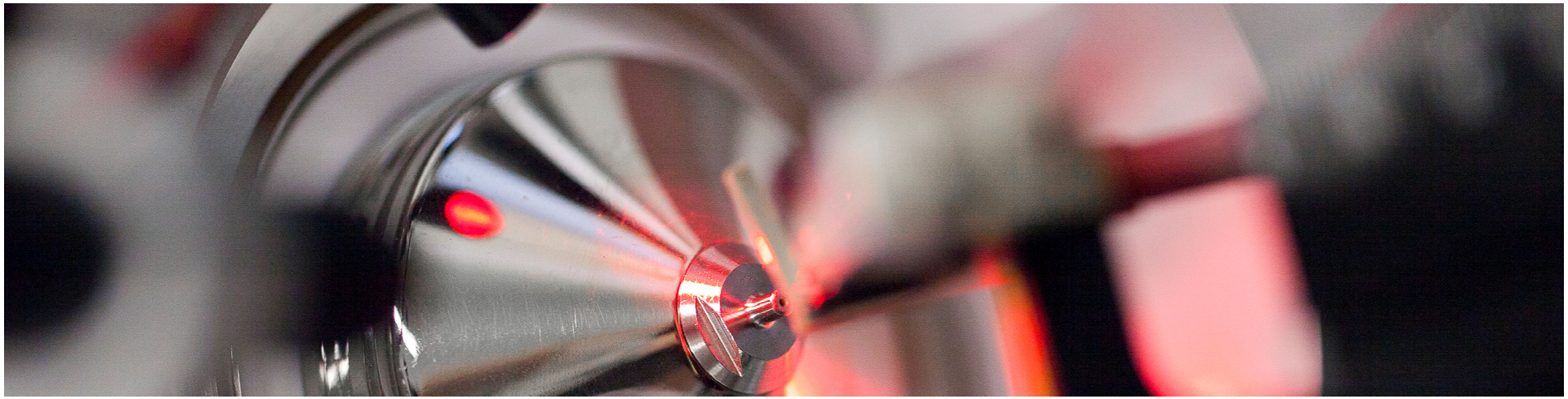
The Impact of Immunoglobulin G1 Fc Sialylation on Backbone Amide H/D Exchange

Felix Kuhne

Institute of Hygiene, University of Muenster, Robert-Koch-Strasse 41, 48149, Muenster, Germany

Pharma Technical Development, Roche Diagnostics GmbH, Nonnenwald 2, 82377 Penzberg, Germany



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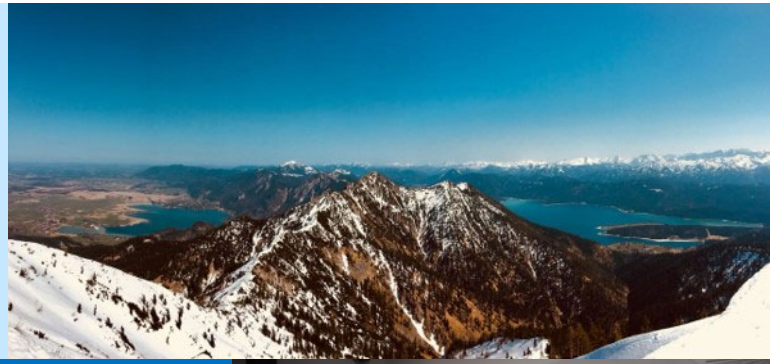

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Immunoglobulin G1 Fc glycosylation

- *critical quality attributes (CQAs) affect the mAb structure and function*

In vitro glycoengineering (IVGE)

- *how to generate specifically glycosylated mAbs in vitro*
- *mAb quality attributes and glycan quantification*

Structure-function analysis of trastuzumab IVGE

- *higher-order structure effects elicited by mAb Fc glycosylation*
- *the impact of sialic acid linkages on the mAb conformation*
- *functional testing and structure to function correlations*

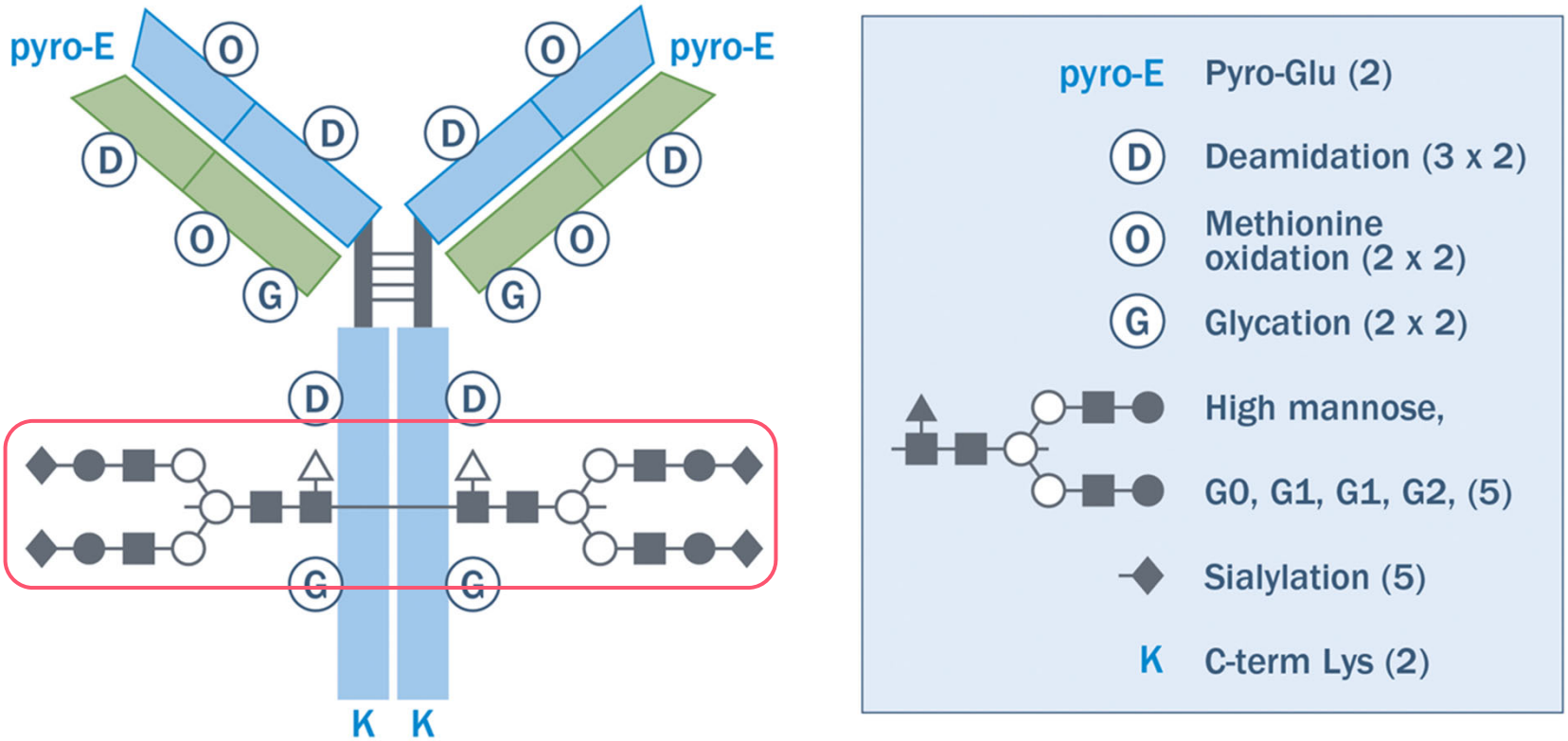
Current status (April 2017) of innovative antibody, Fc fusion protein, and chimeric antigen receptor (CAR) drug candidates

Antibody format	Stage of development			Totals
	Phase I/II	Phase III	Approved for marketing at some point	
Naked IgG	30	51	**52**	493
Naked antibody fragments	7	2	4	13
Immunocytokines	9	2	0	11
Fc fusion proteins	23	3	11	37
Bispecific antibodies	58	1	2	61
Antibody-drug conjugates	75	9	3	87
Radioimmunoglobulins	13	2	2	17
Antibodies only	575	70	74	719
T or NK cells expressing CAR antibodies	145	0	0	145
Totals	720	70	74	864

