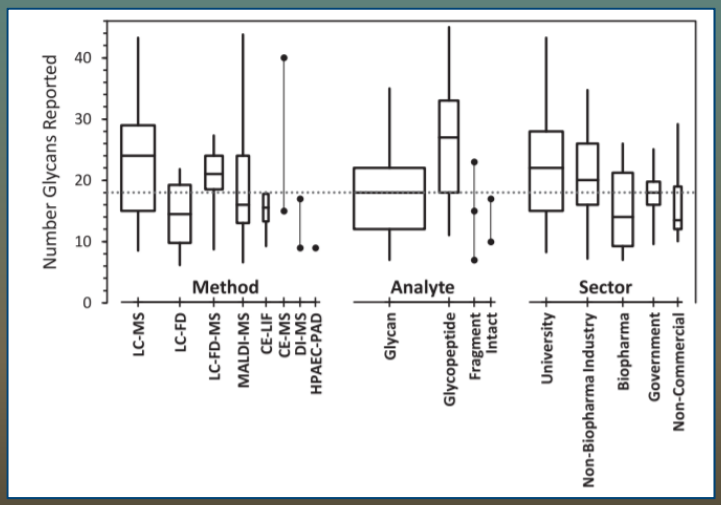
## Challenges for analysis of glycans and glycoconjugates

- Heterogeneity
  - Stereoisomers
  - Anomers
  - Branching
- Glycoform distributions
  - Shape - Interactions
  - Glycan composition
  - Modifications
- Fragile linkages
- Aglycon variations
- No template driver

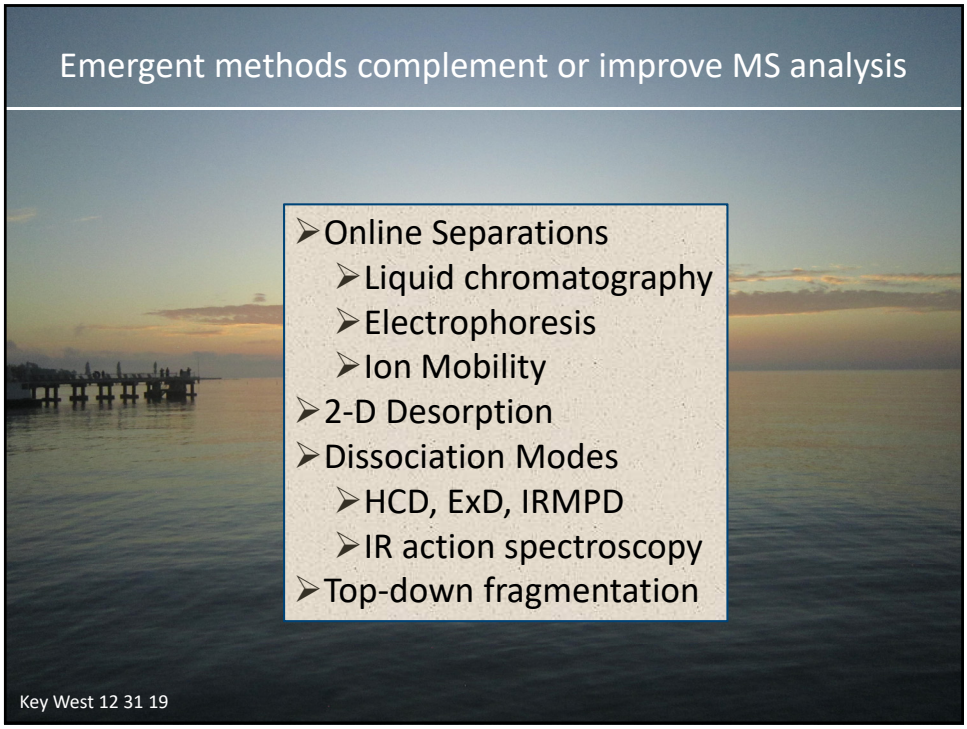
Key West 12 28 19

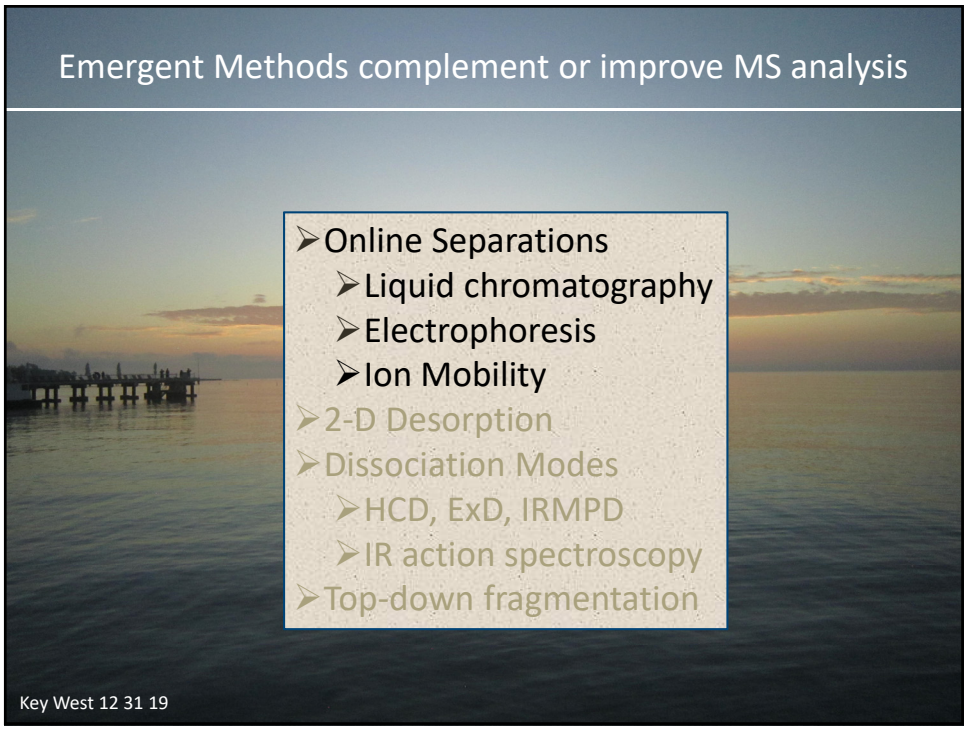
7

## How universal are the methods? Glycoforms detected by 75 labs in 103 analyses of NIST Antibodies




MLA De Leoz et al, 2020, *Molecular & Cellular Proteomics,* 2020, 19, 11–30


Emergent methods complement or improve MS analysis
Online Separations
Liquid chromatography
Electrophoresis
Ion Mobility
2-D Desorption
Dissociation Modes
HCD, ExD, IRMPD
IR action spectroscopy
Top-down fragmentation
Key West 12 31 19


Emergent Methods complement or improve MS analysis
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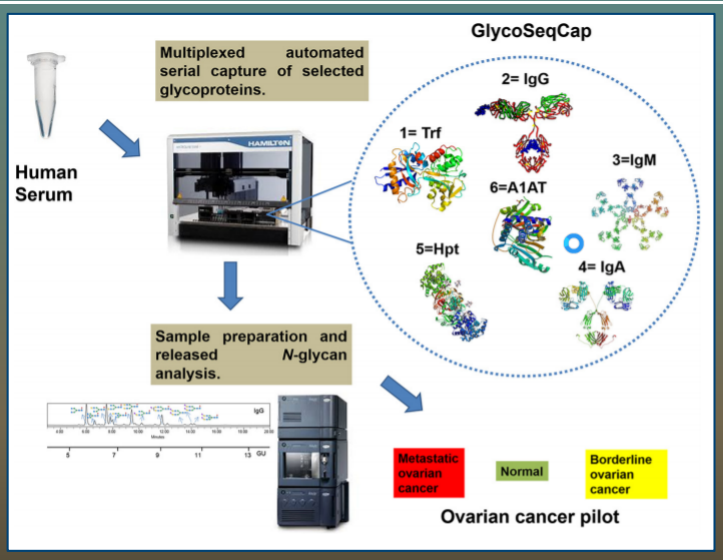
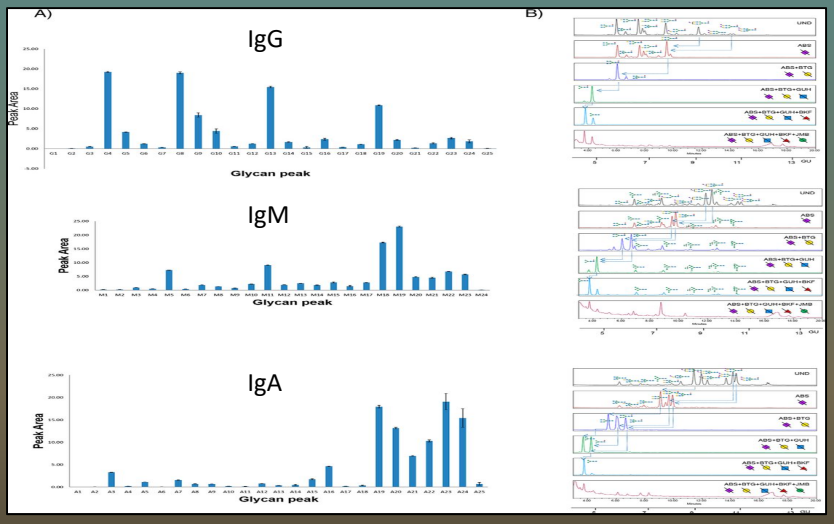
HCD, ExD, IRMPD
IR action spectroscopy
Top-down fragmentation
Key West 12 31 19

## LC-MS: glycoprotein capture, glycan release, glycosidase, sequencing



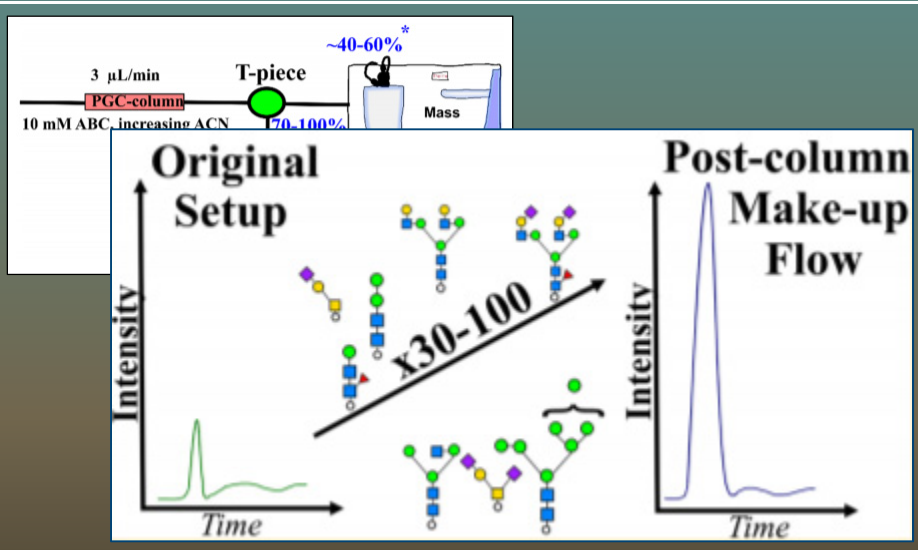

R O'Flaherty, M Muniyappa, I Walsh, H Stöckmann, M Hilliard, R Hutson, R Saldova, PM Rudd. *Mol Cell Proteomics*, 2019, *18*, 2192-2206.

