Biomarker detection: from total serum to tissue specific N-glycan analysis

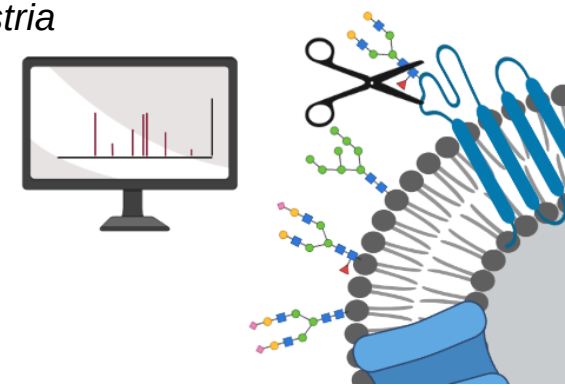
Davide Ret[a,b*], Alessio Gentile[a,b], Daniel García de Otazo Hernández[a,b], Lisa Ying [a], Veronica Monti[a], Haidi Jakovic, Philipp Gritsch[a], Arvand Haschemi[c], Eva Untersmayr[b]

[a] *Institute of Applied Synthetic Chemistry, TU Wien, 1060 Vienna, Austria,*

[b] *Institute of Pathophysiology and Allergy Research, Medical University of Vienna, 1090 Vienna, Austria*

[c] *Department of Laboratory, Medicine Medical University of Vienna, 1090 Vienna, Austria*

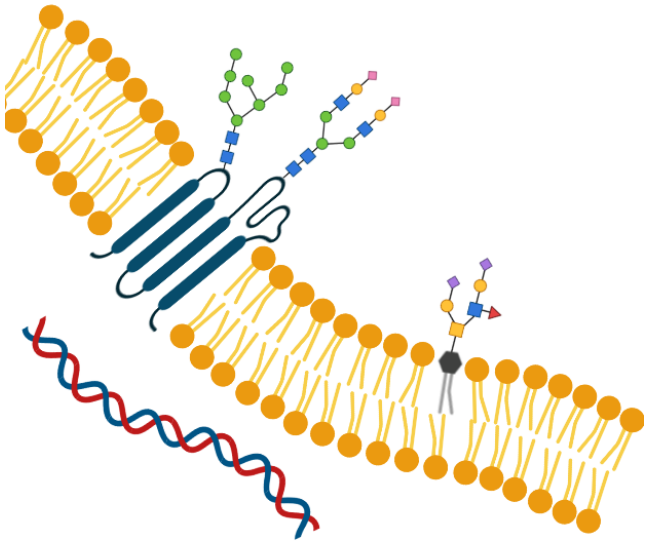
davide.ret@tuwien.ac.at

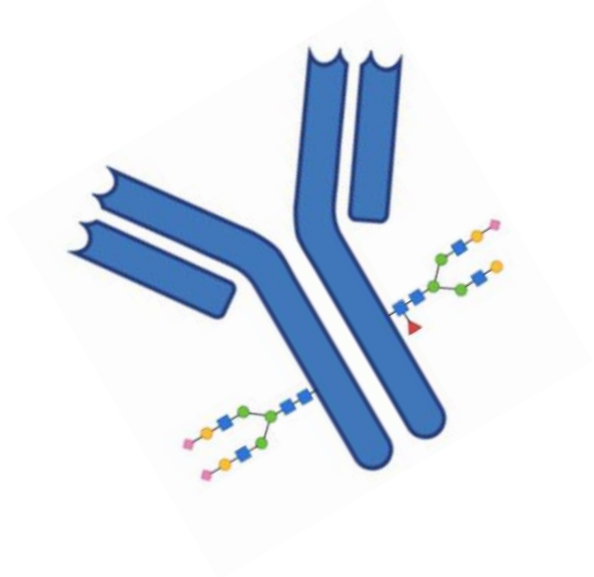


Glycans

key components in most **biological** and **pathological** processes:

- antigen recognition
- inflammation
- cytotoxicity
-cancer
- viral and bacterial infections.





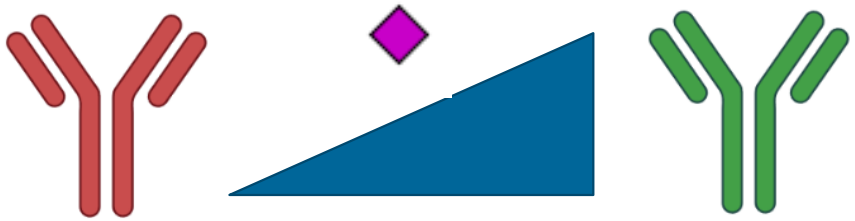
glycosylation:

Biological age

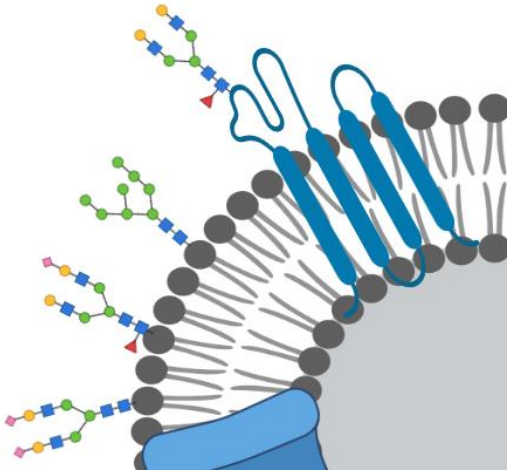
glycan clock

receptors
(glycan-lectins)

inflammatory
regulators



species and
tissue specificity

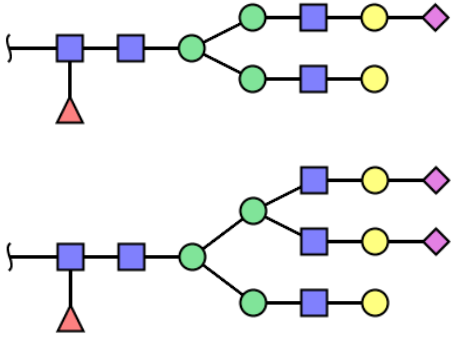


types of glycosylation:

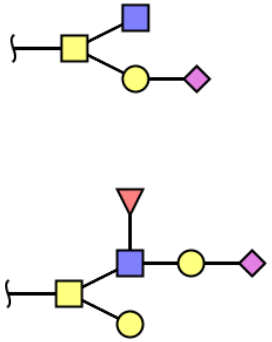
Sugar code:

- N-acetylglucosamine
- mannose
- galactose
- N-acetylneuraminic acid
- N-glycolylneuraminic acid
- fucose