Naked mAbs are glycoproteins (like many other therapeutic proteins, e.g. EPO, IFN-β and Factor VIII)

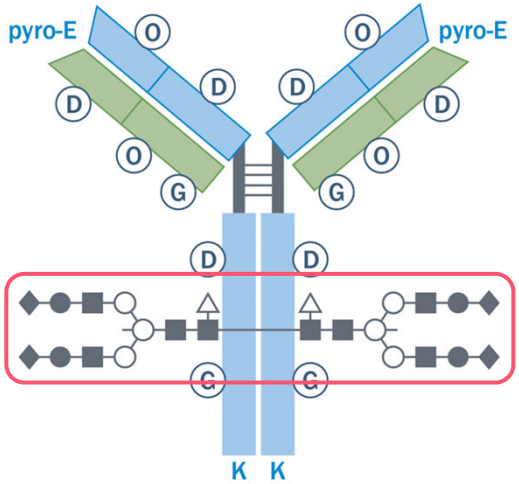
Strohl, W. R. Current progress in innovative engineered antibodies. *Protein Cell* **9**, 86–120, doi:10.1007/s13238-017-0457-8 (2018).

mAb modification sites



Vulto, A. G. & Jaquez, O. A. The process defines the product: what really matters in biosimilar design and production? *Rheumatology (Oxford)* **56**, iv14–iv29, doi:10.1093/rheumatology/kex278 (2017).

mAb Fc glycosylation



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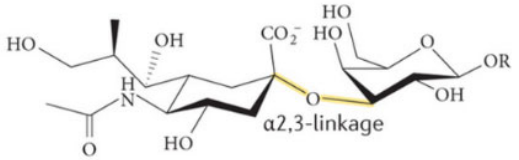

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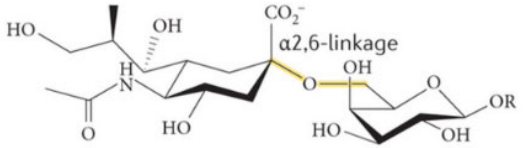

Glycans/glycosylation	Impacts
α1–3-galactose; *N*-glycolylneuraminic acid	Immunogenicity
Terminal sialylation	↓ binding to FcγRIIIa, ↓ ADCC; ↑ PK/PD
Afucosylation	↑ binding to FcγRIIIa, ↑ ADCC, ↑ ADCP
Galactosylation	↑ binding to C1q, ↑ CDC, moderate effect on ADCC
High-mannose	↓ PK/PD; ↑ binding to FcγRIIIa, ↑ ADCC; ↓ binding to C1q, ↓ CDC

↑ positive impact; ↓ negative impact.

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Zhang, L., Luo, S. & Zhang, B. Glycan analysis of therapeutic glycoproteins. *MAbs* **8**, 205–215, doi:10.1080/19420862.2015.1117719 (2016).

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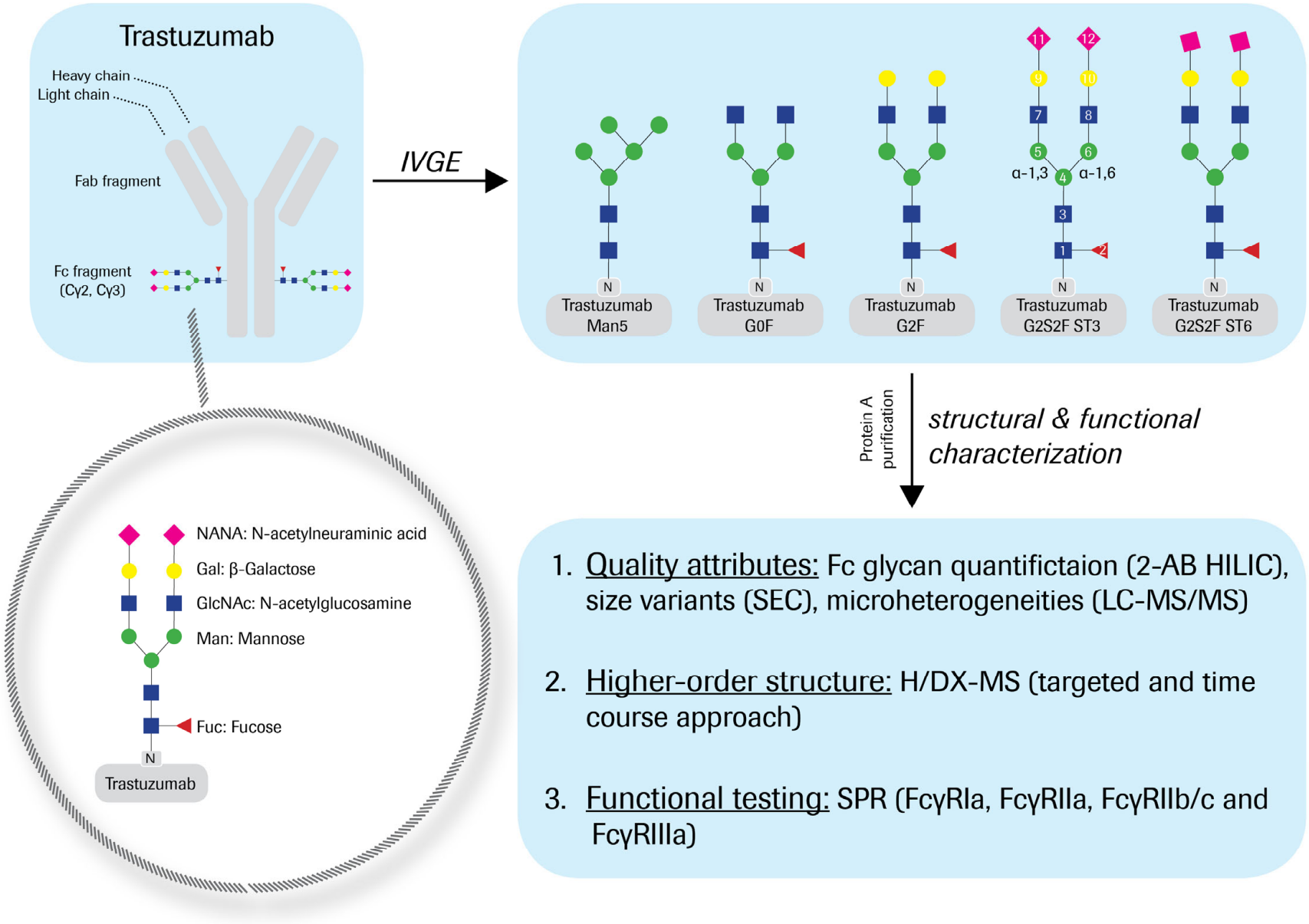

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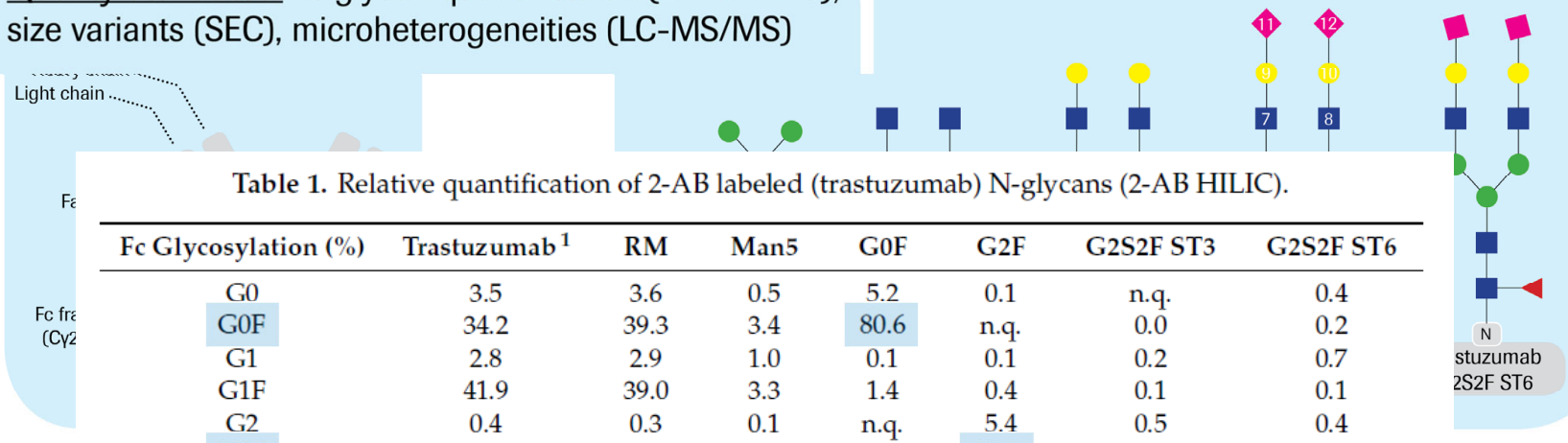

Table 1. Relative quantification of 2-AB labeled (trastuzumab) N-glycans (2-AB HILIC).

