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N-glycome signatures in human plasma: associations with physiology and major diseases

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***N*-glycome analysis in total plasma or serum yields information about the levels and glycosylation patterns of major plasma glycoproteins, including immunoglobulins, acute-phase proteins, and apolipoproteins. Until recently, glycomic studies in disease settings largely suffered from small cohort sizes, poor analytical resolution, and poor comparability of results owing to the diversity of analytical techniques. Here, we report on recent advances in high-throughput mass spectrometry glycomics technology that enabled elucidation of *N*-glycome signatures in the plasma of patients with type 2 diabetes, inflammatory bowel disease, or colorectal cancer. Use of this technology revealed both commonalities and differences among disease fingerprints. Moreover, we summarize findings on glycomic signatures associated with age, sex, and body mass index. High-throughput, high-resolution glycomics technologies, together with robust data analysis workflows, will advance clinical translation.**

Keywords: biomarker; cancer; diabetes; glycomics; inflammation; inflammatory bowel diseases; mass spectrometry; *N*-glycosylation

The approximately 20 000 human genes code for proteins that exist in multiple variants which results in an enormous complexity. Genetic diversity and splice variants at the transcriptome level contribute to this complexity. Subsequently, many variants or proteoforms arise during and after translation *via* enzymatic or nonenzymatic processes. Of these post-translational modifications, protein glycosylation is a particularly abundant and highly heterogeneous one, and is known to often strongly determine protein structure and function [1]. Functional changes include the modulation of receptor affinities as well as effects on protein half-life and targeting. Most glycosylation events occur in the endoplasmic reticulum and the Golgi apparatus, and consequently, protein glycosylation is particularly abundant on proteins that pass through these organelles, such as cell-surface proteins and secreted proteins. The key biosynthetic steps of protein glycosylation are textbook knowledge, with the initial steps of *N*-glycosylation occurring in the endoplasmic reticulum, while further enzymatic processing of the *N*-glycans as well as step-wise *O*-glycosylation occurs further along the secretory pathway at the luminal side of the endoplasmic reticulum and Golgi. This enzymatic processing of *N*-glycans can generate a vast diversity of glycan structures, and dozens if not hundreds of different glycans can be found at a specific glycosylation site of a human protein, a phenomenon for which the term microheterogeneity has been coined [2]. Together with the often only partial occupancy of glycosylation sites (macroheterogeneity), this results in an immense complexity of the human glycoproteome.

Protein glycosylation has been described to change with many biological and pathological processes such as cellular (de-)differentiation as well as in cellular degeneration in the course of malignancies [1,3–5]. Consequently, glycosylation changes and/or signatures have been described for many diseases including

Abbreviations
BMI, body mass index; CD, Crohn's disease; CEA, carcinoembryonic antigen; CRC, colorectal cancer; HNF1α, hepatocyte nuclear factor alpha; IBD, inflammatory bowel diseases; Ig, immunoglobulin; T2D, type 2 diabetes; TPSNG, total plasma/serum *N*-glycome; UC, ulcerative colitis.

various types of cancer, autoimmune diseases, and infectious diseases. In addition, many physiological and behavioral parameters such as sex, age, body mass index (BMI), and smoking have been shown to be associated with protein glycosylation [5–7].

Although glycosylation has been recognized in recent years as an important phenotypic feature of most disease processes, our knowledge on glycosylation changes and signatures of diseases is fragmented and suffers from the following limitations. (a) Hitherto performed disease glycomics studies applied a diverse set of analytical techniques. This difference in methodologies compromises the comparison of results between studies. (b) Often only small cohorts were analyzed, with the consequent risk of false-positive findings. This was mostly due to the rather low throughput of glycomics technologies used [8]. Furthermore, replication of major findings has not commonly been performed and (c) The techniques often featured low resolution, and consequently, only a limited set of glycan features was reported. Hence, information on key glycan structural characteristics is often missing.

Recently, these limitations have been addressed: Methods have been developed and validated allowing the analysis of larger sample sets in a robust and reproducible manner, facilitating the comparison of results between laboratories and disease-specific cohorts [9–11]. Notably, next to fluorescence detection of glycans, also methods with mass spectrometric detection have been proven suitable. Importantly, these methods allow to capture many structural features of *N*-glycans that are known to change with various diseases, such as antennarity of complex-type *N*-glycans, levels of fucosylation, levels and linkage type of sialic acids, as well as levels of galactosylation and bisection (Fig. 1).