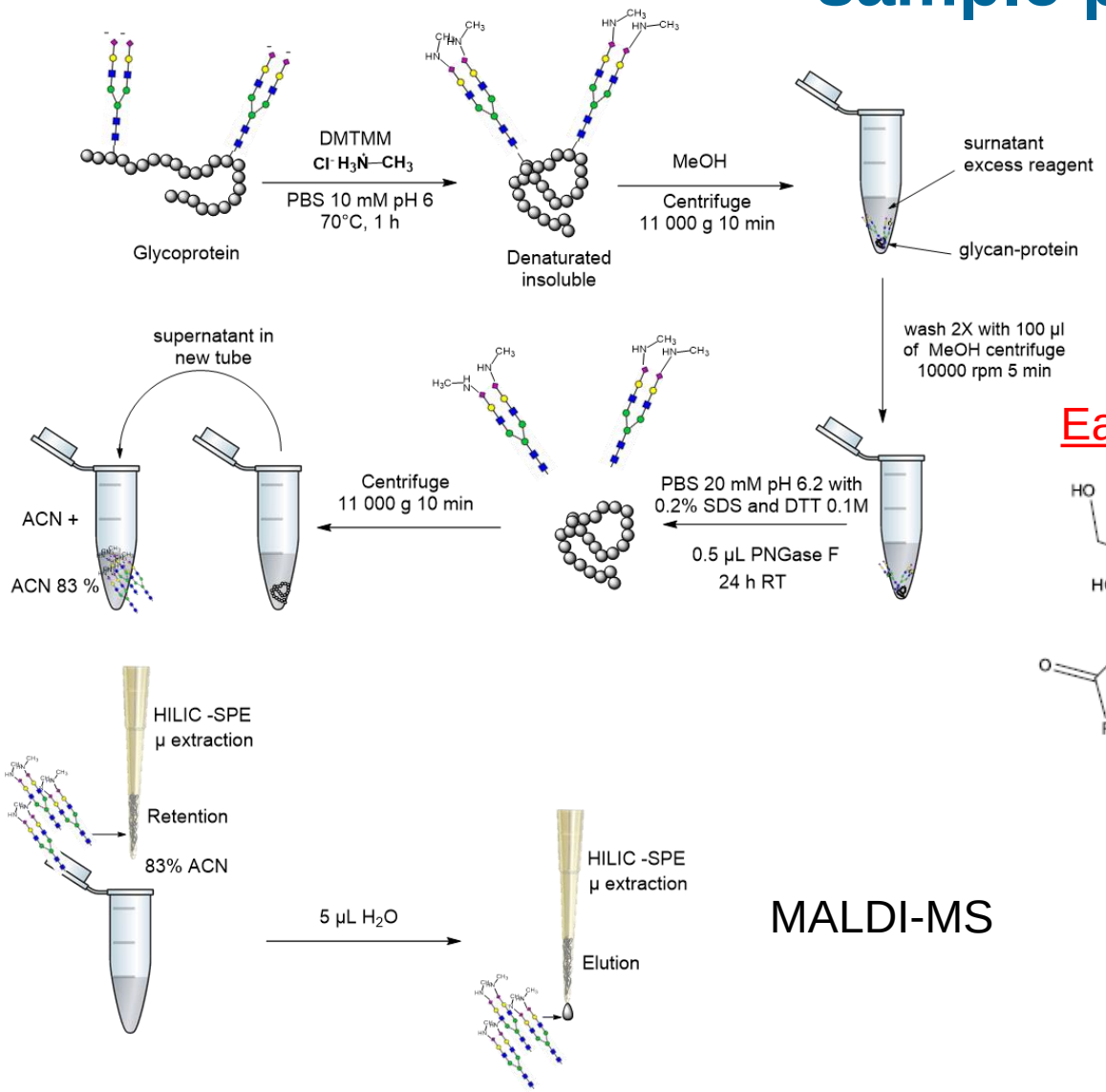
N-Glycan



O-Glycan



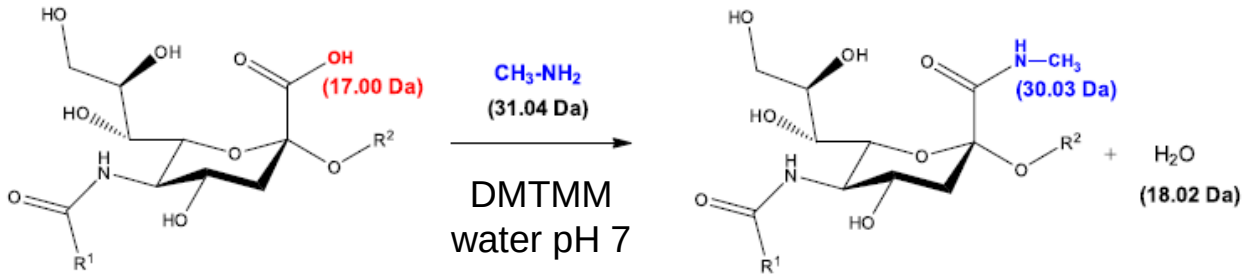
N-Glycan analysis: sample preparation

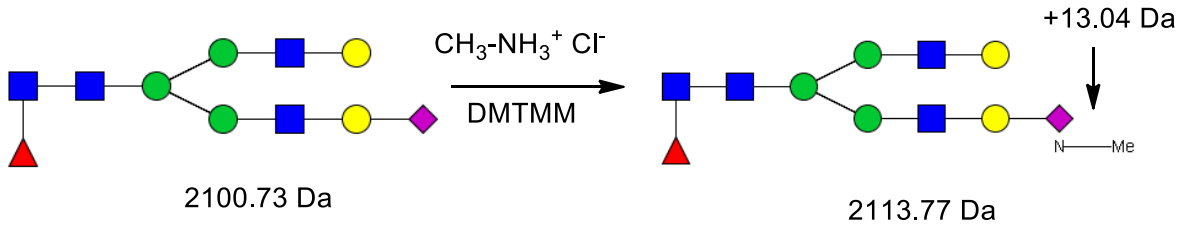


MALDI-MS

The stabilization of sialic acid by DMTMM-mediated methylamidation enables a conservation of the glycan structures, in contrast to other methods where the labile sialic acids are partially lost (e.g. methylation)

Each method highlight different aspects = difficult comparison

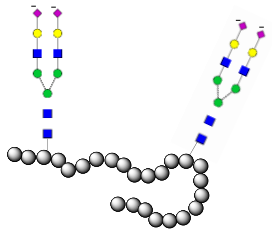




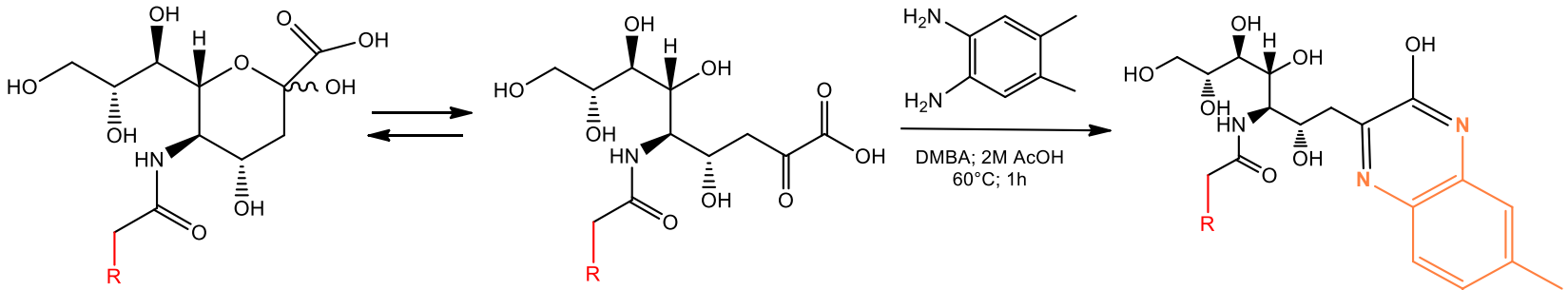
1. Ret D., et al., *DMTMM-mediated methylamidation for MALDI mass spectrometry analysis of N-glycans with structurally conserved sialic acid residues in biological fluids "via direttissima".* Talanta, 2022. **242**: p. 123326