Fc Glycosylation (%)	Trastuzumab [1]	RM	Man5	G0F	G2F	G2S2F ST3	G2S2F ST6
G0	3.5	3.6	0.5	5.2	0.1	n.q.	0.4
G0F	34.2	39.3	3.4	80.6	n.q.	0.0	0.2
G1	2.8	2.9	1.0	0.1	0.1	0.2	0.7
G1F	41.9	39.0	3.3	1.4	0.4	0.1	0.1
G2	0.4	0.3	0.1	n.q.	5.4	0.5	0.4
G2F	9.3	7.2	0.8	0.7	83.1	1.6	1.8
G2S1	0.1	0.1	n.q.	0.2	n.q.	1.7	3.4
G2S1F	0.9	0.6	0.2	0.2	1.7	13.8	26.5
G2S2	0.1	n.q.	n.q.	n.q.	0.5	3.9	2.8
G2S2F	0.2	0.3	n.q.	0.1	n.q.	60.3	42.9
M3	n.q.	n.q.	0.1	n.q.	n.q.	n.q.	n.q.
M3F	n.q.	n.q.	0.1	n.q.	n.q.	n.q.	n.q.
hM3	0.4	0.6	0.2	1.0	0.0	0.1	0.3
hM3F	0.6	1.2	0.3	2.2	0.0	n.q.	0.2
hM3G1S1	0.1	0.1	n.q.	0.1	0.1	1.9	2.5
hM3G1S1F	0.3	0.4	n.q.	0.6	0.5	4.4	3.7
M4	n.q.	n.q.	0.1	n.q.	n.q.	n.q.	n.q.
hM4	0.3	0.3	n.q.	n.q.	1.0	0.1	1.2
hM4F	0.0	n.q.	n.q.	0.0	2.2	n.q.	n.q.
M5	1.3	1.3	88.3	1.4	1.4	3.1	3.3
hM5	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
M6	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
M7	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
not assigned	3.9	2.8	1.4	6.3	3.5	8.3	9.7

[1] Method reference standard; n.q. = not quantifiable.

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Table 2. Relative quantification of chemical amino acid modifications (LC-MS peptide mapping).

Chemical Mod. (%)	Trastuzumab [1]	RM	Degly	Man5	G0F	G2F	G2S2F ST3	G2S2F ST6
LC N30 [2] deamidation	9.6	8.7	10.9	9.1	9.1	9.0	9.6	10.0
LC N30 [2] succinimide	0.6	0.7	0.7	0.6	0.8	0.7	1.1	1.1
HC N54 [2] deamidation	1.5	1.6	1.7	2.2	1.7	2.0	1.8	1.6
HC N54 [2] succinimide	3.9	4.0	3.9	4.1	3.9	3.9	3.7	3.8
HC D98 [2] isomerization	7.7	7.6	8.0	10.0	7.0	7.7	7.9	8.7
HC D98 [2] succinimide	3.5	4.1	3.6	2.5	4.3	3.8	4.3	4.3
HC N389/390 [3] deam.	2.0	1.9	2.2	1.6	1.9	1.9	2.1	2.0
HC N389/390 [3] succ.	1.7	1.7	1.7	2.0	1.8	1.7	1.8	1.8
HC M252 [3] oxidation	2.3	2.6	3.4	5.2	3.0	3.8	3.8	3.8

[1] Method reference standard; [2] Kabat numbering [59]; [3] EU numbering [4].

Table 3. Relative quantification of trastuzumab size variants (SEC-UV).

Mol. Weight Species (%)	Trastuzumab [1]	RM	Degly	Man5	G0F	G2F	G2S2F ST3	G2S2F ST6
Monomer	99.8	99.6	99.6	99.4	99.6	99.3	99.2	99.1
total HMW [2]	0.2	0.4	0.4	0.5	0.3	0.7	0.8	0.9
total LMW [3]	0.0	0.0	0.1	0.1	0.1	0.0	0.0	0.1

[1] Method reference standard; [2] high molecular weight species; [3] low molecular weight species.

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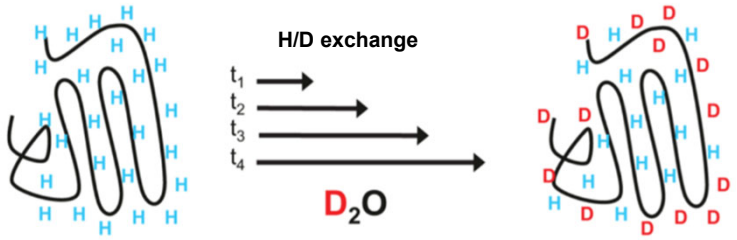
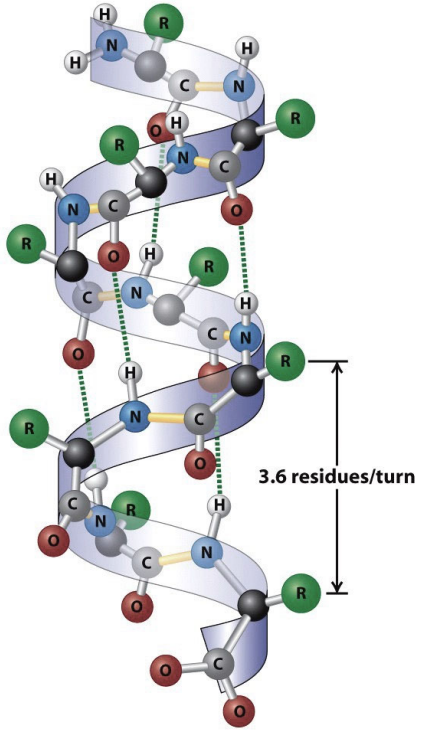
2. <u>Higher-order structure:</u> H/DX-MS (targeted and time course approach)

- according to the protein dynamics the intramolecular H-bonds of the petide backbone open and close more or less frequently

- dependent on molecular dynamics and interactions H-bonds can be more or less solvent-accessible

H/D exchange correlates with the conformational dynamics.