## LC-MS: glycoprotein capture, glycan release, glycosidase, sequencing



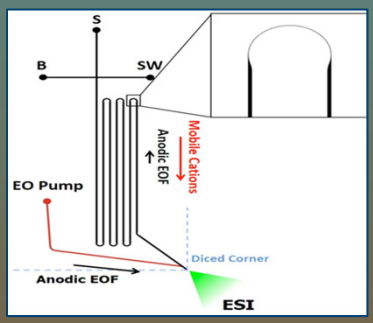

R O'Flaherty, M Muniyappa, I Walsh, H Stöckmann, M Hilliard, R Hutson, R Saldova, PM Rudd. *Mol Cell Proteomics*, 2019, *18*, 2192-2206.

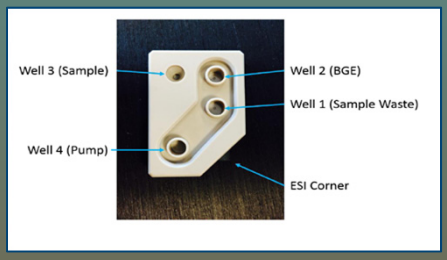
## Post-column make-up flow enhances PGC-LC-MS/MS for glycomics




H Hinneburg, S Chatterjee, F Schirmeister, T Nguyen-Khuong, NH Packer, E Rapp, M Thaysen-Andersen. *Anal Chem*, 2019, *91*, 4559-4567

## Chip-based Capillary Electrophoresis Mass Spectrometry



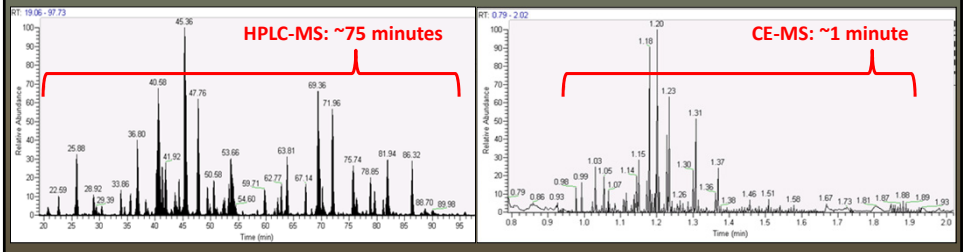




Redman, Batz, Mellors, Ramsey. *Anal Chem* 2015

*collaborators - JM Ramsey, UNC & KJ Knopp, 908 Devices*

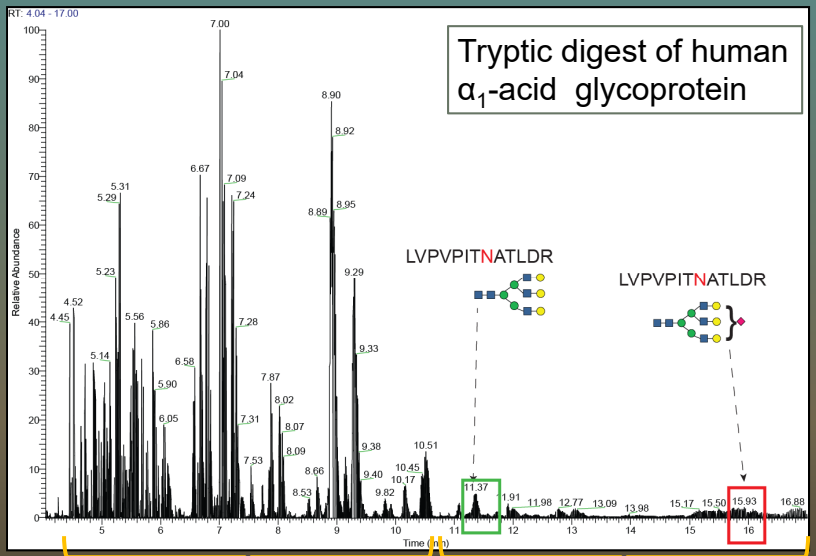
## CE-MS *vs*. LC-MS: in-gel digest of 8-protein-standards mix

| | CE-MS | | | | HPLC | | | |
|---|---|---|---|---|---|---|---|---|
| **Protein** | **# peptides** | **# unique** | **# MS2** | **coverage (%)** | **# peptides** | **# unique** | **# MS2** | **coverage (%)** |
| Ferritin LC | 9 | 10 | 89 | 50 | 8 | 12 | 57 | 50 |
| Glucose oxidase | 18 | 20 | 49 | 40 | 17 | 24 | 69 | 36 |
| Catalase | 34 | 46 | 155 | 60 | 39 | 64 | 235 | 54 |
| GAPDH | 14 | 16 | 70 | 44 | 22 | 34 | 100 | 53 |
| Trypsin Inhibitor | 12 | 18 | 143 | 49 | 11 | 17 | 115 | 45 |
| B-Lactoglobulin | 13 | 17 | 191 | 60 | 18 | 28 | 144 | 75 |
| Myoglobin | 15 | 25 | 140 | 82 | 19 | 37 | 124 | 90 |
| Cytochrome C | 19 | 26 | 57 | 71 | 16 | 26 | 78 | 62 |

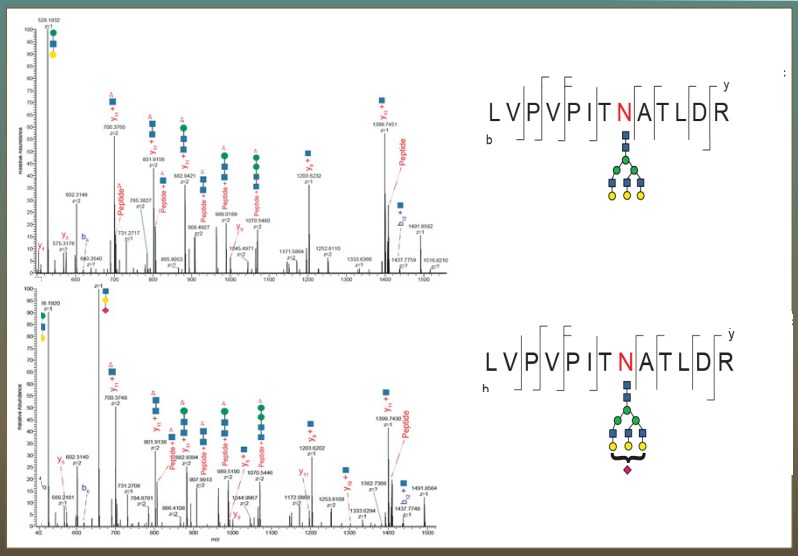



K Khtari, J Klein, JR Haserick, DR Leon, CE Costello, ME McComb, J Zaia.
*Anal Chem*, 2017, *89*, 6645-6655

## CE-MS separation of peptides and glycopeptides

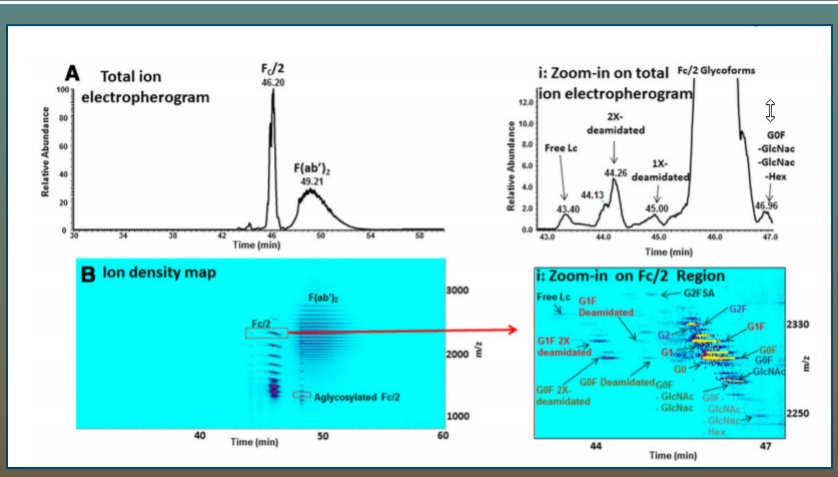


## CE-HCD-MS/MS: glycoforms of LVPVPITNATLDR




HCD MS/MS spectra recorded with 908 Devices CE chip mounted on ThermoFisher QE-Orbitrap MS