Sialic acid quantification
DMBA HPLC-FL detection

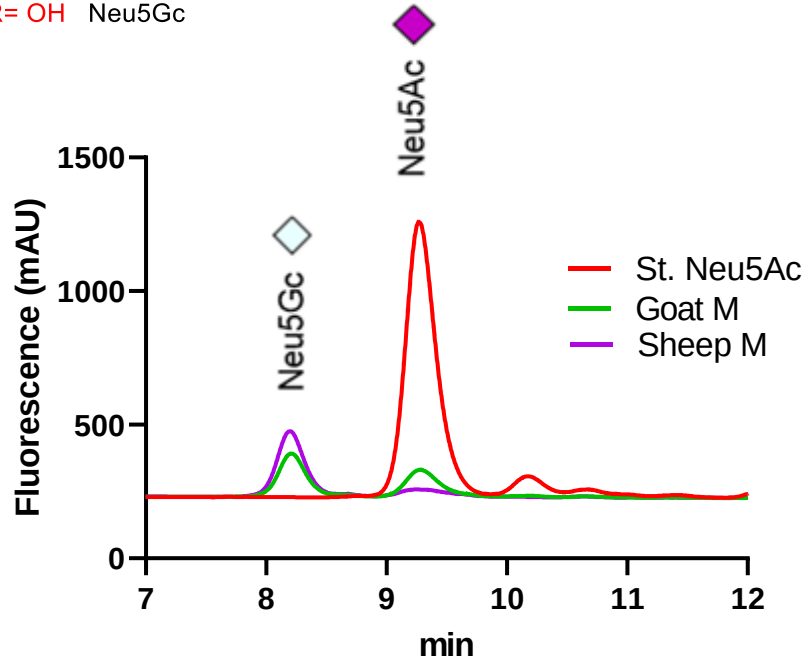
Glycoprotein



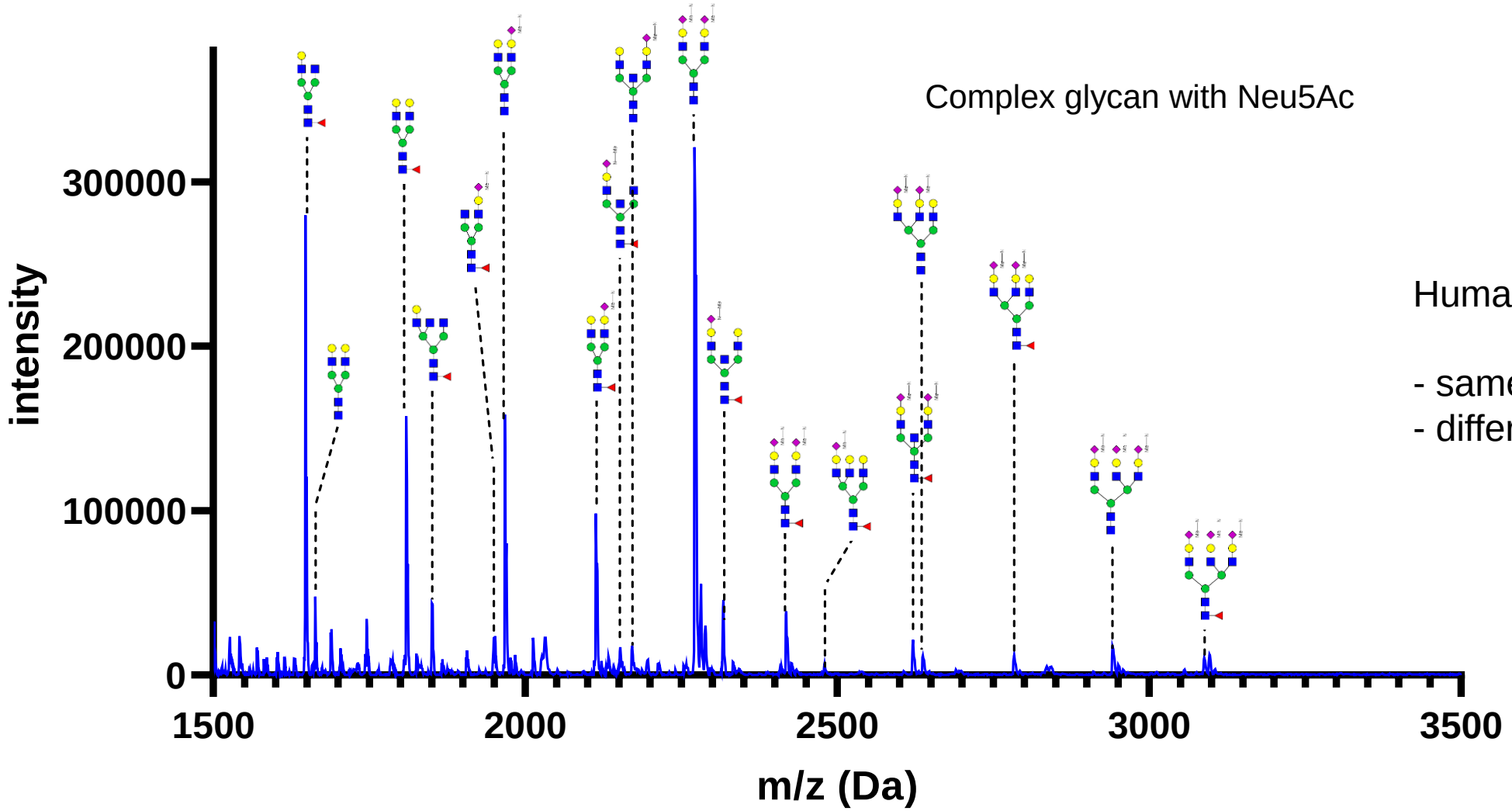
DMBA label
HPLC-FL



R = H Neu5Ac
R= OH Neu5Gc



Human total serum N-Glycans



Human Serum N-glycans

- same glycans pattern
- different amount

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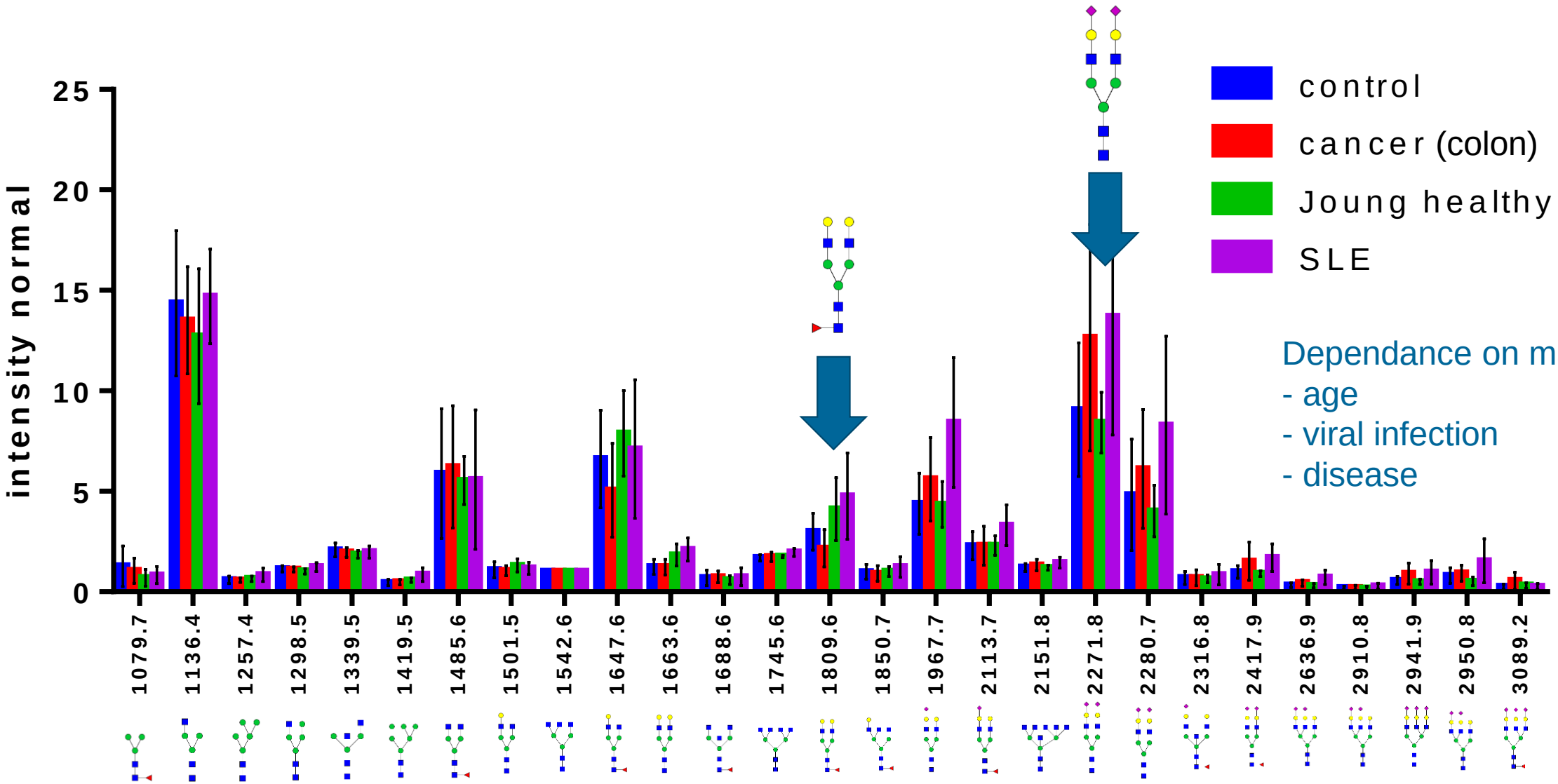
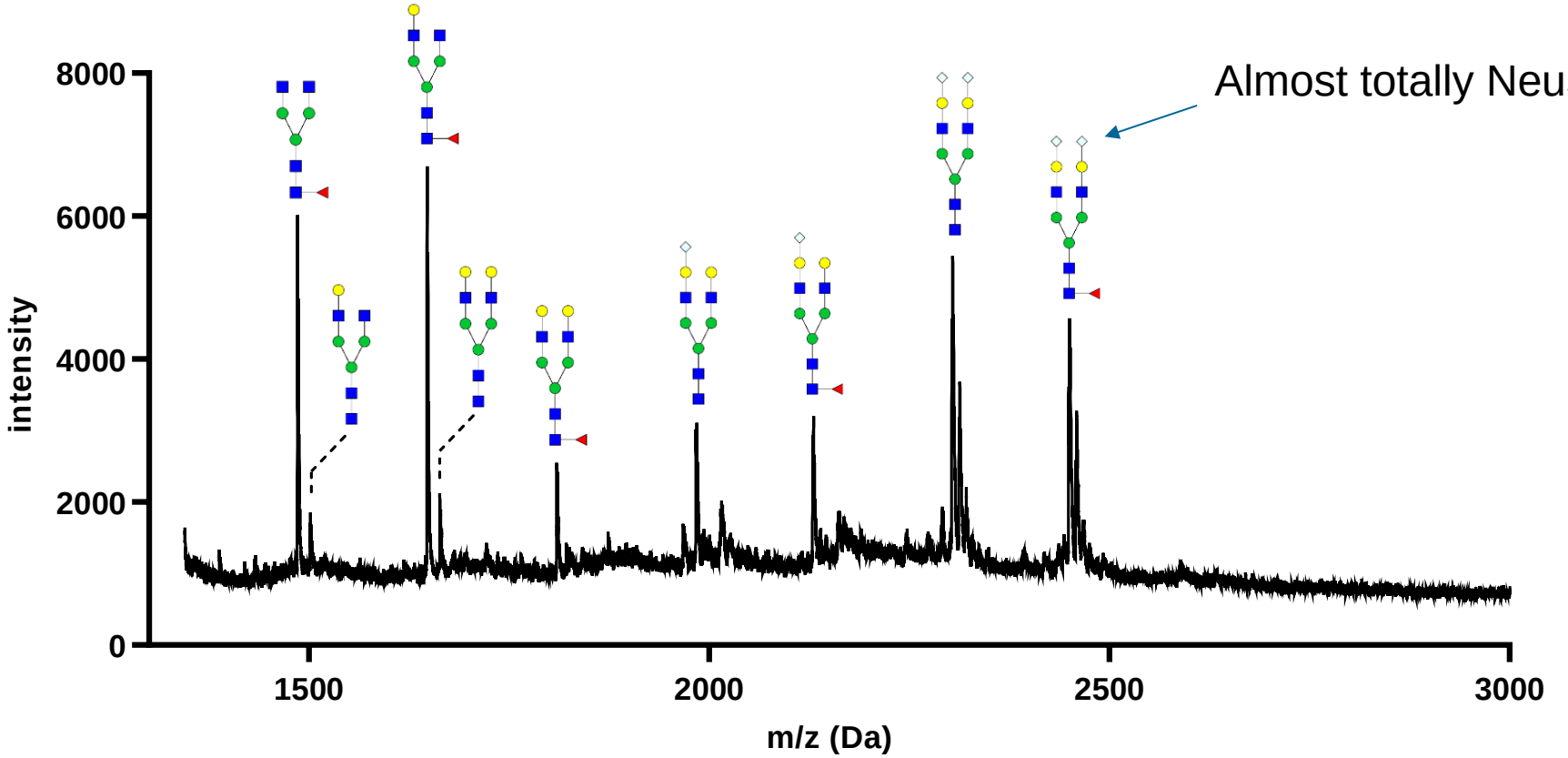
Serum N-glycan: Cancer, Systemic Lupus Erythematosus (SLE)