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Berg, J. M., Stryer, L. & Tymoczko, J. L. Stryer Biochemie. (Springer-Verlag, 2015)

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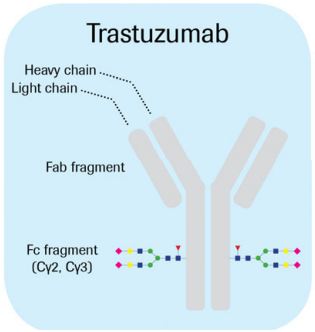

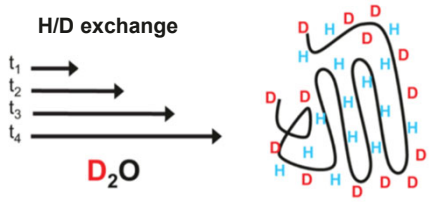

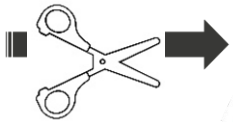

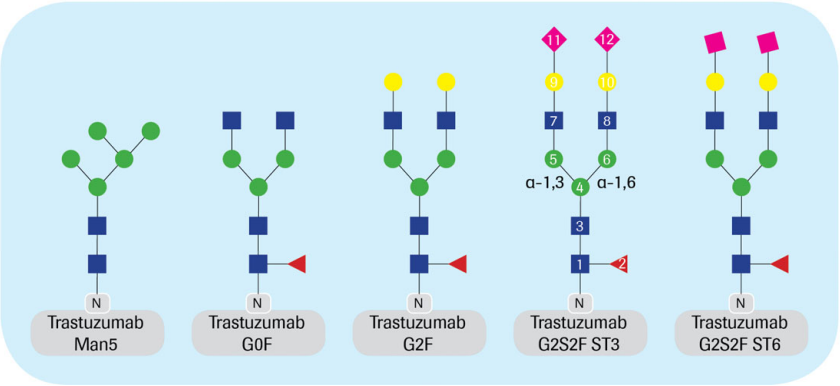

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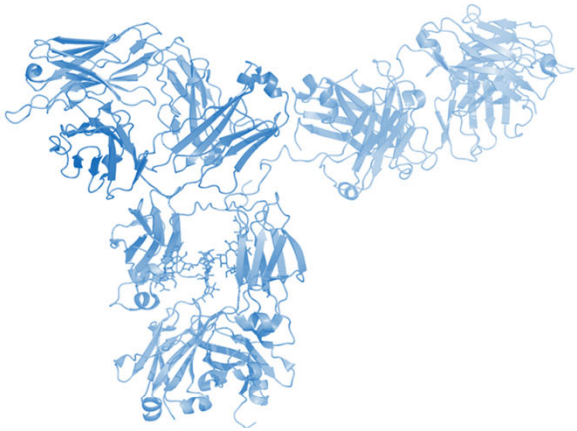
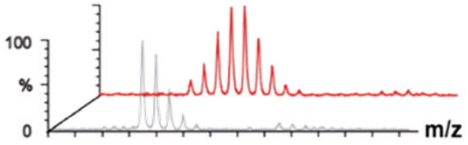
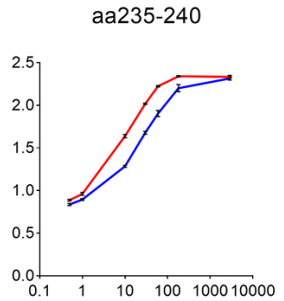
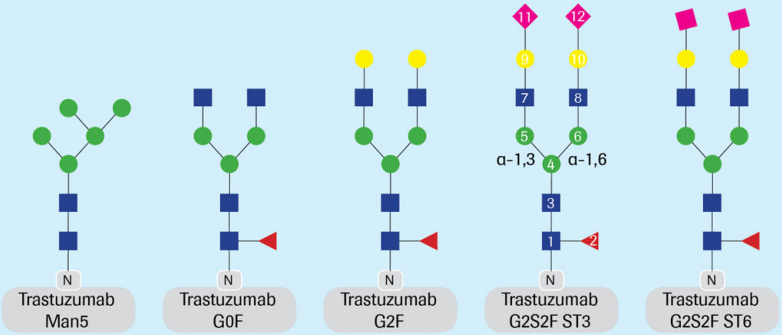
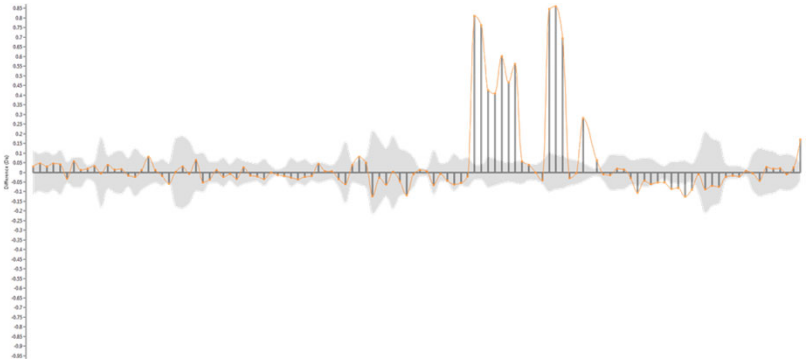