Using these methods, glycomic signatures of various physiological parameters and human diseases have recently been described for human blood plasma and serum samples [10,12,13]. Building on these studies, we here present human total plasma/serum *N*-glycome (TPSNG) signatures of major diseases and common disease risk factors. Moreover, we provide future directions for promising protein-specific disease glycomic signatures which might have potential in elucidating disease mechanisms and developing disease-specific biomarkers.

N-glycome associations with genetic and common disease risk factors

Influence of genetic variation on the *N*-glycome

Congenital disorders of glycosylation are monogenic diseases with a major defect in protein or lipid glycosylation. Glycosylation and thus the function of affected glycoconjugates are altered by, for example, a defect in the formation of the oligosaccharide precursors, a lack in nucleotide-activated sugars, or a defect of one of the glycan processing enzymes in the endoplasmic reticulum or Golgi, often leading to a range of severe developmental and neurological phenotypes [14]. While the classic diagnostic assay for sialic acid-related congenital disorders of glycosylation involves evaluation of transferrin sialylation levels *via* isoelectric focusing, the evaluation of the TPSNG recently gained attention [15]. Notably, many of the above-mentioned defects result in major glycosylation abnormalities of various plasma proteins, not only transferrin, and are detectable in the TPSNG pinpointing the affected biosynthetic steps [16].

Likewise, the TPSNG is influenced by common genetic variations. Large-scale population studies on isolated populations indicated variation in heritability of specific TPSNG traits between populations. Overall, many traits show rather high heritability, contributing significantly to interindividual variation within population, but also to variations between populations [17,18].

The influence of the genetic makeup has been demonstrated in genome-wide association studies of single nucleotide polymorphisms with TPSNG glycosylation features providing insights into the molecular mechanisms regulating glycosylation [17,19,20]. Genome-wide association studies not only revealed the association of genetic variants in glycosyltransferases with the corresponding glycosylation phenotype, but likewise gave insights into the regulation of glycosylation at the level of transcription factors. Moreover, associations with genes were found, where a possible functional relationship with glycosylation is yet unknown. For example, the transcription factor hepatocyte nuclear factor alpha (HNF1α) has been shown to be implicated in the regulation of TPSNG fucosylation [19]. Low expression levels of HNF1α cause maturity-onset diabetes of the young type 3, and the associated TPSNG antennary fucosylation signature has great potential in detecting low HNF1α activity. Consequently, the TPSNG antennary fucosylation signature is currently being evaluated as an indicator of HNF1α activity for the detection of maturity-onset diabetes of the young [21].

N-glycome associations with age, sex, and BMI

Previously, glycan signatures of age and aging-related diseases, as found in small- to medium-sized clinical cohorts mainly using lower-resolution techniques, were reviewed by Miura and Endo [6]. Our recent TPSNG