Dependance on many factors:
- age
- viral infection
- disease

Mouse total serum N-Glycans



Almost totally Neu5Gc (> 95%)

➔ Mouse not good model for study human disease
(not used for human virus study)

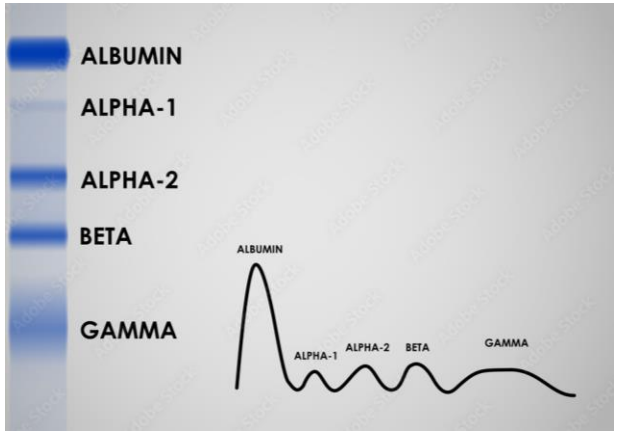
N-glycan analysis

From: total serum

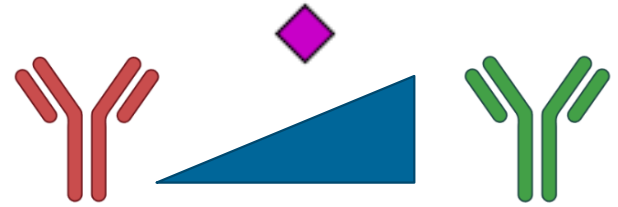
We observed limited, but disease-specific variations between

N-glycosylation of sera of patients with different pathologies.

to tissue specific / fraction specific



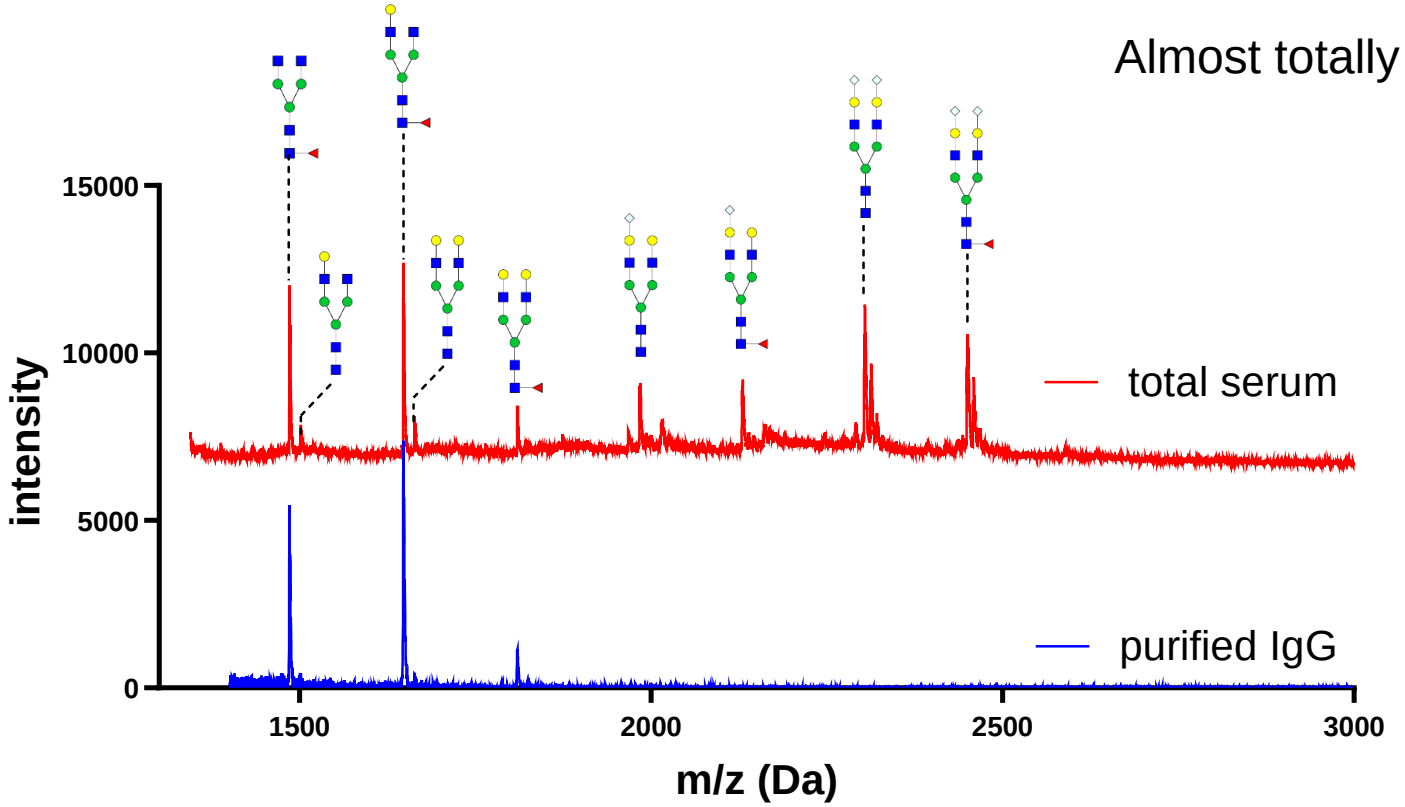
Pro inflammatory

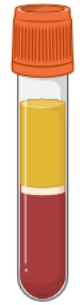


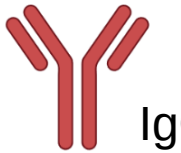
Anti inflammatory

Mouse serum total and IgG fraction N-Glycans

Almost totally Neu5Gc (> 95%)