data analysis









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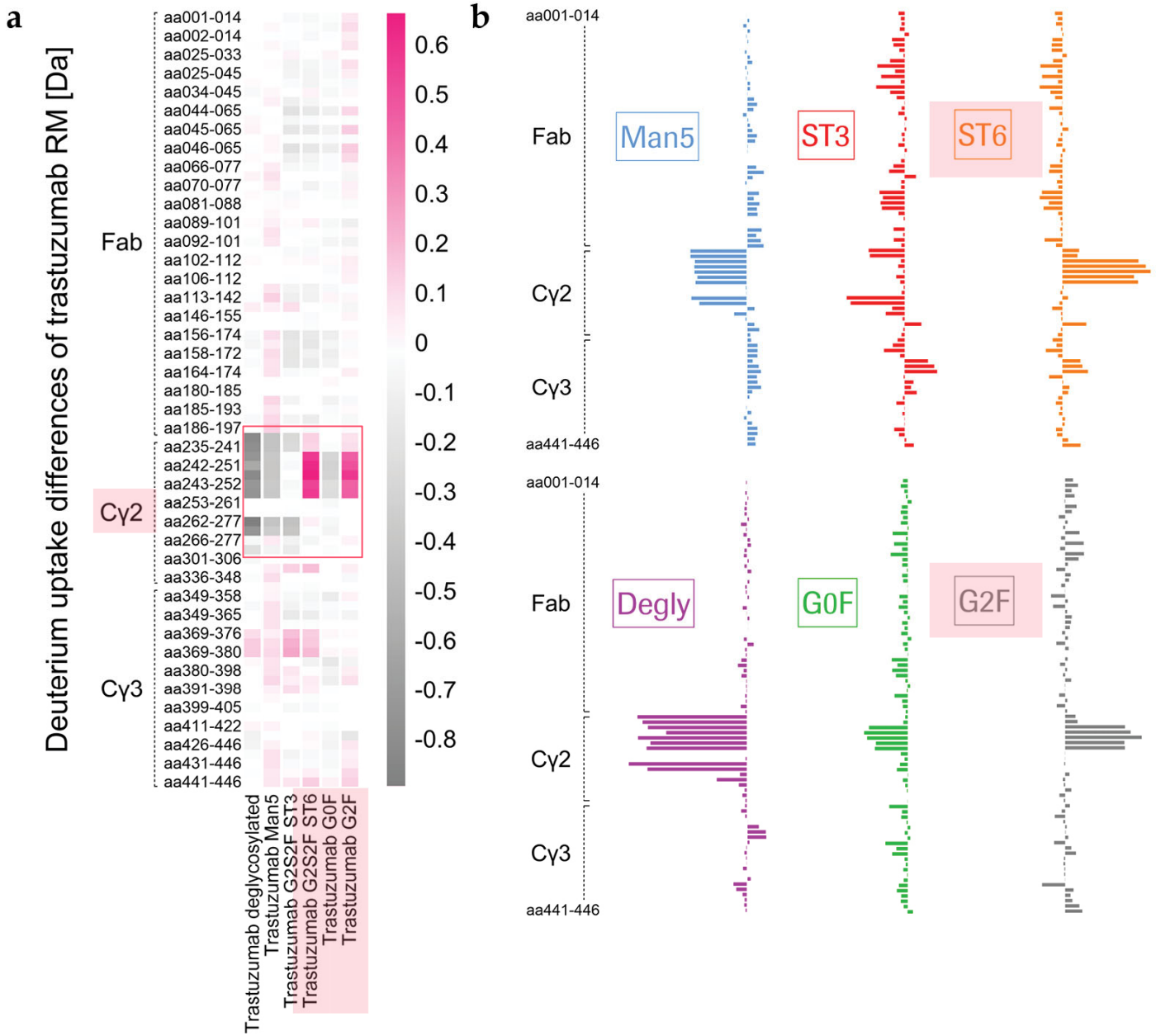
a
Deuterium uptake differences of trastuzumab RM [Da]
Fab
Cγ2
Cγ3
aa001-014
aa002-014
aa025-033
aa025-045
aa034-045
aa044-065
aa045-065
aa046-065
aa066-077
aa070-077
aa081-088
aa089-101
aa092-101
aa102-112
aa106-112
aa113-142
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aa380-398
aa391-398
aa399-405
aa411-422
aa426-446
aa431-446
aa441-446
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
Trastuzumab deglycosylated
Trastuzumab Man5
Trastuzumab G2S2F ST3
Trastuzumab G2S2F ST6
Trastuzumab G0F
Trastuzumab G2F
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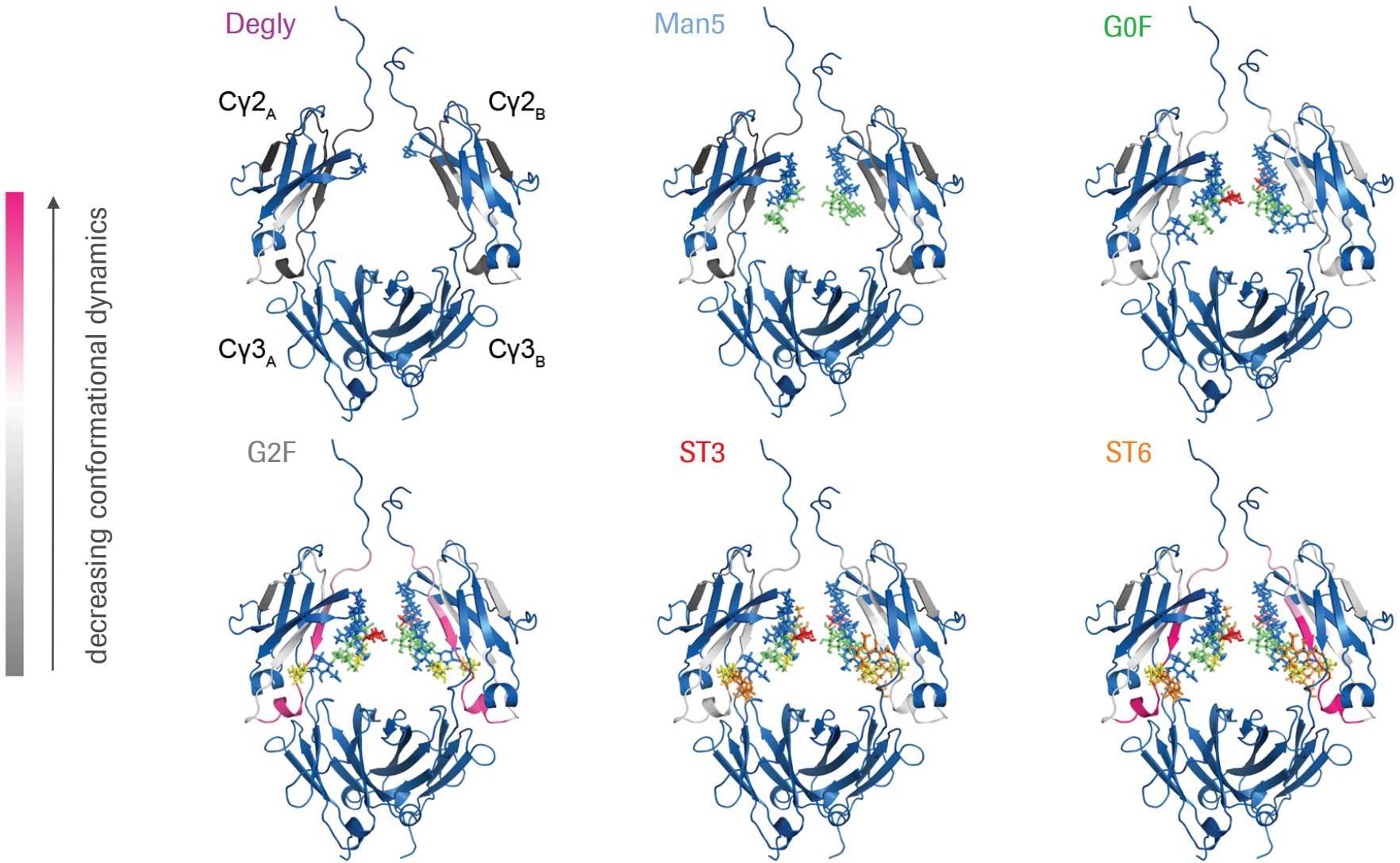
Fab
Cγ2
Cγ3
aa441-446
Man5
ST3
ST6
Degly
G0F
G2F



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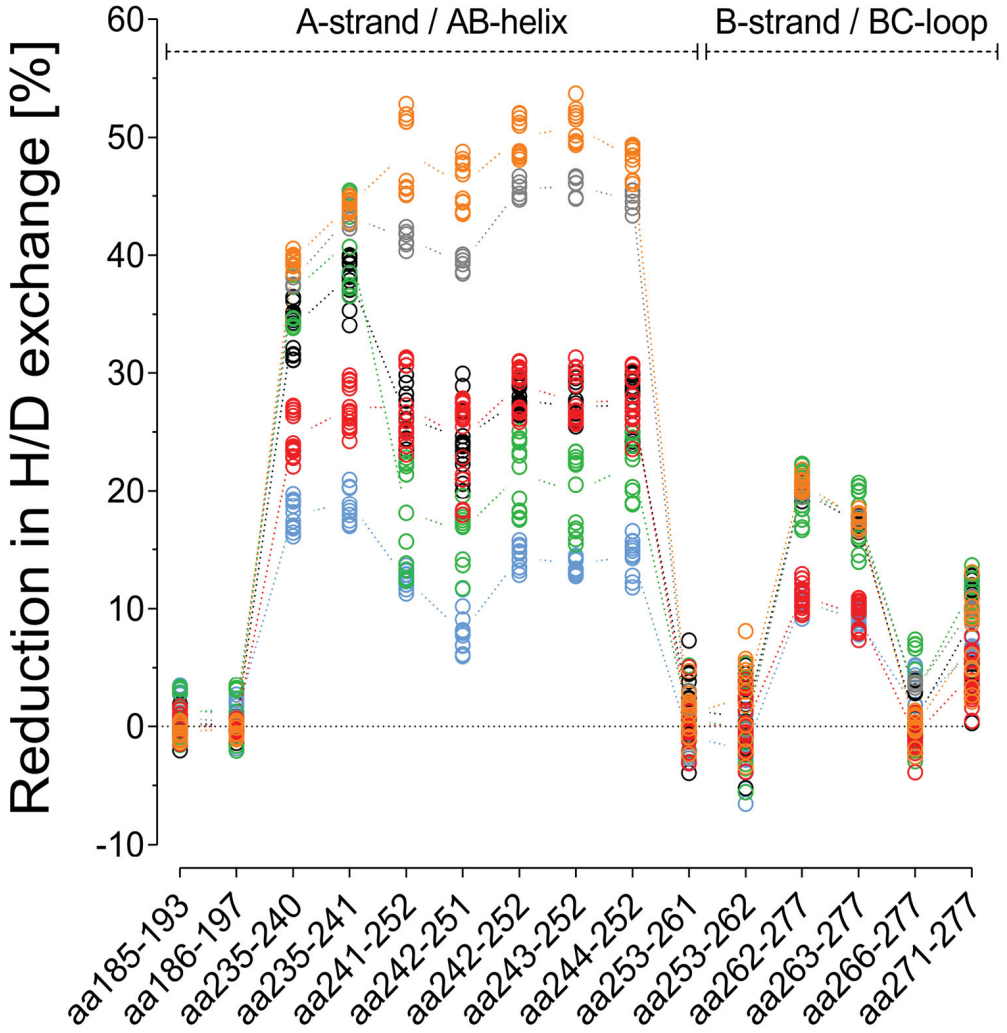
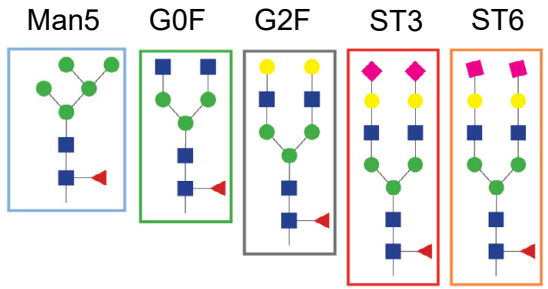
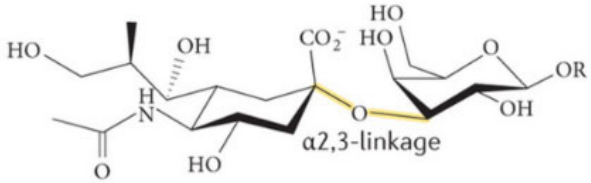
Antibody heavy chain (Cγ2 domain) dynamics vary dependent on Fc glycosylation