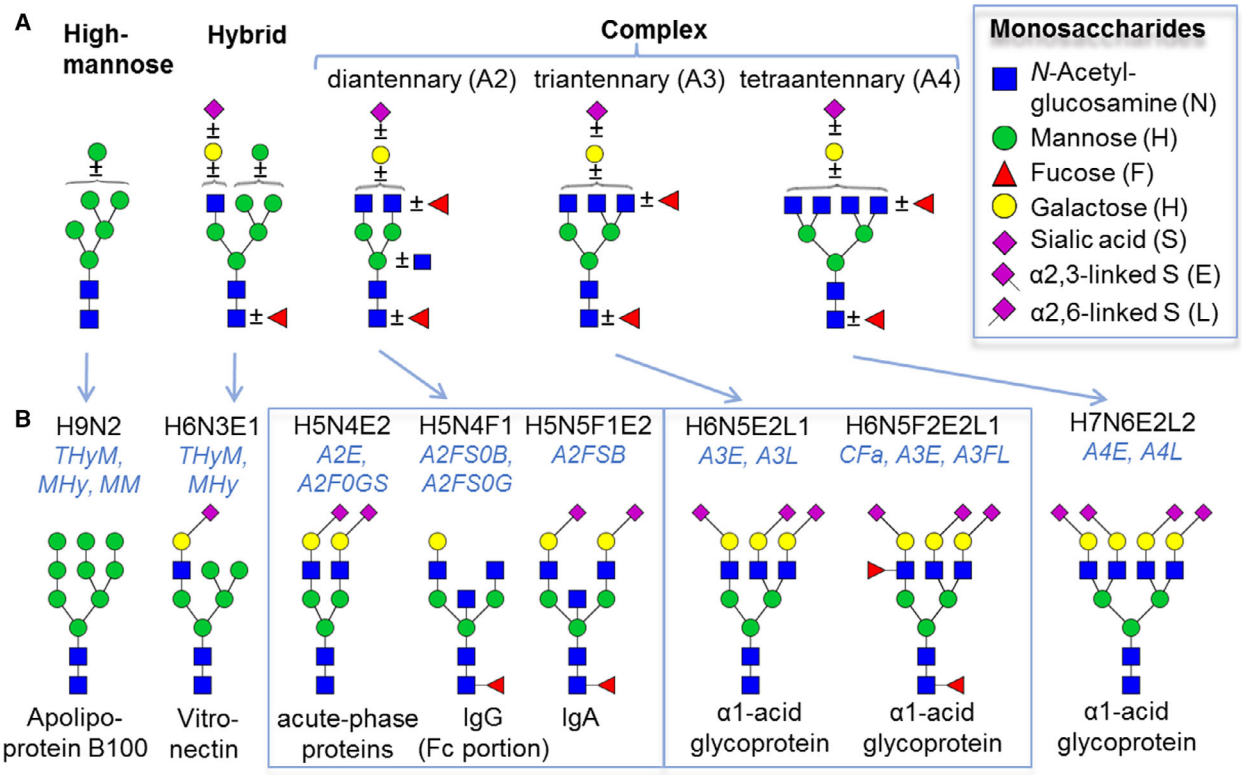

Fig. 1. *N*-Glycan structures present in human plasma. *N*-Acetylglucosamine and mannose monosaccharide units build the core of all *N*-glycans, including high-mannose, hybrid, and complex-type structures, which can further be elongated/modified with galactoses, fucoses, bisecting *N*-acetylglucosamine, and/or sialic acids (A). This results in a large variety of structures, for some of which examples are given in (B). The detected relative signal intensities of direct traits, after total area normalization, can be summed into structurally related derived traits (examples in blue font; see Fig. 2 for details), featuring a higher method repeatability and providing hints on global as well as protein-related glycosylation signatures [24,42].

studies in larger sample sets including healthy controls employed high-resolution mass spectrometry and revealed novel TPSNG associations with sex, age, and BMI due to the added layer of information on sialic acid linkages as well as the ability to detect up to 80 different glycan structures [10,12,13]. Here, we give an overview of these results (Fig. 2) and relate them to the literature.

These compiled data of control individuals from three different cohorts showed a higher antennarity of complex-type *N*-glycans in females as compared to males (Fig. 2A, trait CA; $P = 2.58E\text{-}10$), which is in line with a previous study by Knezevic *et al.* [22] using HPLC fluorescence. Moreover, Knezevic *et al.* [22] found an increase in antennarity with age in women only and an increase of antennarity with BMI. In our studies, the association with BMI did not reach study-wide significance (Fig. 2B and [23]).

With respect to fucosylation, all related traits given in Fig. 2A showed a higher level of fucosylation in males as compared to females. This holds true for the fucosylation of diantennary as well as tri- and tetraantennary glycans (A2F and A34F, respectively). Higher levels of fucosylation were found both within glycans carrying α2,6-linked sialic acids and lacking α2,6-sialylation. While the previous study of Reiding *et al.* [23] did not discriminate between sialic acid linkages, it likewise revealed higher levels of fucosylation in di-, tri-, and tetraantennary glycan traits in males vs. females. On direct trait level, this was most pronounced in the rise of H6N5F1S3 and H7N6F1S4 (fully sialylated, tri- and tetraantennary species carrying a fucose) in males, while the nonfucosylated counterparts H6N5S3 and H7N6S4 showed a reverse association and were higher in females. According to the report by Knezevic *et al.* [22] who used HPLC with fluorescence detection, the increase in fucosylation was mainly due to antennary, not core, fucosylation. This is supported by our observation in the proxy trait for antennary fucosylation CFa (doubly fucosylated complex glycans).