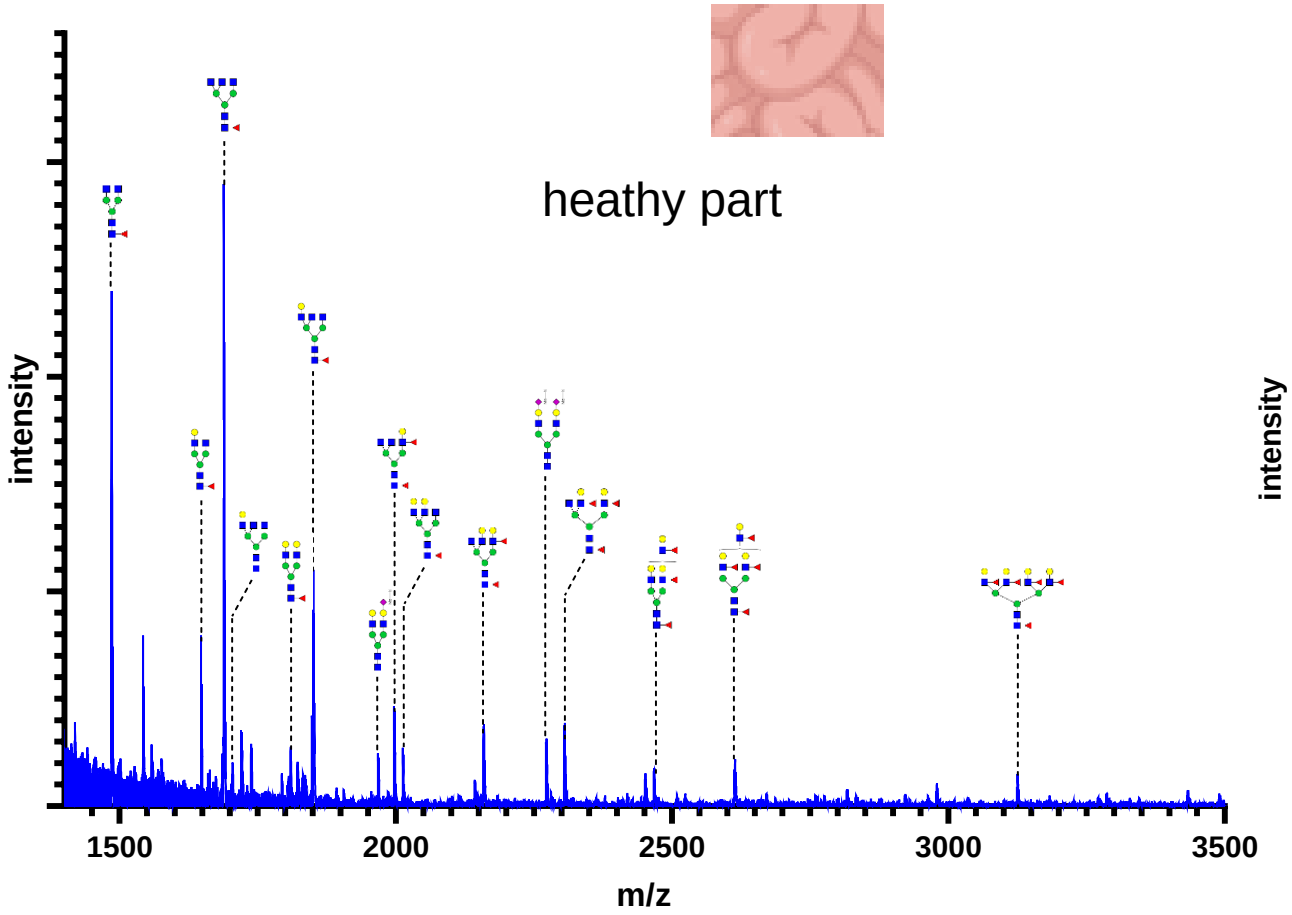


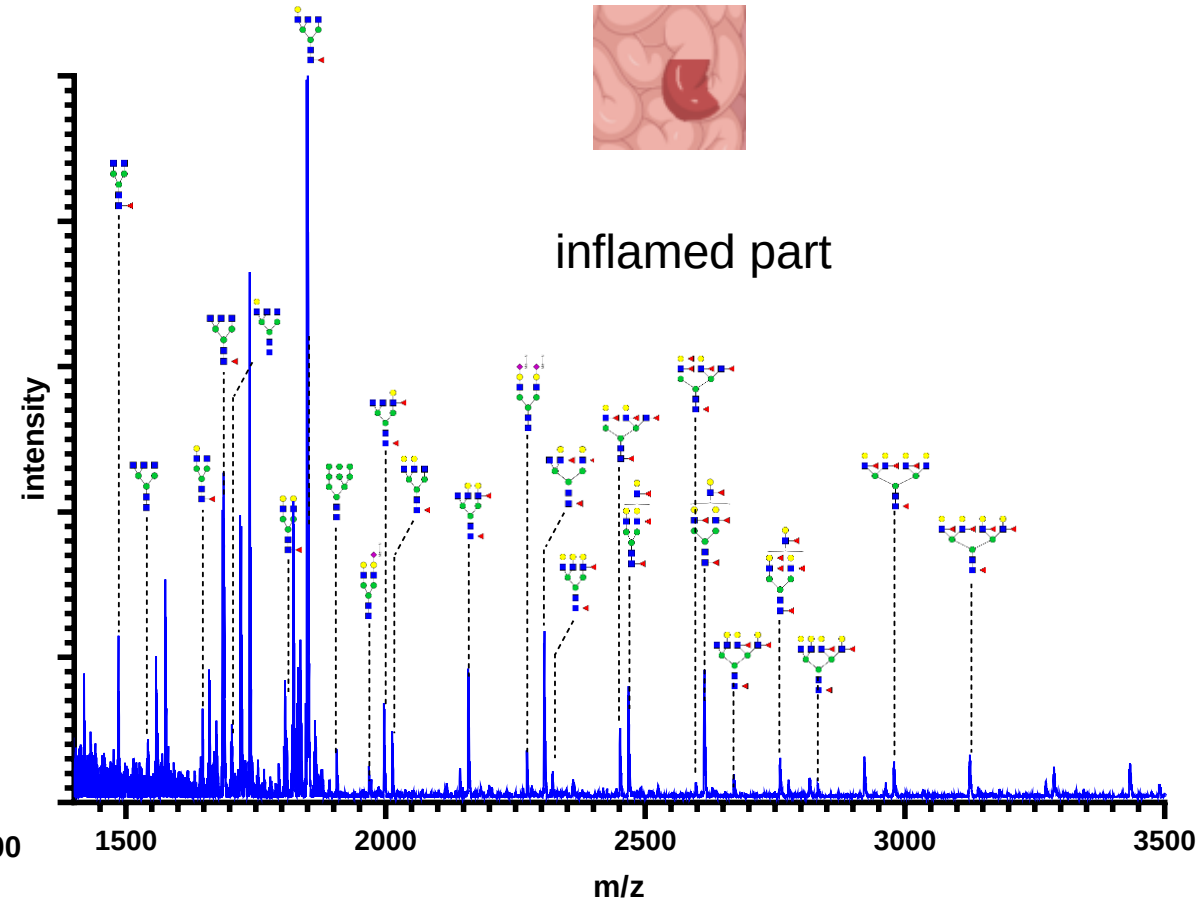


IgG

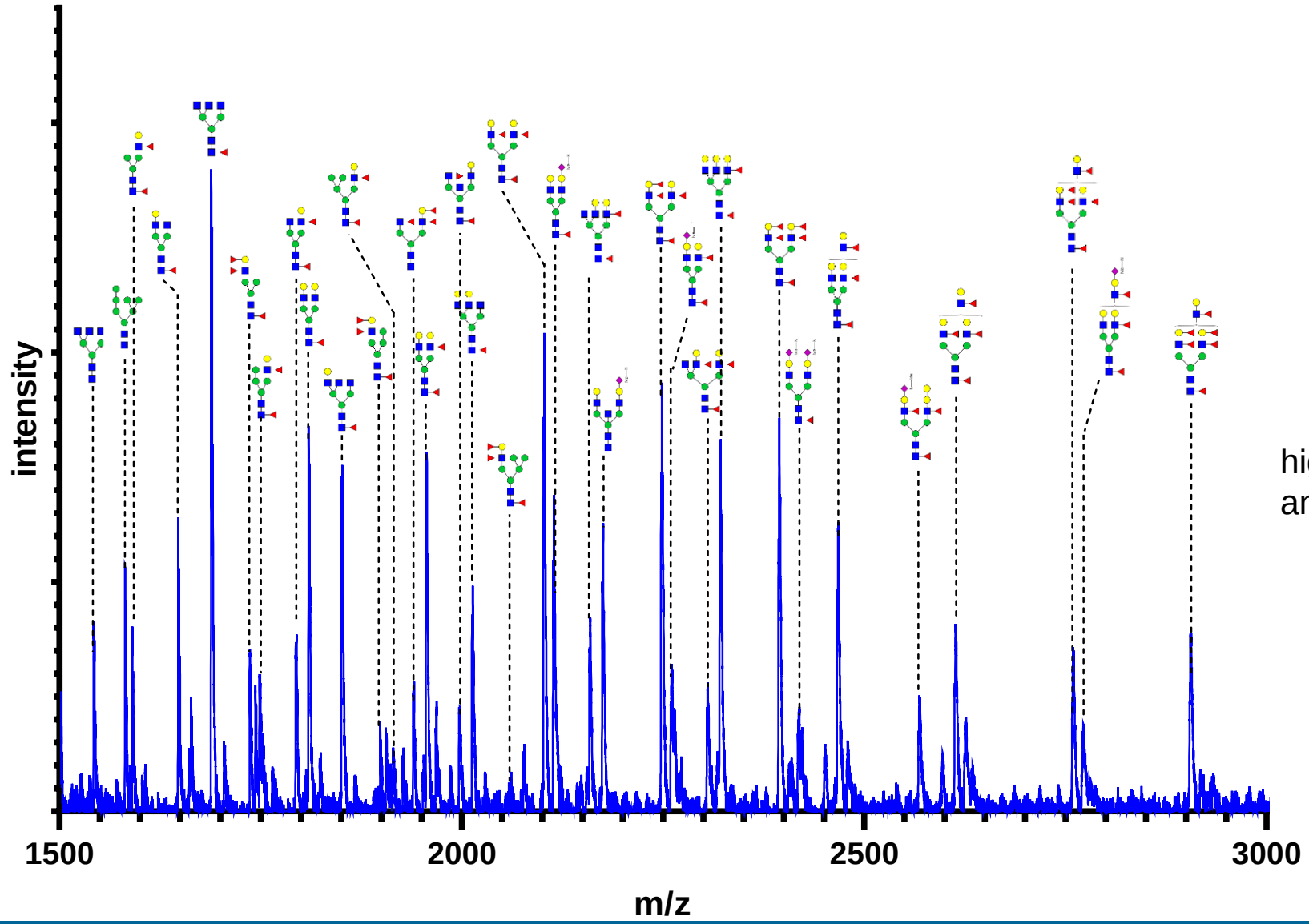
➔ Mouse not good model for study human disease
(e.g. not used for human virus study)