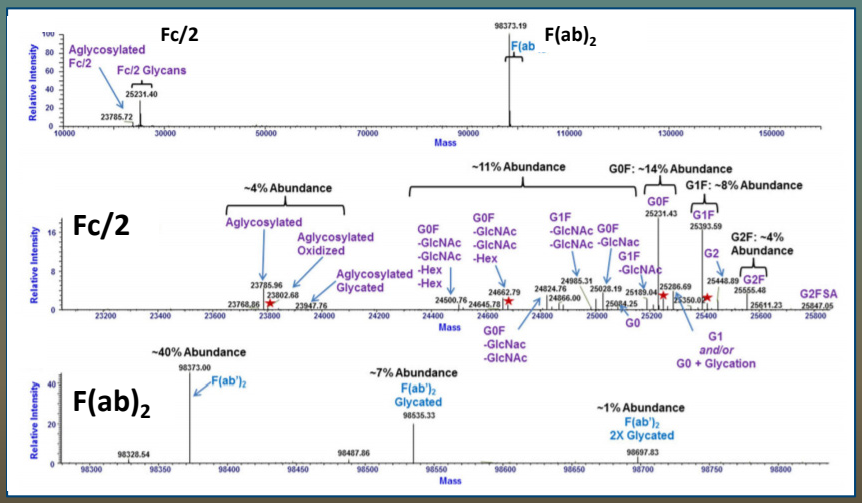
## CZE-MS: top-down and middle-down analysis of mAbs



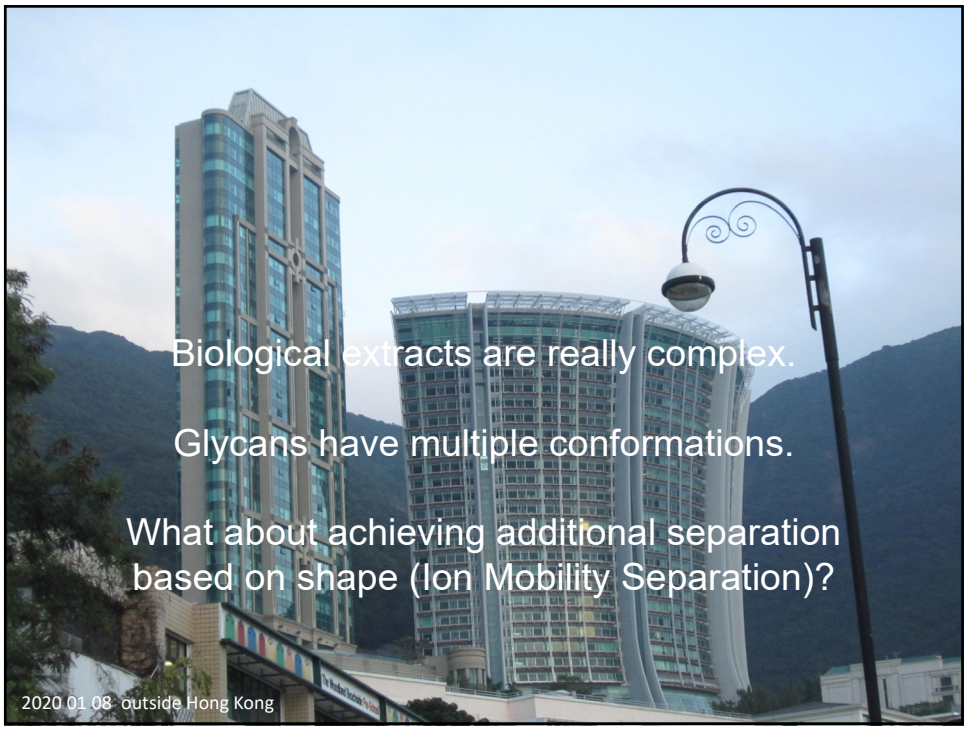

Electropherogram of IdeS-digested biotherapeutic antibody, and PTM assignments. CESI sheathless interface to ThermoFisher QE Orbitrap MS.

AM Belov, L Zang, R Sebastiano, MR Santos, DR Bush, BL Karger, AR Ivanov. *Electrophoresis*. 2018 *39*, 2069-2082.

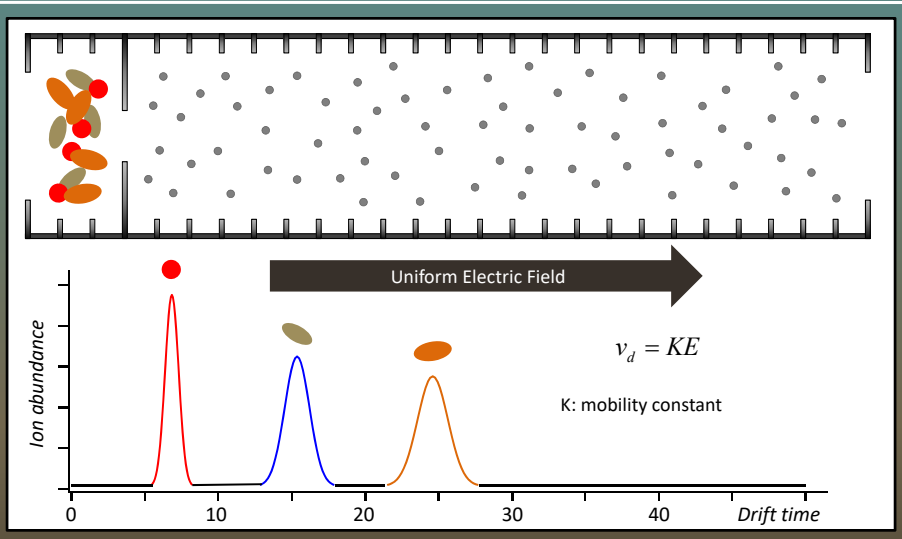
## CZE-MS: assignments of PTMs on *IdeS* products from mAb




AM Belov, L Zang, R Sebastiano, MR Santos, DR Bush, BL Karger, AR Ivanov. *Electrophoresis*. 2018 *39*, 2069-2082.



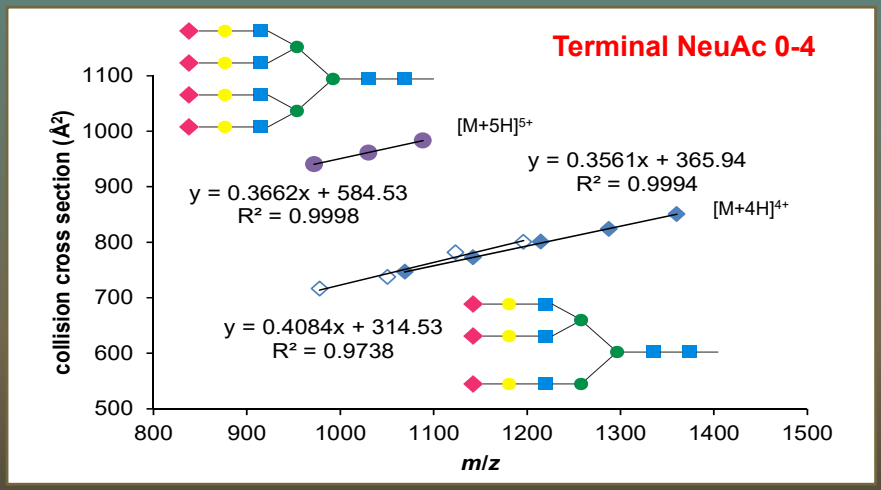
## Drift Tube Ion Mobility Spectrometry (IMS)




Separation occurs on the millisecond time scale, compatible with online LC or nLC (sec/peak) and TOF mass analyzers (100s µsec/spectrum)

## IM-MS: glycopeptides from human $\alpha_1$-acid glycoprotein

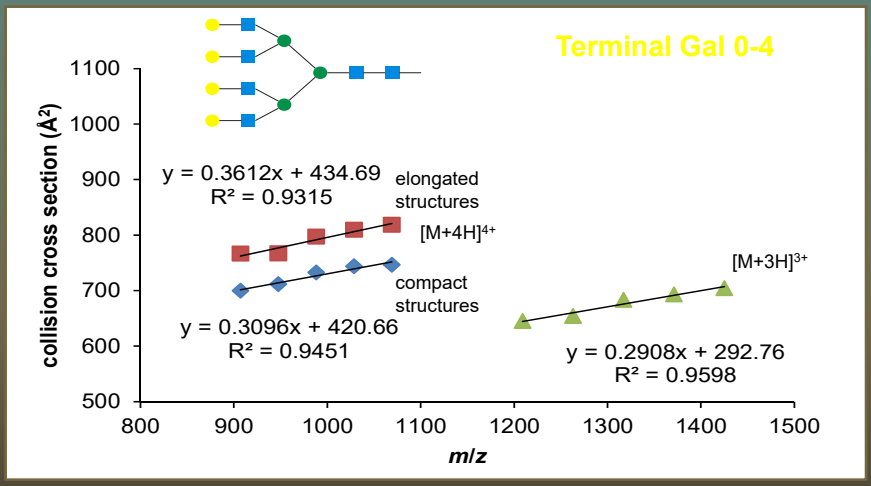
$^{87}$QNQC(Cam)FYN*SSYLNVQR$^{101}$
(N* = occupied glycosylation site)



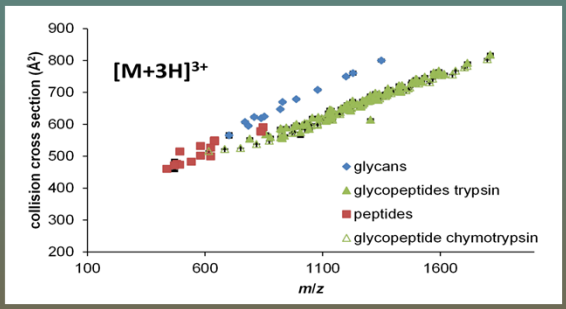

RS Glaskin, K Khatri, Q Wang, J Zaia, CE Costello. *Anal Chem*, 2017, *89*, 4152-4160.