▸ CHO cells



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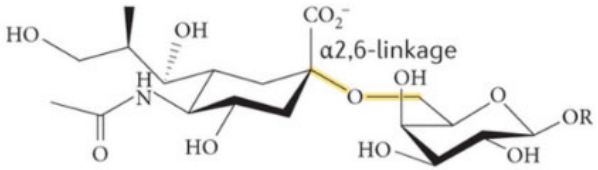

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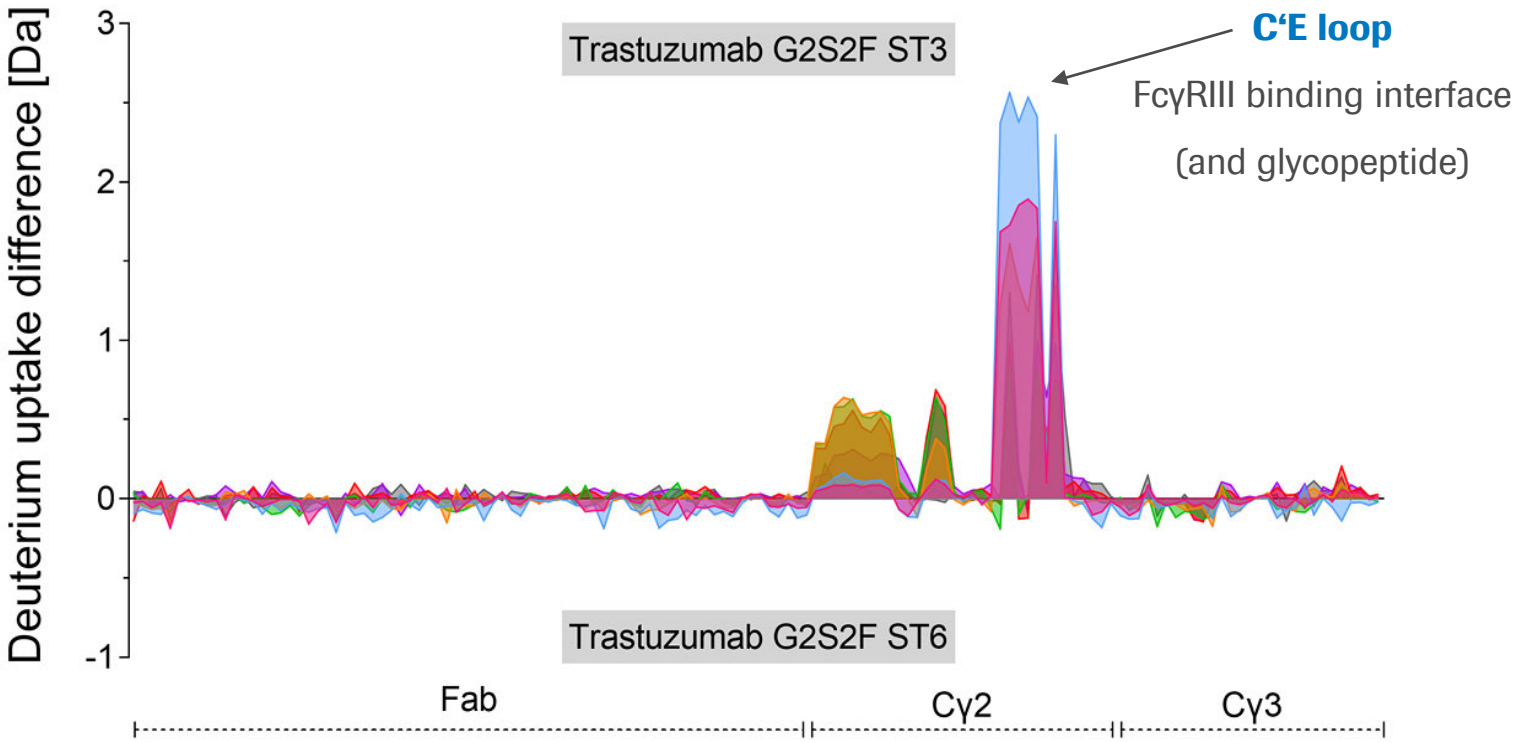
The type of sialic acid linkage impacts the Fc (Cγ2) higher-order structure



Uptake difference plot of H/DX time course experiment performed for:

0.5 min (pink), 1 min (light blue), 10 min (orange), 30 min (green), 1 h (red), 3 h (purple), and 48 h (black).

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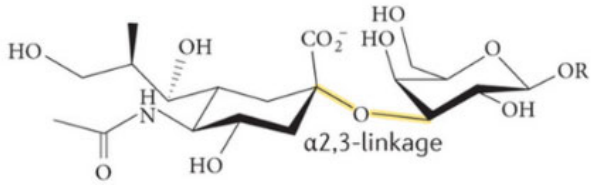
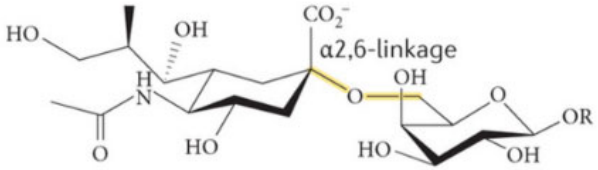


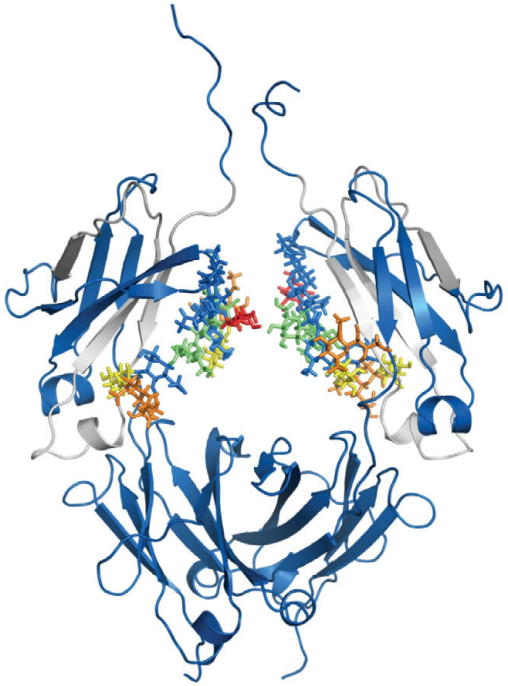


C‘E loop

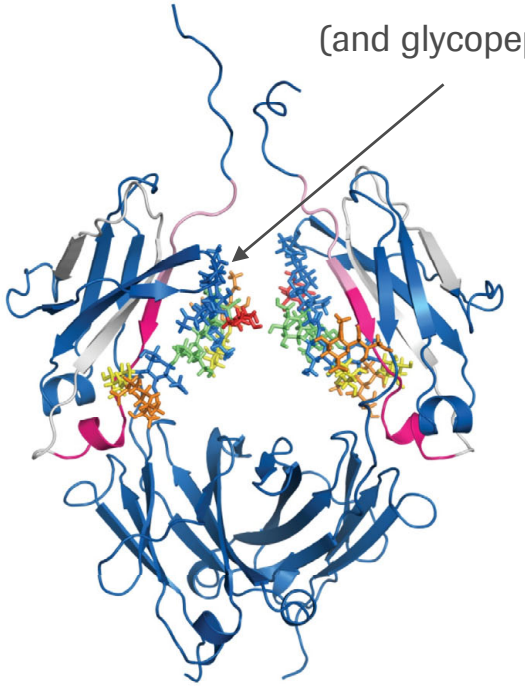
FcγRIII binding interface

(and glycopeptide)