Human intestine tissue N-glycans



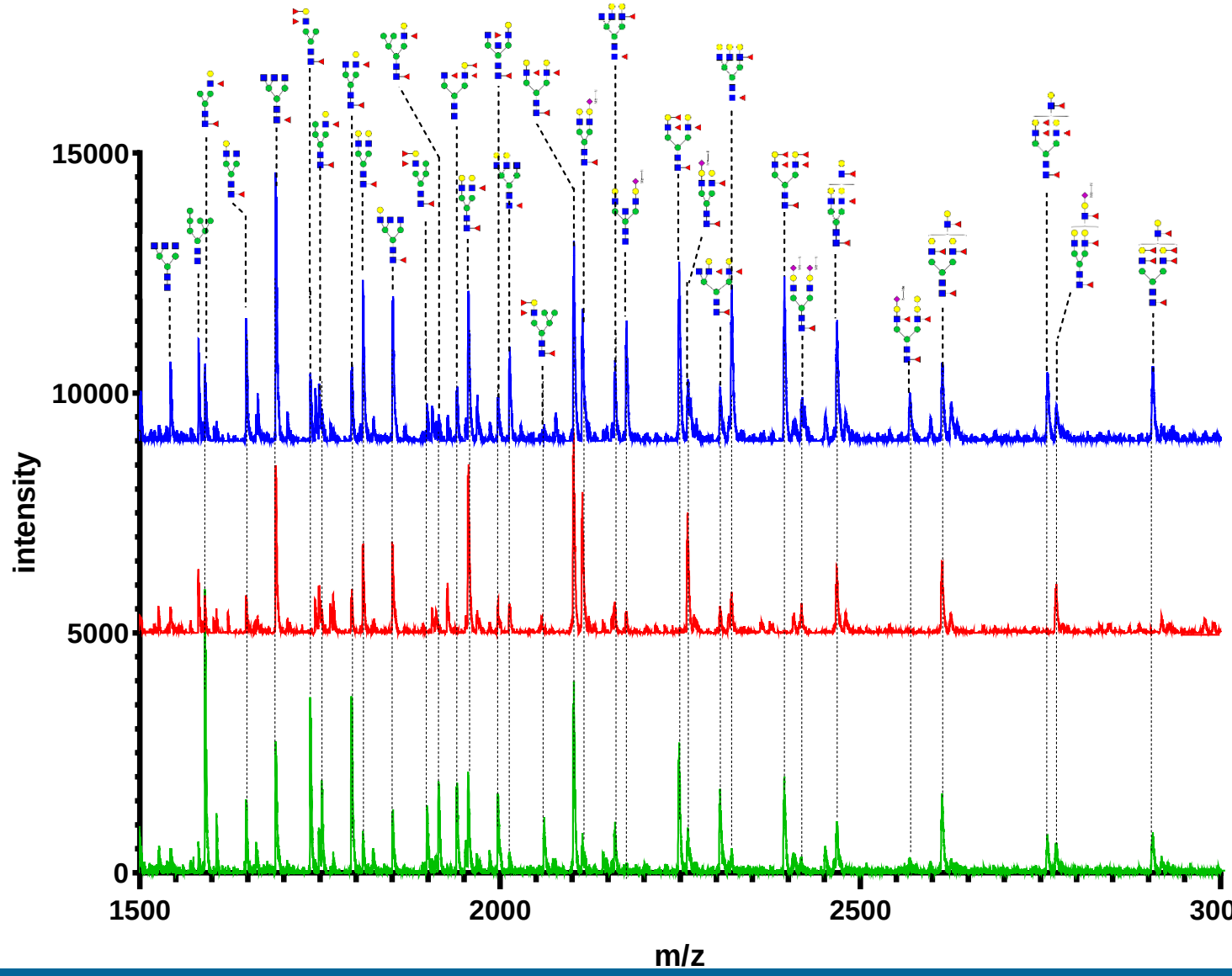


Human saliva N-glycans

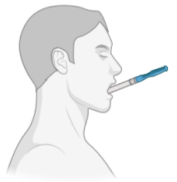


high fucosylation
antennary galactosylation

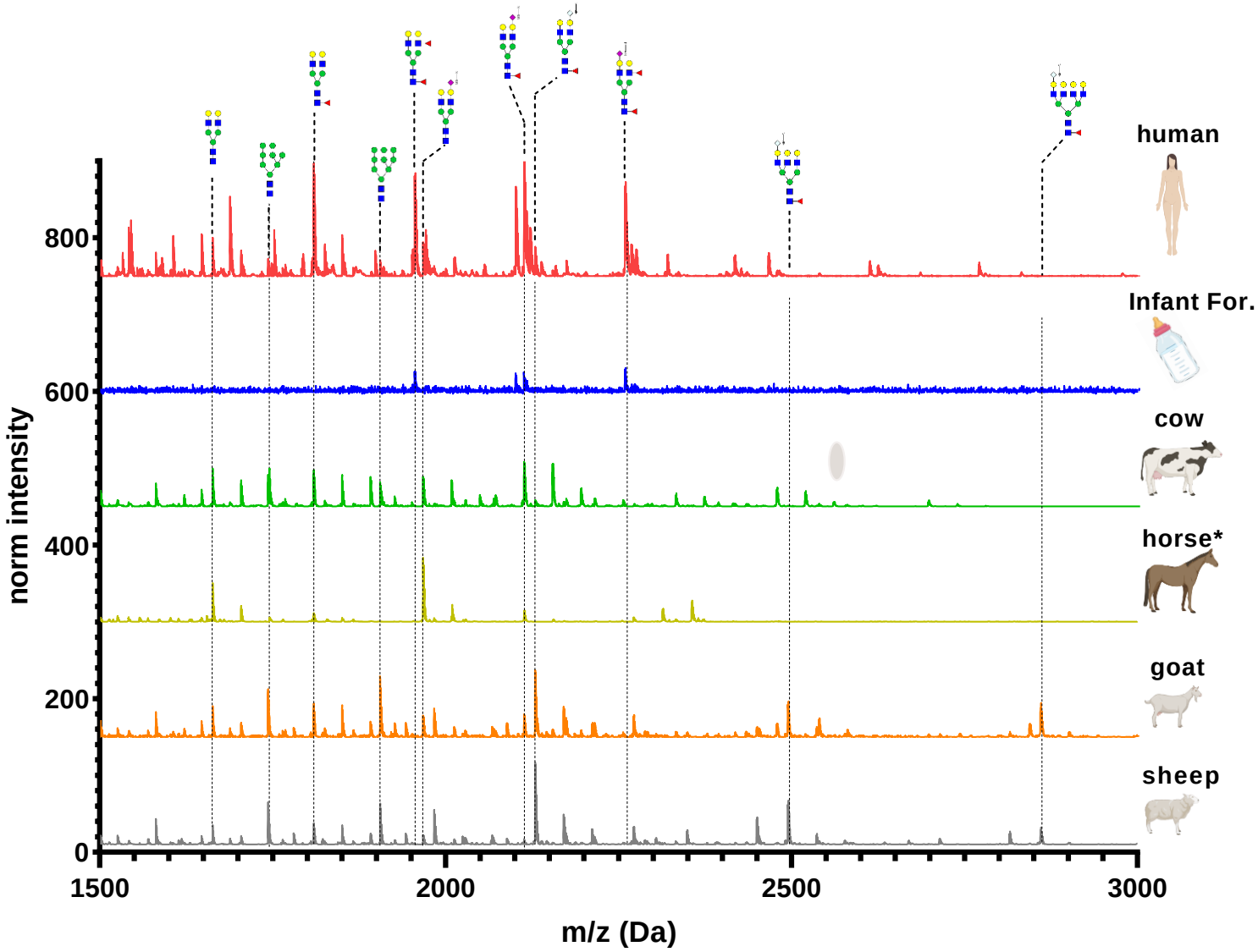
Human saliva N-glycans



Different viral adhesion



MALDI-MS spectra of milk of different species



Variability in the species

Variability between the species

Conclusion

total serum N-glycan analysis

Species dependent -> helpful for understand difference between animals / or animals model

We observed **limited, but disease-specific variations** between

N-glycosylation of sera of patients with different pathologies. (peak intensity)

tissue specific / fraction specific

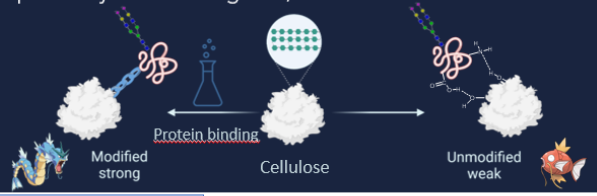
biopsies, body fluid, antibody fraction, cell tissue show a **greater N-glycan pattern diversity** between different patients and patients with different pathologies

-> best candidate for biomarker

Covalent protein immobilization on modified cellulose for N-glycan purification

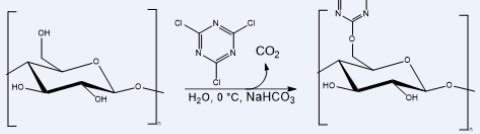
Haidi Jakovic[a], Salvatore Alessio Gentile[a,b], Daniel García de Otazo Hernández[a,b], Lisa Ying[a], Eva Untersmayr[b], Davide Ret[a,b]

Proteins are usually physically adsorbed onto supports, however they can spontaneously undergo to desorption. By considering this, here is described a **covalent immobilization method.**



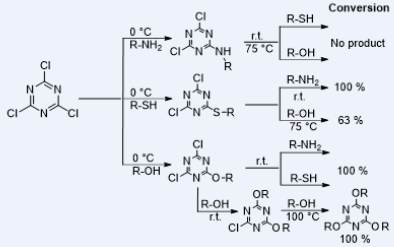
• Step 1: Modify

Cellulose functionalization with ***trichloro triazine*** for ***covalent immobilization*** of ***proteins*** and purification of ***N-glycans***.