## IM-MS: glycoforms from hAGP after sialidase

$^{87}$QNQC(Cam)FYN*SSYLNVQR$^{101}$
(N* = occupied glycosylation site)




## CCS values for reduced permethylated glycan, glycopeptide, and peptide ions from hAGP & hFetuin; monosaccharide CCS increments



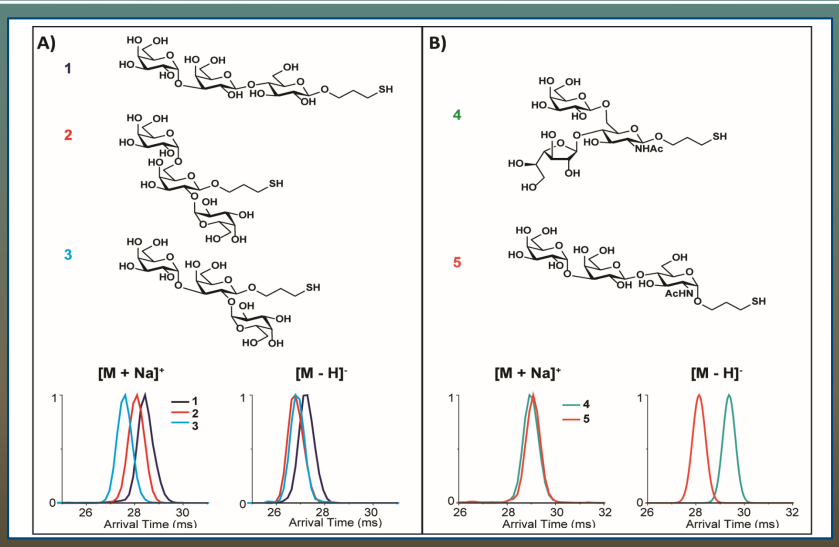

**Glycopeptide increments**

| | CCS (Å²) |
|---|---|
| mannose | 14 to 28 (±5) |
| sialic acid | 22 to 30 (±5) |
| galactose | 11 to 19 (±5) |
| *N*-acetylhexosamine | 13 to 15 (±5) |

**Permethylated glycan increments**

| | CCS (Å²) |
|---|---|
| mannose | 19 to 44 (±9) |
| galactose | 23 to 28 (±2) |
| sialic acid | 32 to 49 (±7) |

## Context matters: (+) and (-)-ion mode IM-MS separations of glycans related to immune response to *T cruzi* mucins in Chagas disease



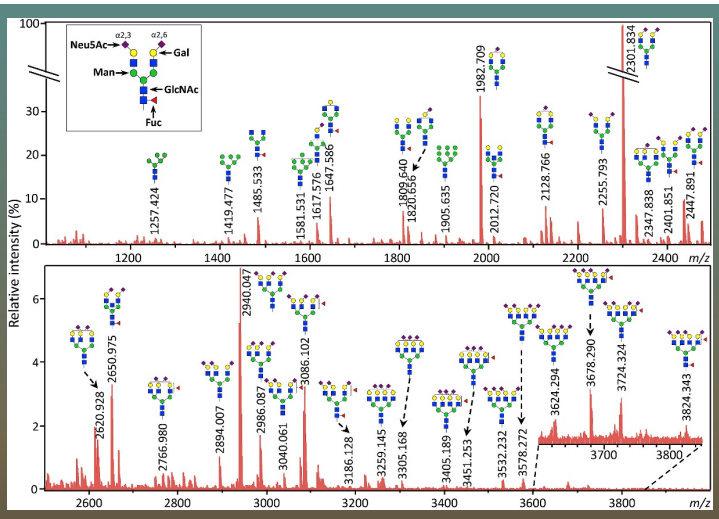

X Zheng, X Zhang, NS Schocker, RS Renslow, DJ Orton, J Khamsi, RA Ashmus, IC Almeida, K Tang, CE Costello, RD Smith, K Michael, ES Baker. *Anyl Bioanyl Chem*, 2017, *409*, 467-476

## Emergent Methods Complement or Improve MS Analysis

- Online Separations
  - Liquid chromatography
  - Electrophoresis
  - Ion Mobility
- 2-D Desorption
- Dissociation Modes
  - HCD, ExD, IRMPD
  - IR action spectroscopy
- Top-down fragmentation

Key West 12 31 19

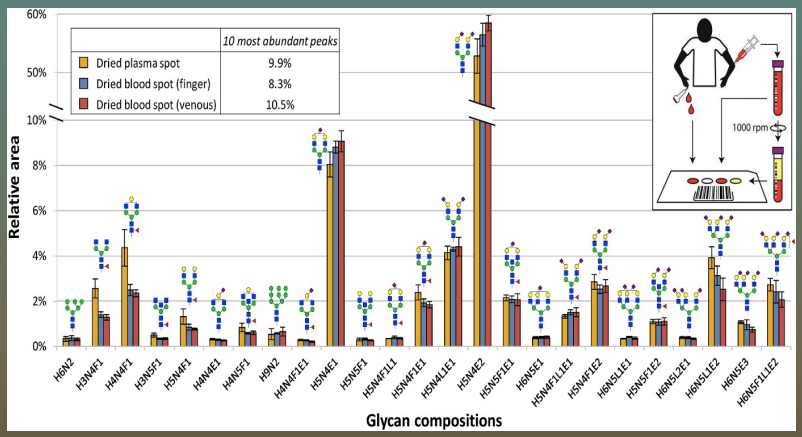
## MALDI-FTICR MS analysis of a dried blood spot




Glycans were released by PNGase F and sialic acids were derivatized to preserve linkage information. α2,3-linked = lactonized NeuAc; α2,6-linked = ethyl esterified NeuAc.

GCMVreeker, MR Bladergroen, S Nicolardi, WE Mesker, RAEM Tollenaar, YEM van der Burgt, M Wuhrer. *Talanta, 2019, 205, 120104*

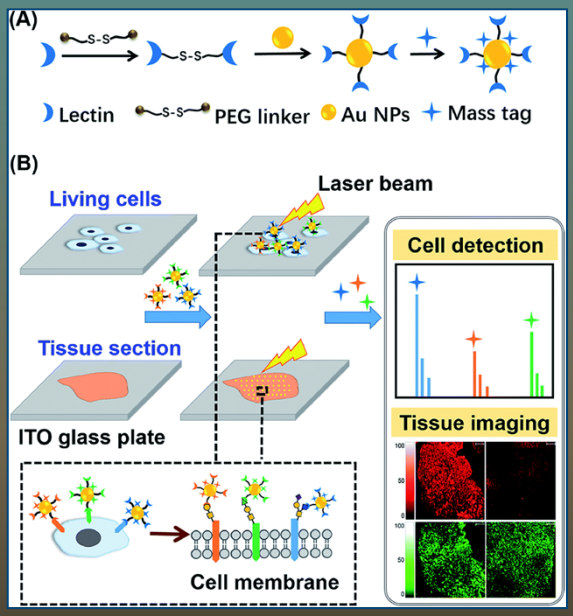
## MALDI-FTICR MS analysis of dried plasma and blood spots




Glycans were released by PNGase F and sialic acids were derivatized to preserve linkage information. α2,3-linked = lactonized NeuAc; α2,6-linked = ethyl esterified NeuAc.

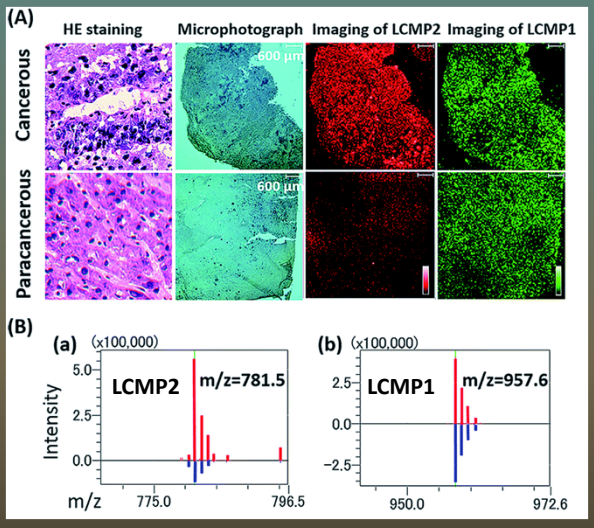
Tissue source affects glycoform distribution. Pattern is stable upon storage.

## Photocleavable dye tags on lectin-loaded nanoparticles facilitate 2-D MS imaging of cell and tissue glycans




W Ma, Su, H Nie, D Hu, Y Bai, H Liu. *Chem Sci*, 2019, *10*, 2320-2325

## Photocleavable dye tags on lectin-loaded NPs enable 2-D MS imaging of LCMP2 (NeuAc) and LCMP1 (Man) in liver tumors




HCC epithelial cells

LCMP2 NPs: +SNA

LCMP1 NPs: +ConA

W Ma, Su, H Nie, D Hu, Y Bai, H Liu. *Chem Sci*, 2019, *10*, 2320-2325

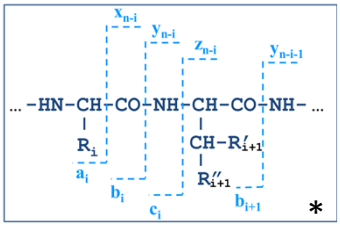
## Emergent Methods Complement or Improve MS Analysis

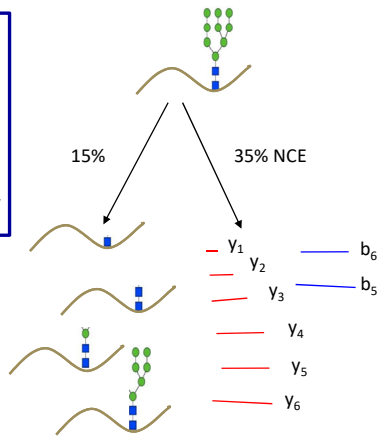
- Online Separations
  - Liquid chromatography
  - Electrophoresis
  - Ion Mobility
- 2-D Desorption
- Dissociation Modes
  - HCD, ExD, IRMPD
  - IR action spectroscopy
- Top-down fragmentation

Key West 12 31 19

## Gentle ionization conditions, Higher Energy Collisional Dissociation (HCD) with stepped collision energy

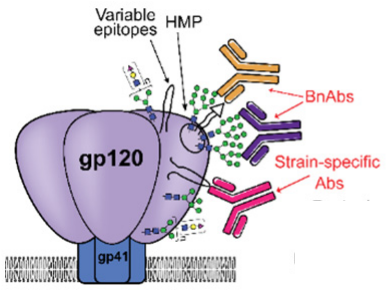
- Site-specific *N*-glycosylation analysis of glycoproteins
  - Clear evidence of peptide sequence
  - Information regarding *N*-glycan composition, topology