			A				B		C							
			Sex		Age		BMI		T2D		UC		CD		CRC	
Description	Related structures	Trait	OR	*P*	beta	*P*	beta	*P*	OR	*P*	OR	*P*	OR	*P*	OR	*P*
Type / Complexity																
Hybrid and high-mannose in total		THyM	0.89	1.21E-01	−0.02	9.65E-01	0.02	8.98E-01	0.84	**1.66E-05**	0.71	**1.77E-08**	0.57	**1.08E-15**	0.69	9.45E-04
Ratio of high-mannose to hybrid		MHy	0.96	4.71E-01	0.05	8.84E-01	0.38	2.62E-03	0.90	1.10E-02	1.15	1.97E-02	1.62	**6.72E-12**	0.79	3.05E-02
Average number of mannoses in high-mannose		MM	1.16	5.03E-02	−1.77	**5.80E-05**	0.44	6.92E-04	1.07	1.12E-01	0.60	**3.88E-14**	0.60	**6.00E-13**	1.35	6.82E-03
Average number of antennas in complex	2-4x	CA	0.57	**2.58E-10**	0.14	7.75E-01	0.16	2.37E-01	1.35	**5.21E-11**	1.38	**4.96E-07**	1.80	**2.05E-14**	1.77	**1.97E-06**
Fucosylation		F														
of diantennary (A2)		A2F	1.40	**1.95E-07**	1.53	**9.85E-06**	−0.12	3.60E-01	0.75	**2.62E-11**	0.82	8.78E-04	0.66	**8.23E-09**	0.58	**5.83E-06**
of A2 with α2,6 sialic acid (SA)		A2EF	1.43	**2.55E-09**	1.55	**1.07E-06**	0.00	9.82E-01	0.85	**1.01E-04**	0.83	1.12E-03	0.71	**8.32E-07**	0.80	4.39E-02
of A2 without α2,6 SA		A2E0F	1.46	**1.21E-09**	0.10	7.72E-01	0.20	1.31E-01	0.79	**1.06E-07**	0.90	8.13E-02	0.76	**1.11E-04**	0.57	**2.45E-06**
of tri- & tetraantennary (A3&A4)		A34F	2.31	**1.62E-38**	3.09	**9.90E-22**	−0.01	9.36E-01	0.88	3.54E-03	0.78	**7.16E-05**	1.11	1.07E-01	1.67	**1.59E-05**
of A3 with α2,6 SA		A3EF	2.30	**2.98E-38**	3.12	**3.66E-22**	−0.01	9.70E-01	0.88	4.39E-03	0.80	**2.98E-04**	1.15	3.12E-02	1.69	**9.01E-06**
of A3 with α2,3 SA		A3LF	2.19	**7.12E-34**	3.41	**1.35E-26**	0.11	4.15E-01	0.97	4.75E-01	0.76	**9.65E-06**	1.08	2.31E-01	1.75	**3.01E-06**
antennary F in complex (doubly fucosylated)	2x	CFa	3.12	**2.30E-10**	4.71	**8.72E-07**	0.20	1.27E-01	1.17	**4.52E-04**	0.81	**2.50E-04**	0.94	3.52E-01	1.62	**3.61E-05**
Bisection		B														
of afucosylated, sialylated A2		A2F0SB	1.04	5.05E-01	2.85	**4.09E-16**	−0.47	**2.66E-04**	0.87	8.72E-04	0.85	7.01E-03	0.88	5.16E-02	0.84	1.07E-01
of fucosylated, asialylated A2		A2FS0B	0.81	**4.03E-04**	4.12	**2.17E-37**	0.16	2.29E-01	1.69	**4.74E-27**	0.68	**7.40E-09**	0.85	2.59E-02	1.24	5.60E-02
of fucosylated, sialylated A2		A2FSB	0.81	7.35E-04	3.69	**5.38E-27**	−0.24	6.01E-02	0.82	**2.07E-06**	1.23	1.12E-03	1.36	**8.12E-06**	1.11	3.54E-01
Galactosylation		G														
of afucosylated, sialylated A2		A2F0SG	1.18	1.28E-02	−2.99	**2.39E-15**	0.70	**6.53E-08**	1.47	**4.87E-18**	1.32	**3.69E-06**	1.43	**1.32E-07**	1.50	1.44E-03
of fucosylated, asialylated A2		A2FS0G	1.08	2.50E-01	−6.93	**8.41E-97**	−0.67	**6.85E-07**	0.67	**3.84E-16**	0.71	**1.86E-06**	0.40	**1.15E-26**	0.54	**1.86E-06**
of fucosylated, sialylated A2		A2FSG	1.25	6.35E-04	−2.24	**2.12E-09**	0.02	8.90E-01	1.09	3.28E-02	1.00	9.54E-01	0.94	3.28E-01	1.22	6.87E-02
Sialylation		S														
per galactose in afucosylated A2		A2F0GS	1.31	**2.98E-06**	−0.95	3.61E-03	0.45	5.86E-04	1.24	**4.23E-07**	1.04	4.70E-01	1.10	1.27E-01	1.48	**4.16E-04**
per galactose in fucosylated A2		A2FGS	1.12	7.34E-02	3.97	**2.57E-29**	0.58	**8.89E-06**	1.89	**1.66E-40**	1.24	8.22E-04	2.14	**1.13E-22**	2.39	**2.77E-11**
α2,6-linked sialylation		E														
of A2		A2E	0.95	3.63E-01	−0.70	4.90E-02	0.59	**4.23E-06**	1.71	**3.29E-32**	1.14	2.42E-02	1.38	**1.74E-06**	1.89	**8.68E-08**
per galactose in afucosylated A2		A2F0GE	1.36	**1.96E-07**	0.88	8.52E-03	0.79	**1.03E-09**	1.50	**5.88E-20**	0.87	1.77E-02	0.91	1.75E-01	1.47	**5.46E-04**
per galactose in fucosylated A2		A2FGE	1.09	2.11E-01	3.78	**8.92E-26**	0.79	**8.81E-10**	1.82	**7.45E-36**	1.24	5.99E-04	1.88	**4.06E-18**	2.12	**3.68E-09**
of A3	3-4x	A3E	0.99	8.73E-01	2.93	**7.58E-18**	1.24	**7.49E-23**	2.28	**5.49E-59**	0.92	1.67E-01	1.12	9.37E-02	1.81	**7.66E-07**
of A4		A4E	1.17	6.75E-02	2.65	**3.62E-08**	0.73	**1.28E-08**	1.74	**3.72E-31**	0.79	**1.06E-04**	0.74	**5.31E-06**	1.53	**1.86E-04**
α2,3-linked sialylation		L														
of A2		A2L	0.99	8.43E-01	−1.91	**1.72E-08**	−0.92	**3.99E-13**	0.68	**3.74E-19**	1.20	2.80E-03	1.29	**1.67E-04**	0.99	9.37E-01
per galactose in afucosylated A2		A2F0GL	0.83	7.26E-04	−2.51	**4.03E-15**	−0.85	**3.94E-11**	0.64	**1.67E-24**	1.47	**9.59E-09**	1.40	**2.33E-06**	0.83	7.97E-02
per galactose in fucosylated A2		A2FGL	1.05	3.60E-01	0.96	2.18E-03	−0.31	1.52E-02	1.08	9.05E-02	0.97	6.30E-01	1.40	**1.31E-06**	1.54	**1.33E-04**
of A3	3-4x	A3L	1.00	9.94E-01	−3.70	**3.52E-25**	−1.11	**2.11E-18**	0.43	**2.28E-57**	1.76	**1.03E-16**	1.81	**3.97E-15**	1.01	9.40E-01
of fucosylated A3		A3FL	1.24	**4.71E-04**	−2.08	**1.64E-09**	−0.83	**1.07E-10**	0.53	**6.02E-35**	1.60	**1.55E-13**	2.19	**8.07E-23**	1.42	1.54E-03
of A4		A4L	1.01	9.18E-01	−4.38	**1.92E-11**	−0.90	**1.02E-12**	0.51	**9.34E-44**	1.46	**3.55E-09**	1.37	**3.42E-06**	1.14	2.04E-01