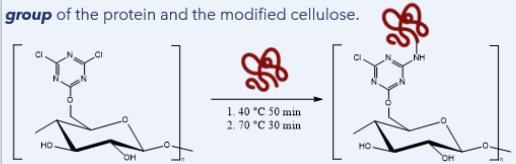
Substitution of chlorine atoms of trichloro triazine is possible at ***different temperatures.***

Below: Temperature of reactions for subsequent substitution of chlorine atoms of trichloro triazine by different nucleophiles [2].

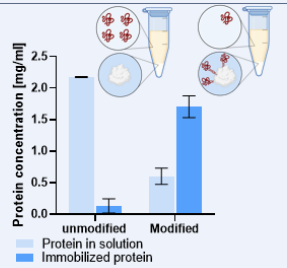


• Step 2: Immobilize

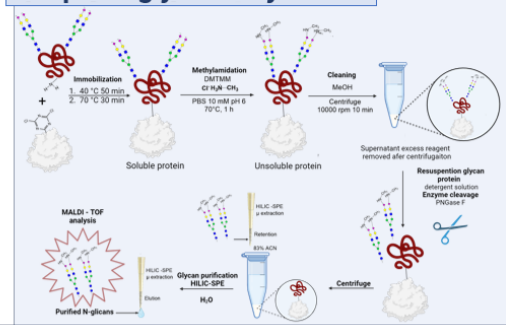
Protein immobilization *via* ***covalent bond*** between the ***ammine group*** of the protein and the modified cellulose.

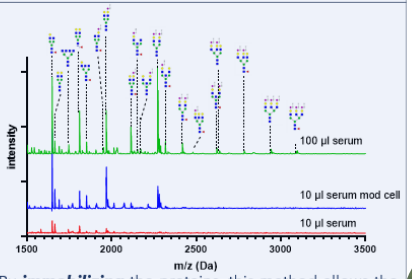


The degree of modification and the temperature of storage of cellulose are of key importance in the immobilization of the protein.



• Step 3: N-glycan analysis





By ***immobilizing*** the proteins, this method allows the analysis and quantification of glycans in ***low amounts of proteins*** (e.g. antibodies).

a) Research Unit Macromolecular Chemistry, Institute of Applied Synthetic Chemistry, TU Wien
b) Institute of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna

Contact information:
haidi.jakovic@tuwien.ac.at
davide.ret@tuwien.ac.at

References:
[1] Blotny G. *Tetrahedron* **2006**, *62* (41), 9507-9522.
[2] Sharma et al. *Front. Chem.* **2018**, *6*.
[3] Ret et al. *Talanta* **2022**, *242*:123326.

Edible Bird's Nest: N-Glycosylation pattern and quantitative analysis of Sialic Acid

Lisa Ying [a], Alessio Salvatore Gentile [b], Daniel Garcia de Otazo Hernandez [a], Haidi Jakovic [a], Eva Untersmayr [b], Philipp Gritsch [c] and Davide Ret [a]

BACKGROUND

In the Chinese community Edible Bird's Nest (EBN) has long been celebrated because of its nutritious properties. It contains a high amount of sialic acid among its various bioactive components. Sialic Acid is known for its nutritious values and has been widely researched. Its immune enhancing, anti-cancer as well as skin whitening properties are well acknowledged. For this reasons Sialic Acid is a promising functional ingredient in various fields. [1]

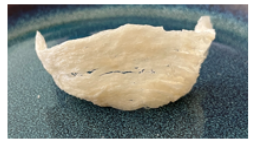


Figure 1: Edible Bird's Nest

Our aims are the quantification of total sialic acid amount in Edible Bird's Nest through HPLC-FL and the analysis of N-Glycosylation pattern with MALDI-ToF.

N-GLYCOSYLATION PATTERN

Method:

1. Delipidation with Hexane and Isopropanol
2. Modification with methylamine using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMTMM) and methylamine hydrochloride

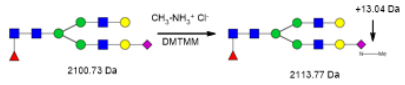


Figure 2: Reaction scheme of Methylamidation of sialic acid.

3. N-Glycan release by PNGase-F
4. Purification by HILIC solid phase extraction
5. Analysis of N-Glycans with MALDI-ToF/MS [2]

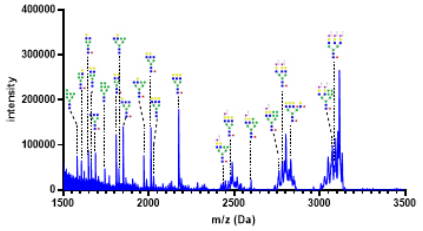


Figure 3: MALDI-ToF/MS spectra of the methylamidated N-glycan profile of EBN.

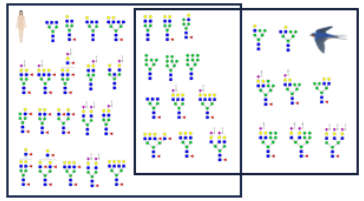


Figure 4: Venn plot of principal N-glycans in EBN compared to Human serum. High amount of hybrid type glycans are found in EBN.

QUANTIFICATION OF SIALIC ACID

Method:

1. Cleavage of sialic acid from glycoconjugate using concentrated CH_3COOH
2. Derivatization of sialic acid with 4,5-Dimethyl-1,2-Phenylendiamin (DMBA) and formation of fluorescent quinoxaline derivatives

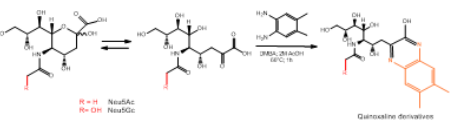


Figure 5: Reaction scheme of sialic acid with DMBA.

3. Analysis with HPLC-FL

Results:

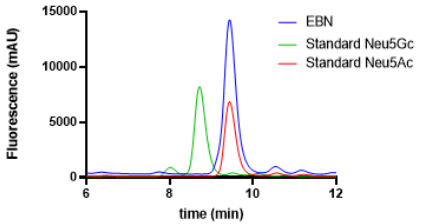


Figure 6: HPLC-Chromatograms of DMBA-tagged sialic acid. In EBN no Neu5Gc could be detected.

Table 1: The amount of Neu5Ac in EBN compared to cow milk.

sample	Neu5Ac	SD	unit
Cow milk	0.349	0.048	mg/mL
EBN	85.6	7.605	mg/g
One glass of cow milk (300 mL)	104.7	14.4	mg
One Leaf of EBN (6 g)	513.5	45.6	mg

References:
[1] Dai, Y. et al. (2021) 'A comprehensive review of Edible Bird's nest', Food Research International, 140, p. 109875.
[2] Ret, D. et al. (2022) 'DMTMM-mediated methylamidation for MALDI mass spectrometry analysis of N-glycans with structurally conserved sialic acid residues in biological fluids: "via direttissima"', Talanta, 242, p. 123326.

a) Research Unit Macromolecular Chemistry, Institute of Applied Synthetic Chemistry, TU Wien
b) Institute of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna
c) Research Unit Stereoselective and sustainable synthesis, Institute of Applied Synthetic Chemistry, TU Wien

Contact information:
lisa.ying@tuwien.ac.at
davide.ret@tuwien.ac.at

A robust quantification method of sialic acids and AGES precursors

Daniel García de Otazo Hernández[ab], Alessio Gentile[b], Lisa Ying[a], Haidi Jakovic[a], Eva Untersmayr[b], Davide Ret[ab]

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Quinoxaline derivate stabilization allows for the reproducible quantification of diketone biomolecules present in raw and cooked foods.

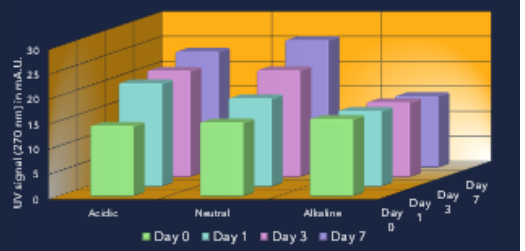


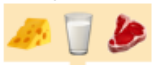
Fig.1: Neu5Ac + DMBA stability study (Quinoxaline derivate peak)

What are diketone biomolecules?

Diketone biomolecules are substances containing two vicinal ketone functional groups. They are commonly found in neuraminic acids (Neu5Ac & Neu5Gc) as well as AGEs precursors (Glyoxal & Methylglyoxal). These diketone biomolecules are of special interest due to their negative impact on digestion in humans [1].

Sialic acids

- Neu5Gc is exogenous in humans
- Foods of animal origin (raw and processed) contain it
- Examples:



General diketone functional group

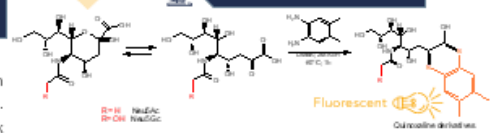
AGEs precursors

- Advanced Glycation End products
- Polymerize during cooking and form AGEs
- Examples:

How do we quantify them?

Starting from an organic matrix, sialic acids are first cleaved from the glycoprotein and then derivatized yielding a quinoxaline. AGEs precursors are extracted dependent on the sample matrix and derivatized the same way. The fluorescent quinoxaline-labelled analyte can now be easily quantified via HPLC-FL.