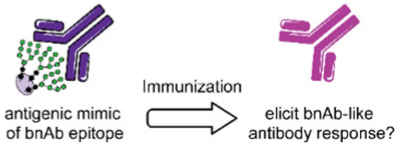




*after P Roepstorff/J Fohlmann and K Biemann

## HIV-Ab Vaccines? Oligomannose glycopeptides should elicit bnAbs targeting gp120 high mannose glycans.



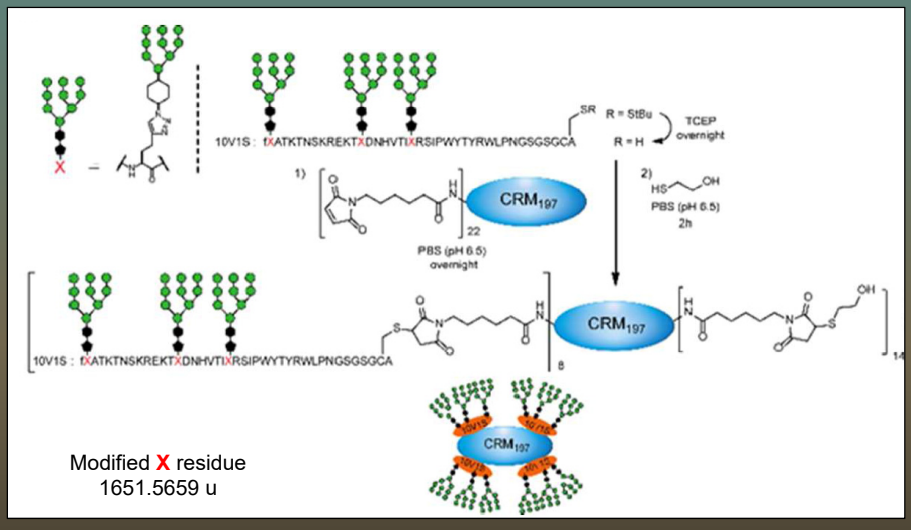

≤20 % of HIV-infected individuals develop broadly neutralizing antibodies (bnAbs); ~40% of bnAbs target the "high-mannose patch" (HMP) on HIV gp120.



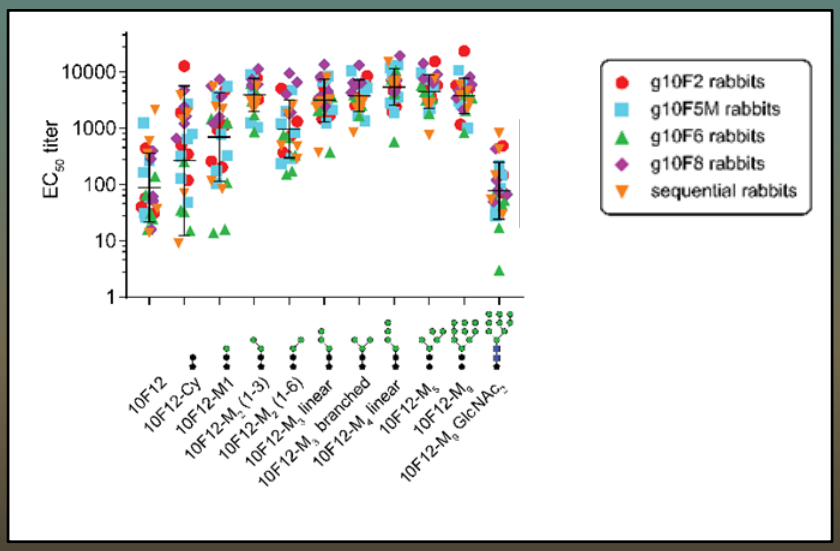

- Synthetic neoglycopeptides, designed by *in vitro* selection, bind tightly to anti-HMP antibody 2G12.

DN Nguyen, B Xu, RL Stanfield, JK Bailey, S Horiya, JS Temme, DR Leon, CC LaBranche, DC Montefiori, CE Costello, IA Wilson, IJ Krauss. *ACS Central Sci.,* **2019**, *5*, 237-249

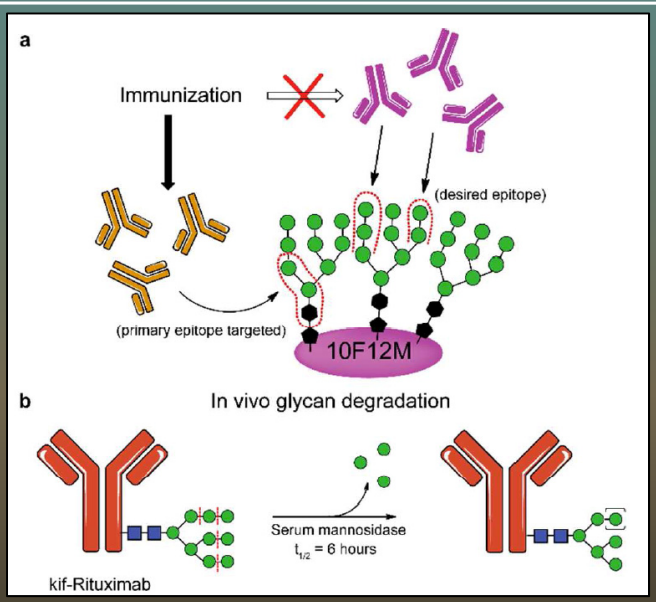
## Cys residue on glycopeptide covalently links to maleimide-functionalized carrier protein (CRM)



## OOOPS! Elicited Abs preferentially target glycan core.




## Serum mannosidase activities may account for preferential targeting of glycan core by elicited Abs



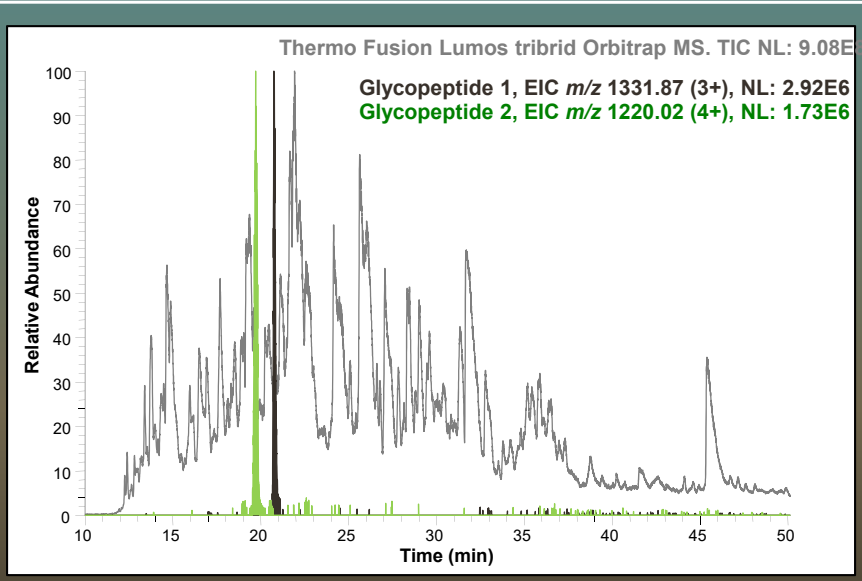
## X*-glycopeptide conjugated to carrier protein, a non-toxic mutant of diptheria toxin. $M_r$ 70-130 kDa

(formylated)**X***L**X***FIRIYPTR**X***QYVYHAPLLT**X***VRGSPTGPLIGSGSGCA

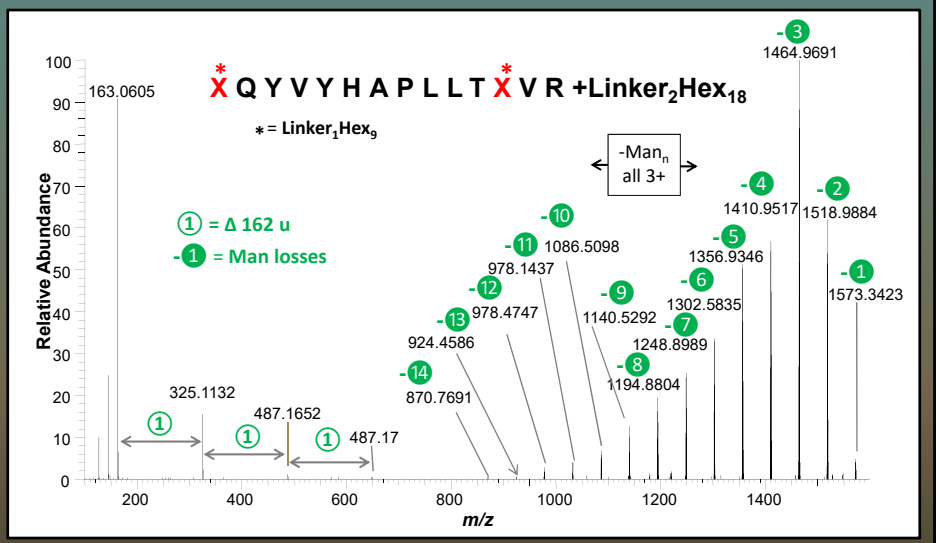
Carrier Protein (CRM):