Fig. 2. Total TPSNG signatures of sex and age (A), BMI (B), and major diseases (C). Glycans were released from total plasma or serum by PNGase F and analyzed by MALDI-TOF-MS after sialic acid derivatization. For details on the cohorts, methods, and extensive results, see [10,12,13]. Here, data are shown for the entire T2D DiaGene dataset (N = 835 controls and 1816 T2D cases), UC and CD Italian cohort (N = 570 controls/1085 UC/905 CD cases), and N = 185 controls and 185 CRC cases. To ensure comparability between cohorts, the total area normalized relative intensities of detected direct traits were here summed into 30 derived traits prior to statistical analysis in R using RStudio, applying the same approach as described in [10,12,13]. Logistic or linear regression models were used for binary or continuous outcome variables, retrieving odds ratios (OR) or beta-values, respectively, which are labeled blue for negative and red for positive associations. Models used: sex (0 = f, 1 = m) vs. glycans adjusted for age and cohort, age vs. glycans adjusted for sex and cohort, BMI vs. glycans as well as case–control status in the four diseases adjusted for age, sex, and age*sex interaction. *P*-value thresholds were defined by Bonferroni correction as α = 5.56E-04 for the three blocks sex, age, and BMI, and α = 4.17E-04 for the four disease datasets. Combined data from healthy controls of the UC/CD, CRC, and T2D cohorts were used for sex and age models, while BMI values were only available in the T2D cohort.