decreasing conformational dynamics



G2S2F ST3



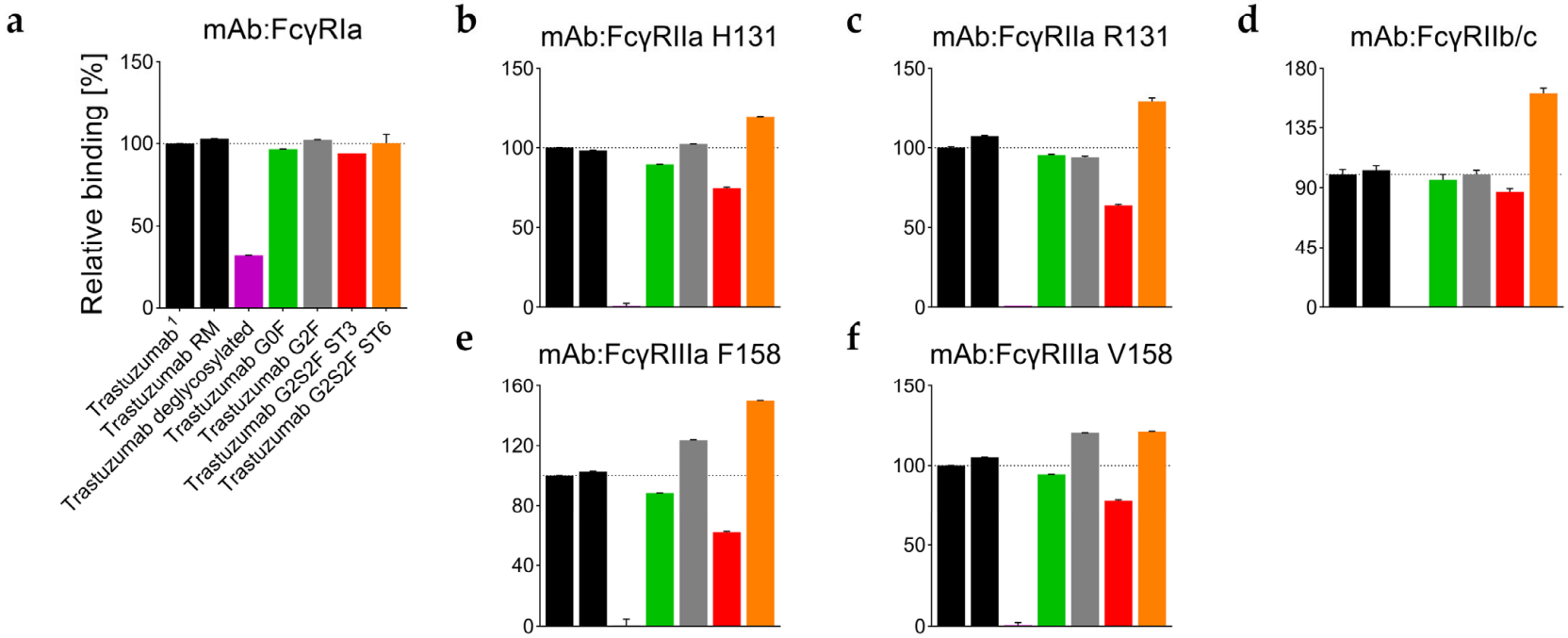
G2S2F ST6

Kuhne, F. *et al.* The Impact of Immunoglobulin G1 Fc Sialylation on Backbone Amide H/D Exchange. *Antibodies (Basel)* **8**, doi:10.3390/antib8040049 (2019).

1. Quality attributes: Fc glycan quantifictaion (2-AB HILIC), size variants (SEC), microheterogeneities (LC-MS/MS)

2. Higher-order structure: H/DX-MS (targeted and time course approach)

3. Functional testing: SPR (FcγRIa, FcγRIIa, FcγRIIb/c and FcγRIIIa)



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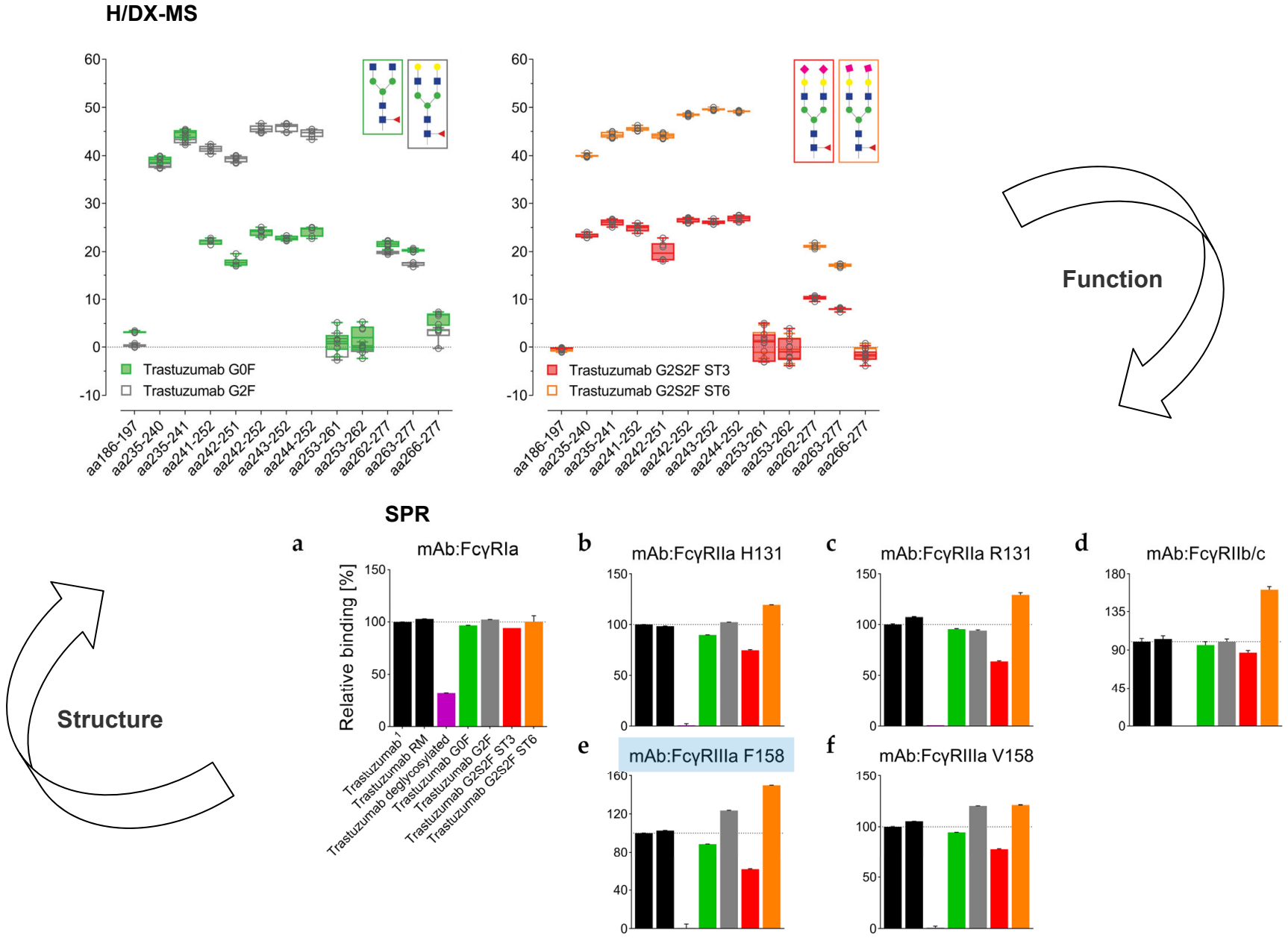