GADDVVDSSKSFVMENFSSYHGTKPGYVDSIQKGIQKPKSGTQGNYDDD
WKEFYSTDNKYDAAGYSVDNENPLSGKAGGVVKVTYPGLTKVLALKVDN
AETIKKELGLSLTEPLMEQVGTEEFIKRFGDGASRVVLSLPFAEGSSSVEYIN
NWEQAKALSVELEINFETRGKRGQDAMYEYMAQACAGNRVRRSVGSSL
SCINLDWDVIRDKTKTKIESLKEHGPIKNKMSESPNKTVSEEKAKQYLEEFH
QTALEHPELSELKTVTGTNPVFAGANYAAWAVNVAQVIDSETADNLEKTTA
ALSILPGIGSVMGIADGAVHHNTEEIVAQSIALSSLMVAQAIPLVGELVDIGF
AAYNFVESIINLFQVVHNSYNRPAYSPGHKTQPFLHDGYAVSWNTVEDSIIR
TGFQGESGHDIKITAENTPLPIAGVLLPTIPGKLDVNKSKTHISVNGRKIRM
RCRAIDGDVTFCRPKSPVYVGNGVHANLHVAFHRSSSEKIHSNEISSDSIGV
LGYQKTVDHTKVNSKLSLFFEIKS

## Elution of $Man_9$ glycopeptides 1 and 2 @ time point 0 h

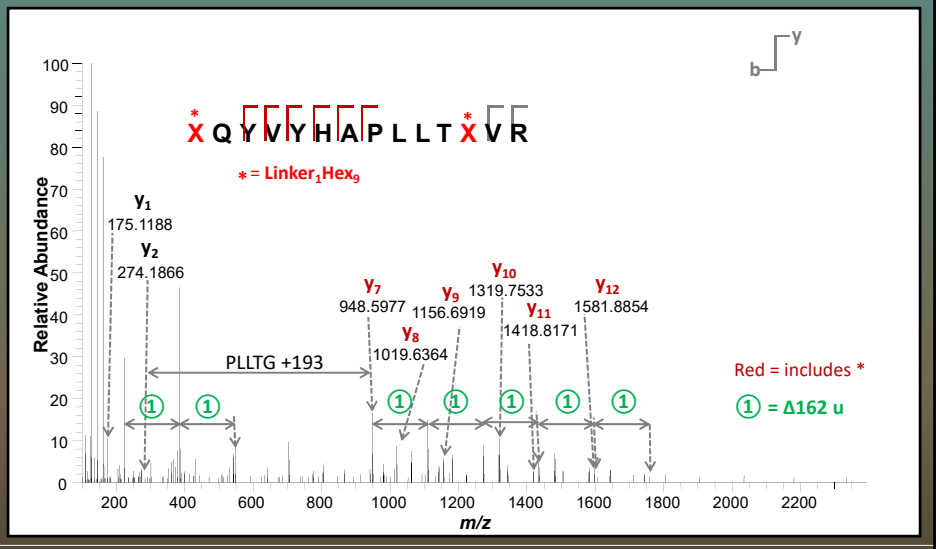


## Dissociation of glycopeptide 2 $[M+4H]^{4+}$ *m/z* 1220.0194



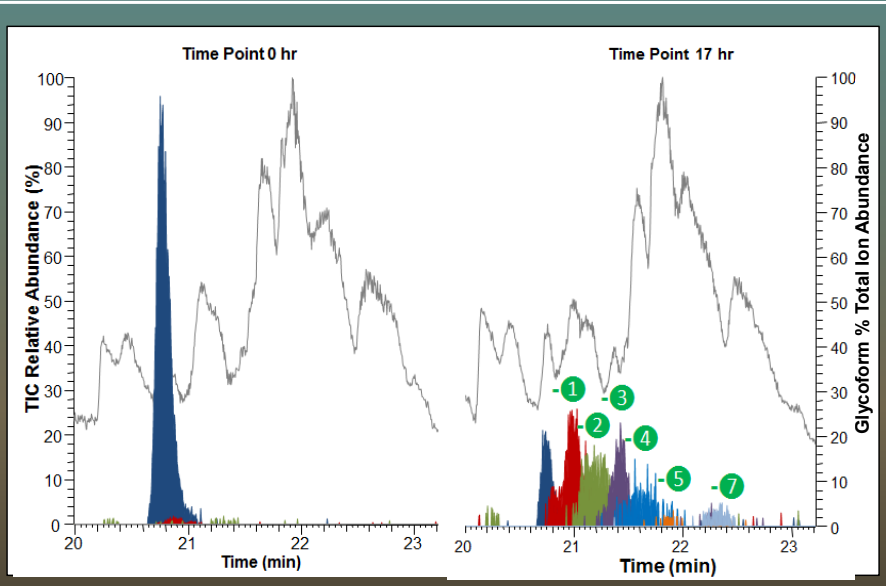

nUPLC-HCD-MS/MS at 15 eV: only glycan fragmentation

## Identification of glycopeptide 2 $[M+4H]^{4+}$ *m/z* 1220.0194

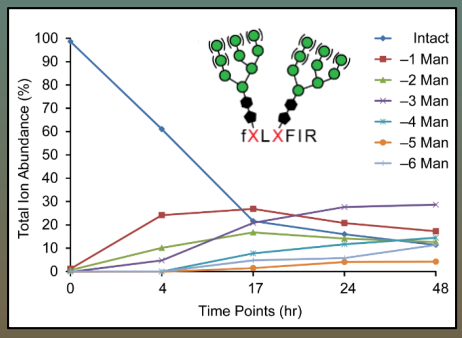


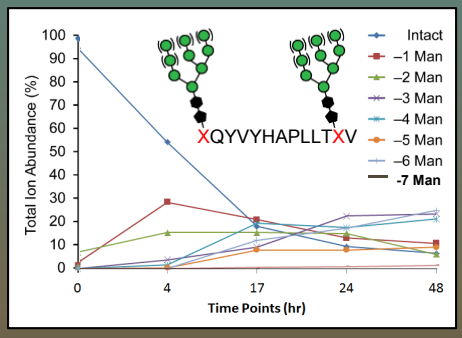

nUPLC-HCD-MS/MS at 45 eV: peptide backbone fragmentation; glycan intervals

## Elution of $Man_n$ glycoforms of glycopeptide 1 @ 0,17 h




## nanoUPLC/MS relative quantification of glycoforms in tryptic digest of g10F6 when g10F6 CRM conjugate is trimmed by serum mannosidases

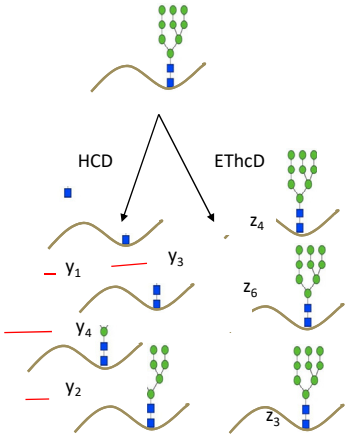



- These findings have broad implications for vaccine design aiming to target glycan-dependent HIV neutralizing antibodies.
- "Soft" $MS^n$ conditions may be required to accurately follow pharmacokinetics.

## HCD *vs.* HCD (Oxonium Ion) - triggered EThcD

- HCD-triggered EThcD
  - Perform exploratory HCD fragmentation of precursors
  - Trigger EThcD when oxonium ions are observed in HCD spectra
  - Supplemental activation
- Preserves the relationship between the *N*-glycan and the glycosylation site

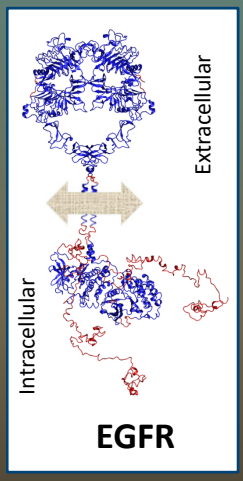



Example: β-catenin/CBP Inhibition Alters Epidermal Growth Factor Receptor Fucosylation Status in Oral Squamous Cell Carcinoma. KB Chandler, KA Alamoud, VL Stahl, B-C Nguyen, VK Kartha, MV Bais, K Nomoto, T Owa, S Monti, MA Kukuruzinska, CE Costello. *Molecular Omics*, **2020**, *16*, 195 – 209.

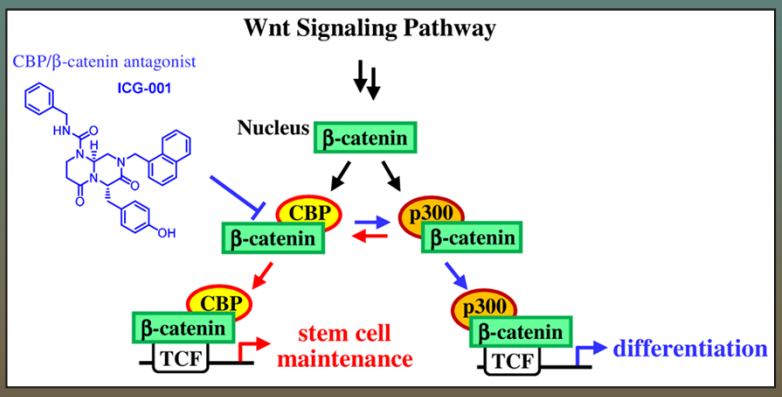
## *N*-glycosylation plays a role in Head and Neck Squamous Cell Carcinoma (HNSCC)

HNSCC is the 6th most common malignancy in the world. Two MAbs targeting the **EGFR** and PD-1, the only available FDA-approved targeted therapies, have < 20% clinical success.

Interaction of the EGFR *N*-glycosylation and Wnt/β-catenin signaling pathways promotes Oral Squamous Cell Carcinoma (OSCC) pathogenesis. **Regulation of EGFR glycosylation impacts stem cell maintenance, proliferation and survival**



## Interference with *N*-glycosylation may offer a treatment for HNSCC




**ICG-001,** a small-molecule inhibitor, binds the **CREB-binding protein (CBP)**, disrupting its interaction with β-catenin and thereby inhibiting the **Wnt pathway**. ICG-001 may be an effective therapeutic for HNSCC.