Reaction scheme



1. Extraction 2. Derivatization 3. Detection

Why is stabilizing necessary?

Previous analysis protocols suffered from a change in signal intensity and peak width when the quinoxaline derivate started to degrade over time. We have discovered that the derivate is stabilized by shifting to an alkaline pH after the reaction concludes. This way all possible side reactions are inhibited.

Before stabilization

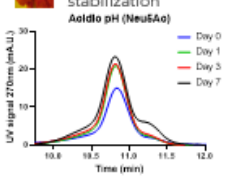


Fig. 2: Change of signal intensity over time in acidic conditions

After stabilization

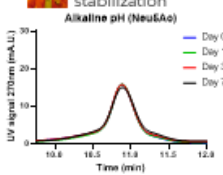
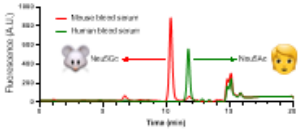


Fig. 3: Change of signal intensity over time in alkaline conditions

Exemplary data:



	Human	Mouse
Neu5Ac (mg/mL)	0.39	0.03
Neu5Gc (mg/mL)	-	0.63

Tab. 1: Sialic acid concentration in serum samples.

Fig. 4: Measurement of sialic acids in human and mouse serum.

a) Research Unit Macromolecular Chemistry, Institute of Applied Synthetic Chemistry, TU Wien
b) Institute of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna
Contact information: daniel.garcia@tuwien.ac.at, @danielgdeoh, davide.ret@tuwien.ac.at

References: [1] Varki, A. (2008). Sialic acids in human health and disease. Trends in molecular medicine, 14(8), 351-360.

MEDIZINISCHE UNIVERSITÄT WIEN

Gastrointestinal Immunology Research Group

TECHNISCHE UNIVERSITÄT WIEN

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AUSTRO CARB NET

Comparative N-Glycosylation Analysis in Human Serum: Insights into COVID-19, Long COVID, and ME/CFS

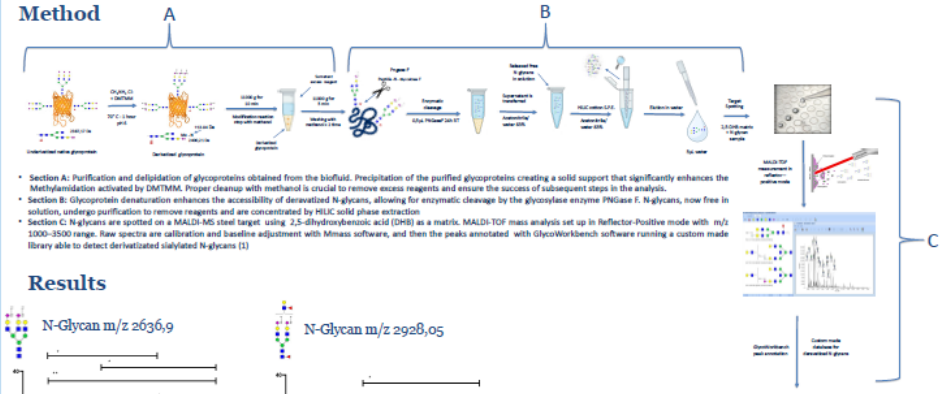
Davide Ret[ab], Salvatore Alessio Gentile[ab], Johanna Rohrhofer[b], Larissa Koidl[b], Daniel García de Otazo Hernández[a], Lisa Ying[a], Haidi Jakovic[a], Simone Knaus[a], Eva Untersmayr[b]

[a] Research Unit Macromolecular Chemistry, Institute of Applied Synthetic Chemistry, TU Wien, 1060 Vienna, Austria,
[b] Institute of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, 1090 Vienna, Austria

Why N-Glycosylation?

Evaluation of N-glycosylation pattern is gaining interest in immunology as it plays a crucial role in the structure and function of molecules relevant for the immune system. N-glycans can modulate the activity and specificity of antibodies, the complement system as well as pathogen-host interactions influencing the ability of antigens recognition and of eliciting an immune response. Alterations in N-glycan structures have implications in a large variety of diseases such as rheumatoid arthritis, cancers and viral infection. Thus, a comprehensive picture of the N-glycosylation profile is pivotal for better understanding of mechanisms associated with poorly understood disorders such as post-viral fatigue.

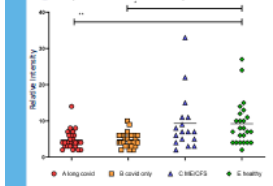
Method



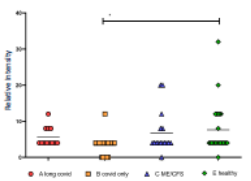
- Section A: Purification and delipidation of glycoproteins obtained from the biofluid. Precipitation of the purified glycoproteins creating a solid support that significantly enhances the Methylamidation activated by DMTMM. Proper cleanup with methanol is crucial to remove excess reagents and ensure the success of subsequent steps in the analysis.
- Section B: Glycoprotein denaturation enhances the accessibility of derivatized N-glycans, allowing for enzymatic cleavage by the glycosylase enzyme PNGase F. N-glycans, now free in solution, undergo purification to remove reagents and are concentrated by HILIC solid phase extraction
- Section C: N-glycans are spotted on a MALDI-MS steel target using 2,5-dihydroxybenzoic acid (DHB) as a matrix. MALDI-TOF mass analysis set up in Reflector-Positive mode with m/z 1000–3500 range. Raw spectra are calibration and baseline adjustment with Mmass software, and then the peaks annotated with GlycoWorkbench software running a custom made library able to detect derivatized sialylated N-glycans (1)

Results

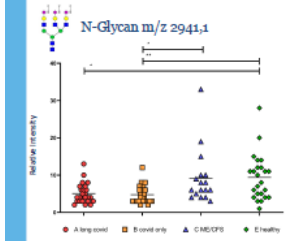
N-Glycan m/z 2636,9



N-Glycan m/z 2928,05

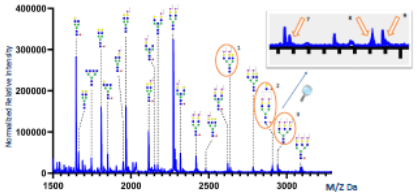


N-Glycan m/z 2941,1

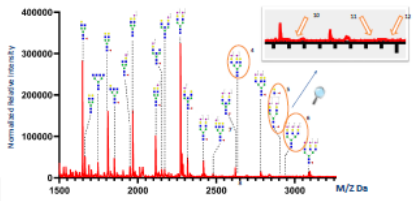


Graphical representation of N-glycans showing significant statistical variation:
Out of 20 distinct types of N-glycans analyzed, 3 of them (i.e. mz 2636,9 mz 2928,05 and mz 2941,1) showed significant statistical variation among the 4 groups (A long covid, B covid only, C ME/CFS and E healthy control group) under consideration. Based on the data obtained, the first two groups i.e. A and B show lower levels of high molecular weight sialylated N-glycans than groups C and E, which never came in contact with N-sars-cov2.
Statistics: Data was tested for normal distribution by Kolmogorov-Smirnov test first (none of the groups are normally distributed), then the not normally distributed data was analyzed by Kruskal-Wallis test with Dunn's multiple comparisons test.
* p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001

Healthy serum spectra



Long Covid serum spectra



MALDI-TOF/MS spectra of methylamidated N-glycans profile of human healthy and long covid serum:
Altered health situations, especially in our case post covid fatigue, can alter the N-glycosylation profile, especially for glycans with sialic acids in the antennary position as can be seen in 1,2,3 (healthy control group) and 4,5,6 (Long Covid group). Notice how the peaks in the spectrum associated to this N-glycans (arrows 7,8,9 for the healthy and arrows 10,11,12 for the Long Covid) are significantly lower in the Long Covid serum spectra.
In contrast, the levels of all the other N-glycans are not altered under pathological conditions

Findings

- Our method allows a rapid, accurate and cost-effective identification of N-glycans in biofluids opening up future developments in diagnostics. New biomarkers can be identified, since distinct N-glycosylation pattern have been observed in many pathophysiological conditions.
- In patients' sera more than 10 weeks after COVID infection we observed significant changes in the N-glycome profile. High molecular weight glycans were reduced significantly in patients after COVID infections being associated with elevated levels of non-sialylated glycans. This was in contrast to the pattern detected in healthy controls indicating a persistent change of the glycome on serum proteins after viral infection.
- Even though the ME/CFS group C presented chronic fatigue symptoms similar to post covid fatigue and had developed disease also after an infection (2), the N-glycosylation patterns were similar to those of the healthy control group. This indicated that N-glycosylation pattern restore over time.

References

1) Ret D. Et al, Talanta, May 2022
2) Rohrhofer J. Et al, Allergy 2023

TEAM MEMBERS TU Wien:

Daniel García de Otazo Hernández
Lisa Ying
Haidi Jakovic
Antonio Ammaturo
Veronica Monti
Sabatino Gianluigi
Philipp Gritsch
Knaus Simone

TEAM MEMBERS Meduni Wien:

Gentile Alessio
Johanna Rohrhofer
Larissa Koidl
Untersmayr Eva

Collaborators Meduni Graz:

Selina Keppler
Matteo Villa

Collaborators Meduni Wien:

Arvand Haschemi

Stefania Garofalo

Thank you for your attention