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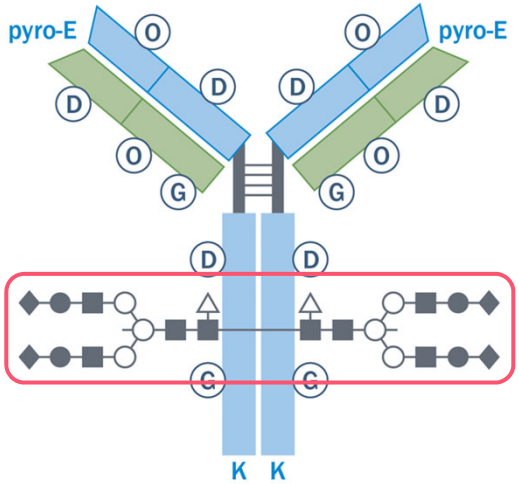
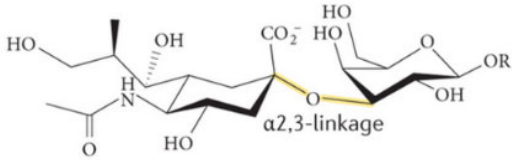
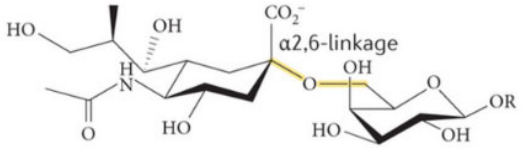
mAb Fc glycosylation



▸ CHO cells



▸ Human



conformational differences & functional differences

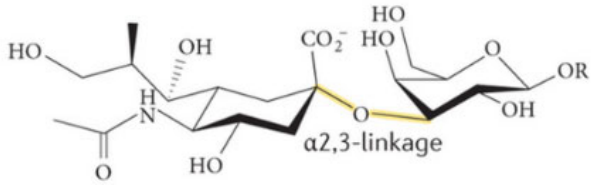
needs to be differentiated

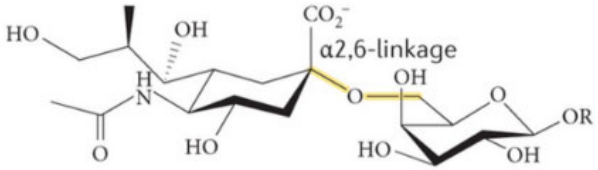
Glycans/glycosylation	Impacts
α1–3-galactose; *N*-glycolylneuraminic acid	Immunogenicity
Terminal sialylation	↓ binding to FcγRIIIa, ↓ ADCC; ↑ PK/PD
Afucosylation	↑ binding to FcγRIIIa, ↑ ADCC, ↑ ADCP
Galactosylation	↑ binding to C1q, ↑ CDC, moderate effect on ADCC
High-mannose	↓ PK/PD; ↑ binding to FcγRIIIa, ↑ ADCC; ↓ binding to C1q, ↓ CDC

↑ positive impact; ↓ negative impact.

Vulto, A. G. & Jaquez, O. A. The process defines the product: what really matters in biosimilar design and production? *Rheumatology (Oxford)* **56**, iv14–iv29, doi:10.1093/rheumatology/kex278 (2017).

Zhang, L., Luo, S. & Zhang, B. Glycan analysis of therapeutic glycoproteins. *MAbs* **8**, 205–215, doi:10.1080/19420862.2015.1117719 (2016).





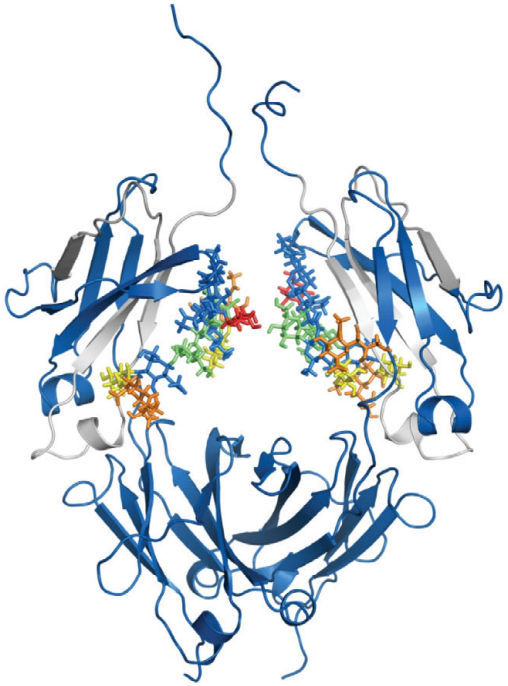
HOW?

C‘E loop

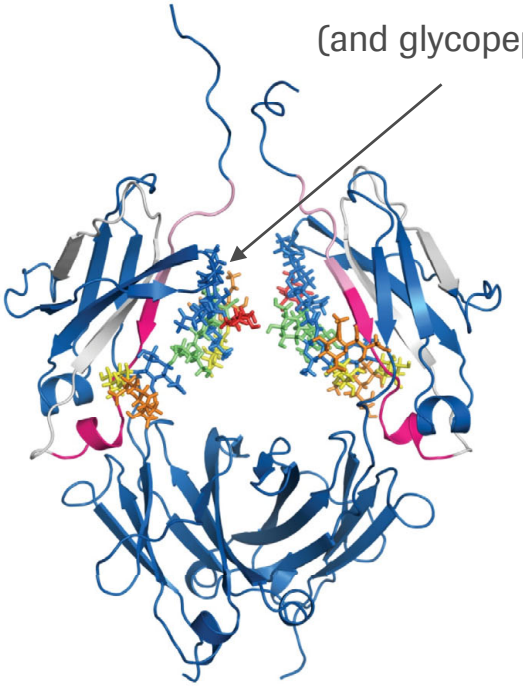
FcγRIII binding interface

(and glycopeptide)

decreasing conformational dynamics



G2S2F ST3



G2S2F ST6

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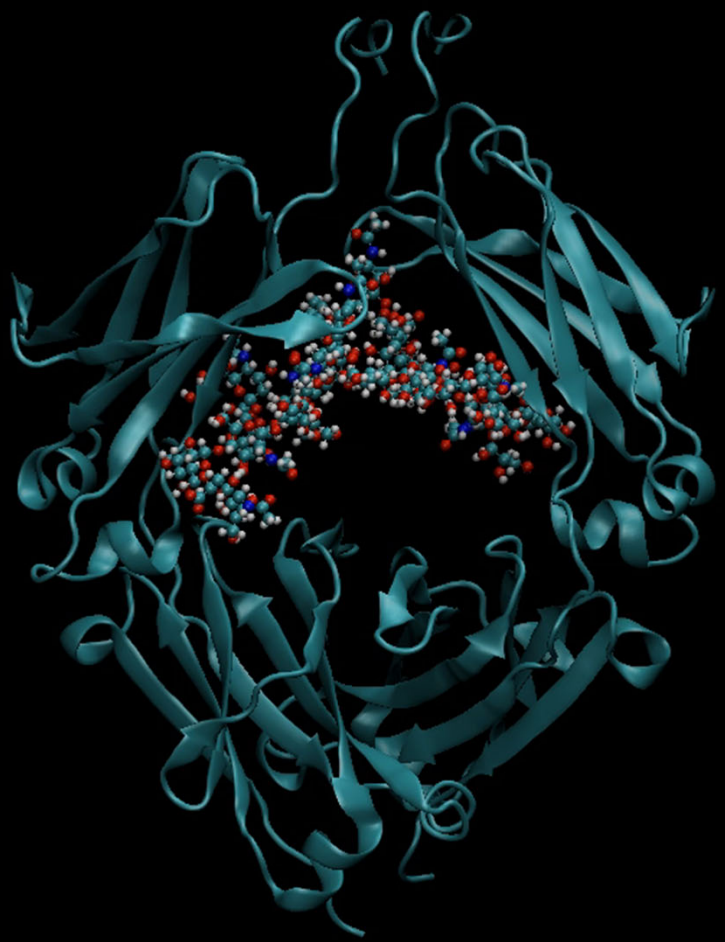
Outlook

- molecular dynamics
- receptor interaction
- covalent labeling



Doing now what patients need next