Teo and Kahn, *Adv Drug Deliv Rev,* 2010

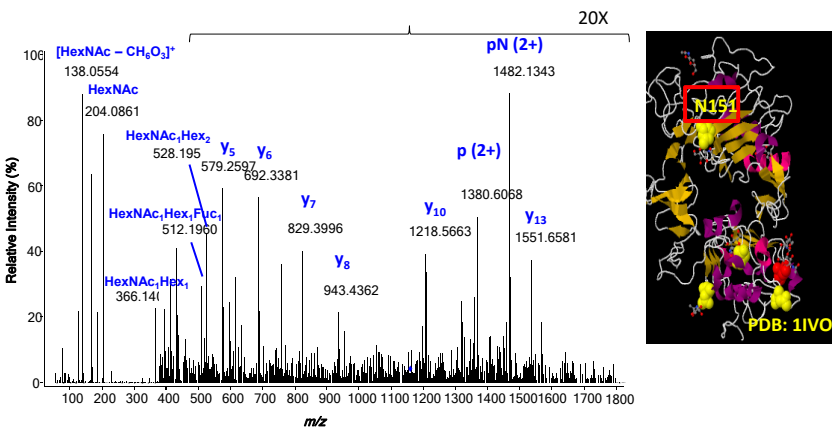
## HCD MS/MS EGFR site N151, $[M + 4H]^{4+}$ *m/z* 1169.6999

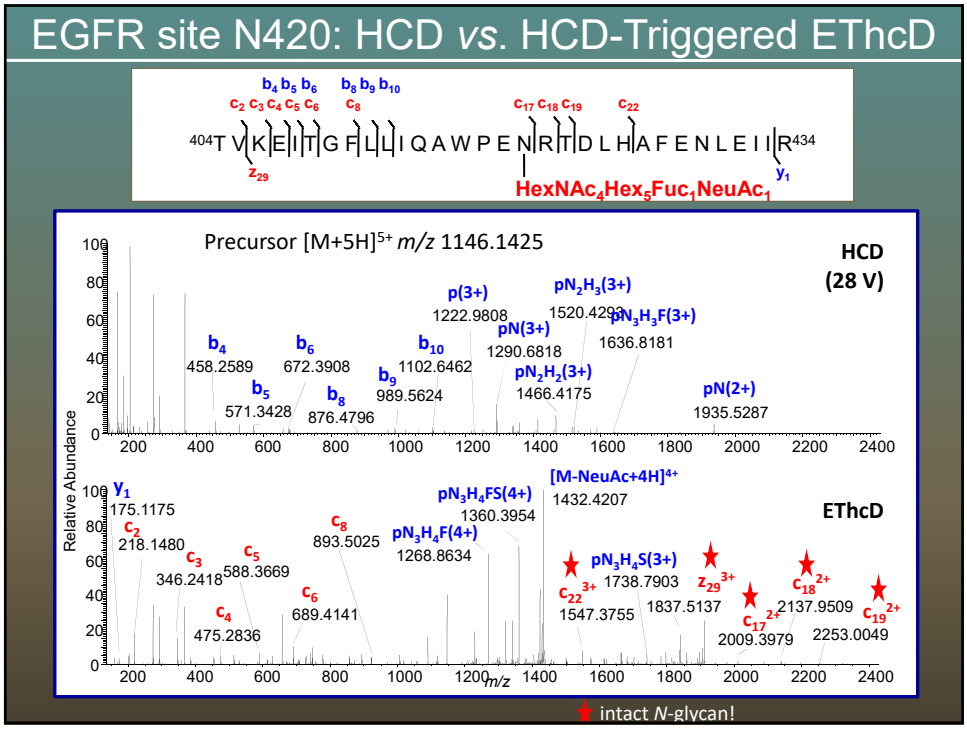
**Legend**
**p** – peptide
**N** – *N*-acetylhexosamine (HexNAc)
**H** – hexose (Hex)
**F** – fucose (Fuc)

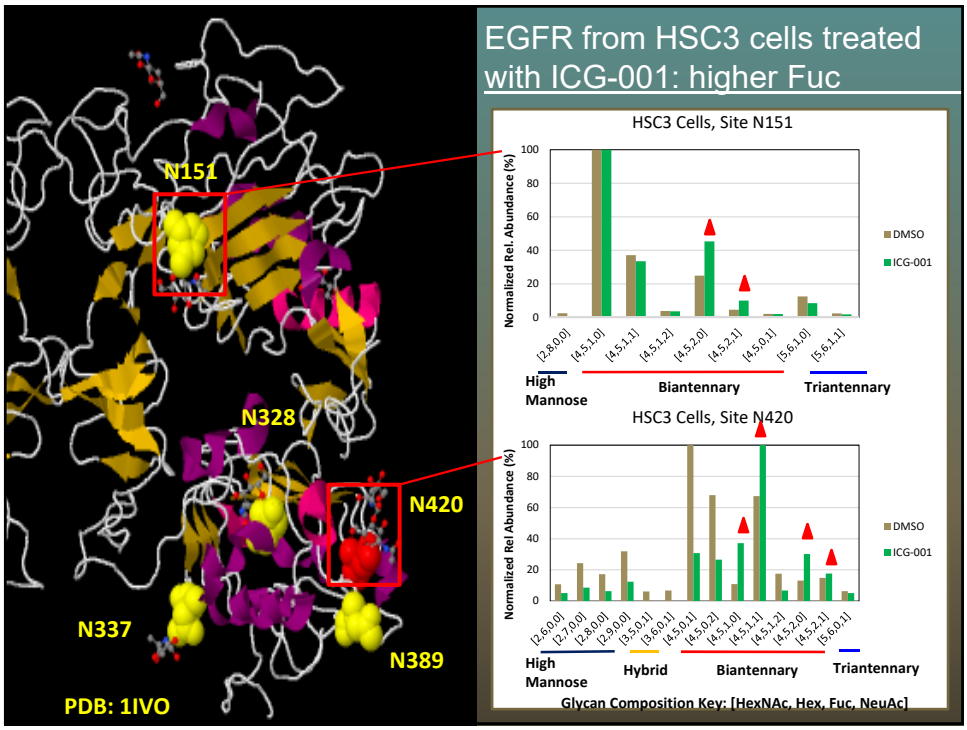
$^{142}$D I V S S D F L S N M S M D F Q N H L G S C Q K$^{165}$

$y_{13}$ $y_{10}$ $y_8$ $y_7$ $y_6$ $y_5$

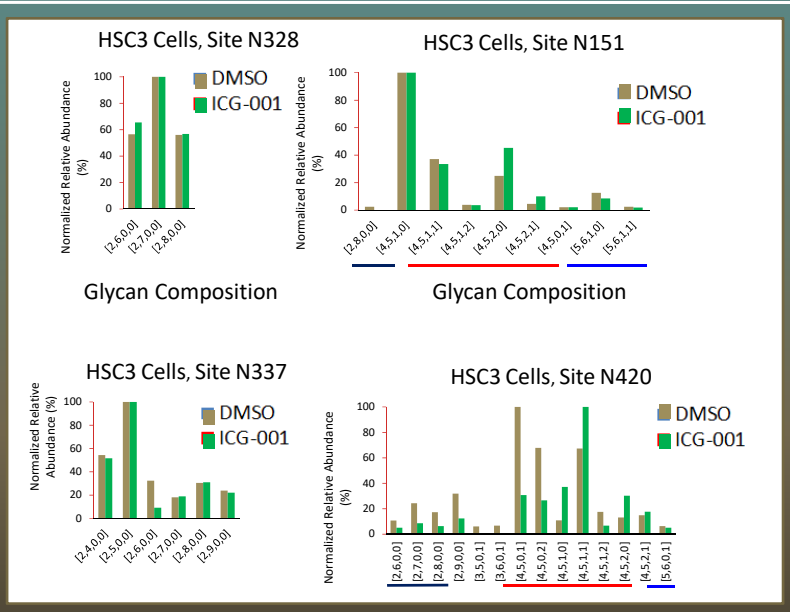
$HexNAc_4Hex_5Fuc_2$




EGFR site N420: HCD vs. HCD-Triggered EThcD
404T V K E I T G F L L I Q A W P E N R T D L H A F E N L E I I R434
HexNAc4Hex5Fuc1NeuAc1
Precursor [M+5H]5+ m/z 1146.1425
HCD
(28 V)
EThcD
Relative Abundance
m/z
intact N-glycan!

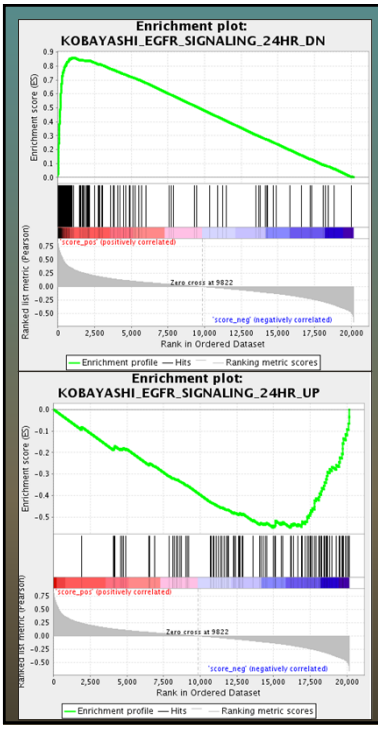

EGFR from HSC3 cells treated with ICG-001: higher Fuc
N151
N328
N420
N337
N389
PDB: 1IVO
HSC3 Cells, Site N151
HSC3 Cells, Site N420
Normalized Rel. Abundance (%)
DMSO
ICG-001
High Mannose
Hybrid
Biantennary
Triantennary
Glycan Composition Key: [HexNAc, Hex, Fuc, NeuAc]

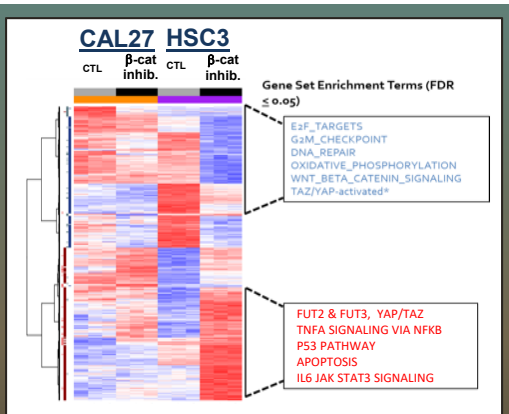
## Site-specific fucosylation modulates EGFR signaling




Glycan composition key: [HexNAc, Hex, Fuc, NeuAc]

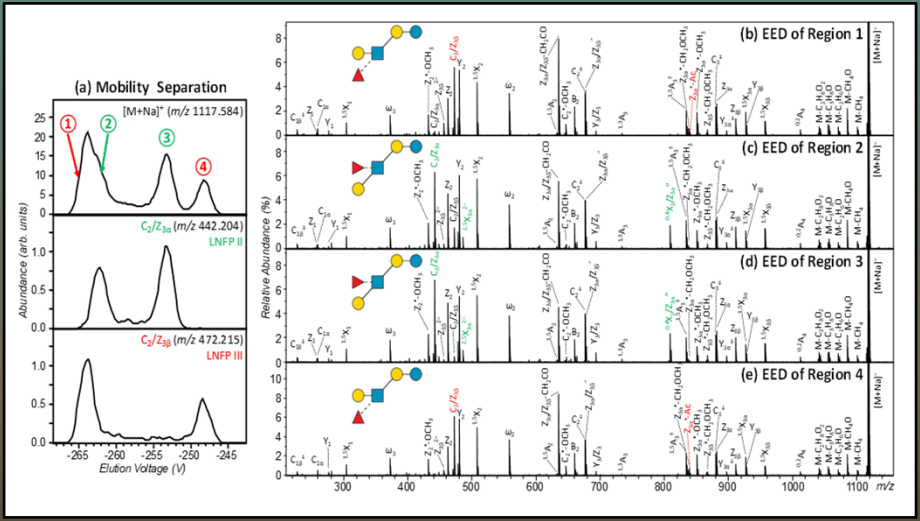
## ICG-001 inhibition positively correlates with EGFR inhibition and increased FUT expression



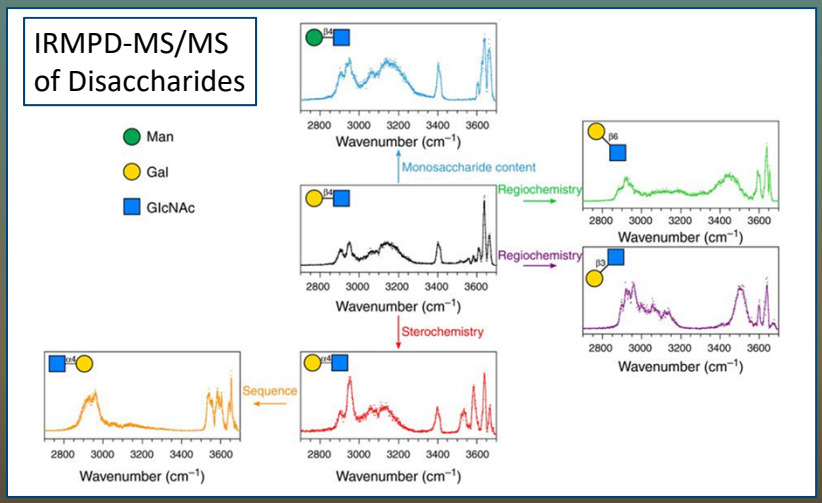

Downregulation of β-catenin/CBP activity with ICG-001 relieves FUT2/FUT3 suppression, leads to increased fucosylation of EGFR, concomitant with its diminished membrane abundance/retention.

## Gated Trapped Ion Mobility Separation with Electronic Excitation Dissociation of isomeric human milk glycans




J Wei, Y Tang, M Ridgeway, M Park, CE Costello, C Lin, *Anal Chem*, 2020, *92*, in press.

## Determination of monosaccharides and glycosidic linkages by Infrared Multiple Photon Dissociation Ion Spectroscopy



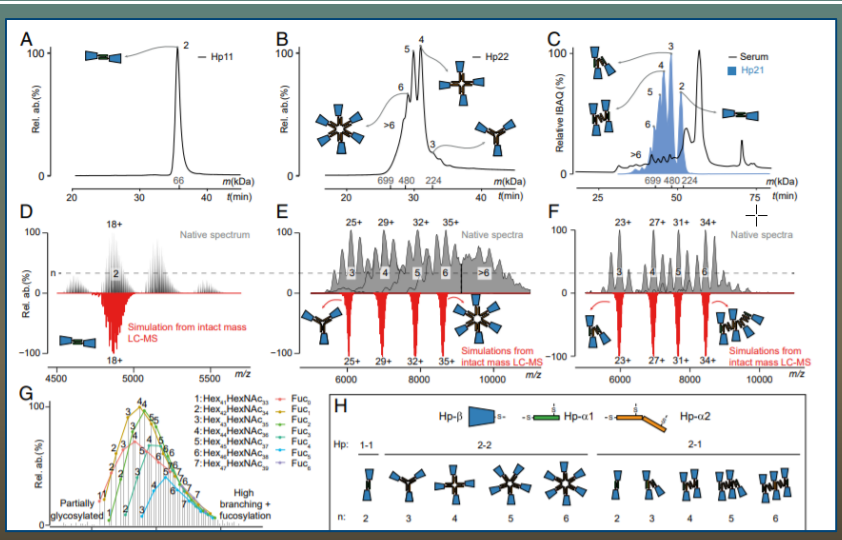

B Schindler, L Barnes, G Renois, C Gray, S Chambert, S Fort, S Flitsch, C Loison, AR Allouche, I Compagnon. *Nat Commun,* 2017, *8*, 973, 1-7

## Emergent Methods Complement or Improve MS Analysis

- Online Separations
  - Liquid chromatography
  - Electrophoresis
  - Ion Mobility
- 2-D Desorption
- Dissociation Modes
  - HCD, ExD, IRMPD
  - IR action spectroscopy
- Top-down fragmentation

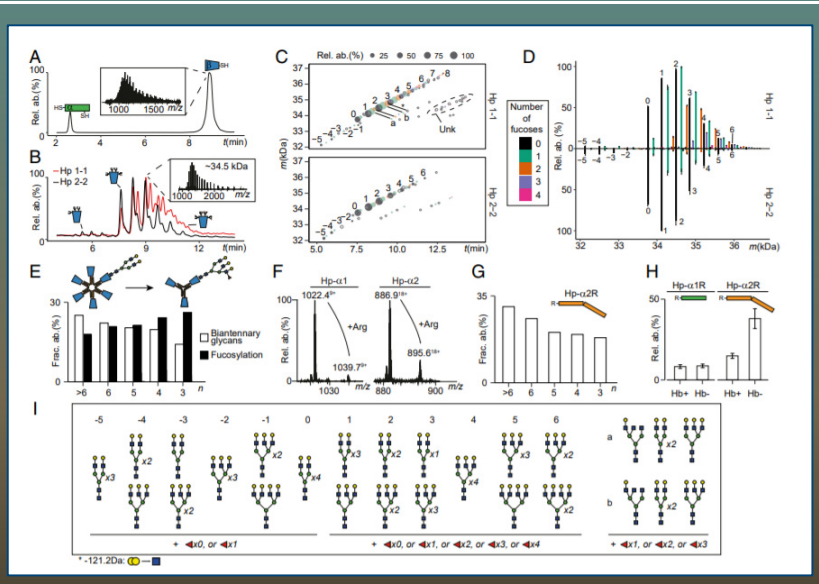
Key West 12 31 19

## Native MS of Glycoproteins: Determinatin of haptoglobin proteoforms and proteoform assemblies in human serum that vary in potential to scavenge hemoglobin



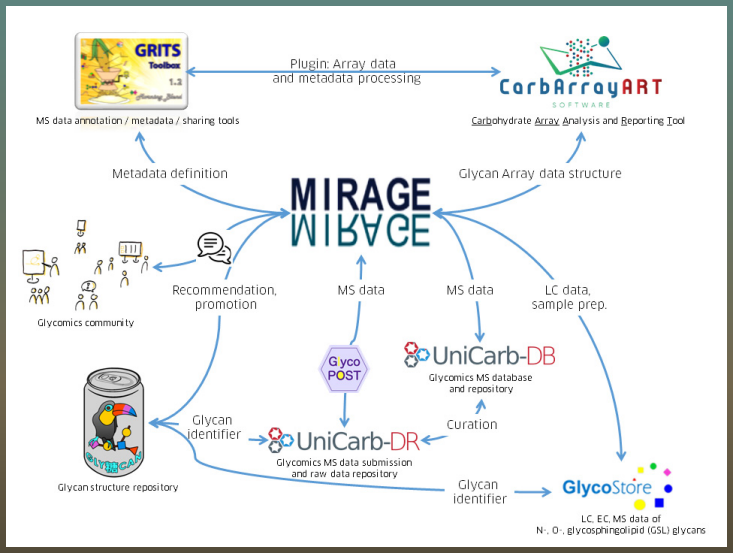

T Sem, F Vojtech, AJR Heck. *Proc Natl Acad Sci* USA, 2020, *117*, 15554-15564.

## LC-MS of native assemblies and individual glycoforms of haptoglobin in human serum



T Sem, F Vojtech, AJR Heck. *Proc Natl Acad Sci* USA, 2020, *117*, 15554-15564.

## Shared resources for MS-based glycoanalysis




https://www.beilstein-institut.de/en/projects/mirage/

## Progress & potential in MS-based methods for clinical analyses of glycans and glycoconjugates

- Progress:
  - Combinations of MS-based characterization with on- and off-line separations, IMS +/- ExD dissociation
  - Judicious choice of ion source and mass analyzer operating conditions.
  - Implementation of multiple techniques, development of new protocols.
- Emergent possibilities:
  - Online CE-MS/MS and IM-MS/MS are becoming user-friendly.
  - nLC-ExD-MS is appropriate for QTOF MS, IM-QTOF MS and FT-MS systems (both FT-ICR and Orbitrap).
  - High throughput automation, advanced data handling, modelling improve efficiency and extract additional information.

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P Hong, Brandeis U
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M Kukuruzinska, BU GSDM

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C Schwartzer
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***Bruker***

M Park
M Ridgeway

***OSU/e-MSion***

D Barofsky
J Beckman
V Voinov

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Slide 1, The Hermitage Museum, Filial Piety (The Paralytic), J-B Greuze, 1725-1805

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