Intriguingly, the association of age with TPSNG signatures likewise showed prominent differences in fucosylation: Older individuals showed a higher level of fucosylation on their TPSNG (Fig. 2A). The signatures appear to be particularly strong for tri- and tetraantennary glycans and are in line with previous findings from Reiding *et al.* [23] in the Leiden Longevity Study. No associations of the BMI with fucosylation were found in the data summarized in Fig. 2 which is again in line with the results of the Leiden Longevity Study (fig. 3 [23]). However, Knezevic *et al.* [22] reported a negative association of core fucosylation with BMI.

The bisection signatures were very diverse, with overall positive associations with age and diverse patterns of associations with sex and BMI (Fig. 2A,B). In older people, bisection of fucosylated diantennary glycans was increased in species with and without sialic acid (traits A2FSB and A2FS0B, respectively). These traits are mainly attributable to immunoglobulin (Ig) A and G bisection (Fig. 1B and [24]). The positive association with age for the presumably IgG-related trait A2FS0B is in line with the Leiden Longevity Study [23]. Likewise, for IgG1 Fc glycosylation, increased levels of bisection have been reported with increasing age [25]. Regarding differences between sexes, the presumably IgG-related trait A2FS0B was found to be lower in men than in women which is in line with findings in the Leiden Longevity Study [23]. In addition, the sialylated diantennary fucosylated glycans showed higher bisection in females (trait A2FSB) [23]. With increasing BMI, afucosylated sialylated

diantennary glycans showed decreased levels of bisection (A2F0SB). It is unclear which proteins would mainly contribute to this feature.

Galactosylation showed a particularly strong aging signature in line with previous reports [22,26,27]. The most prominent finding relates to the trait A2FS0G which was found to be lower with increasing age (Fig. 2A). This trait is known to be dominated by IgG, and accordingly, decreased levels of IgG galactosylation with increasing age have been described in the literature, both at the level of total IgG glycans [28] and in a subclass-specific manner looking at Fc glycans of IgG1 and IgG2 [5,25,29] which is in accordance with the concept of inflammaging [6,30]. In addition, this IgG-related galactosylation trait was negatively associated with BMI (Fig. 2B), possibly reflecting the chronic inflammation observed in obesity [31].

Sialylation showed linkage-specific association patterns with age and with BMI (Fig. 2A,B). Notably, association patterns with age and BMI were similar, implying possibly shared molecular mechanisms underlying the observed associations of the sialylation in TPSNG. In more detail, α2,6-sialylation of triantennary and tetraantennary glycans was found to be increased with higher age as well as BMI. In contrast, the degree of α2,3-sialylation of triantennary and tetraantennary, but also diantennary glycans, showed the reverse association and was decreased with higher age as well as BMI. Notably, sialylation showed only a few differences between males and females. In males, a higher α2,6-sialylation in diantennary nonfucosylated glycans and a higher α2,3-sialylation in triantennary glycans were found (traits A2F0GE and A3FL). Together with the fucosylation trait A3LF which was likewise found to be higher in males than in females, this points toward higher levels of sialyl-Lewis-type structures in males than in females: (Antennary) Fucose and alpha 2,3-linked sialic acid are structural motifs of inflammation-associated sialyl-Lewis X and sialyl-Lewis A structures on acute-phase proteins [32] and should, therefore, translate into the fucosylation and sialylation traits A3LF and A3FL.

N-glycomic signatures of major human diseases

The growing recognition of the immense potential of glycans for personalized medicine has resulted in roughly 10 000 publications in the last decade (source: PubMed, search term 'glycan AND biomarkers', results 2009–2018). Glycomic changes in plasma, selected tissues, organs, and cell models, with cancer, neurological disorders, diabetes, and aging-related diseases were reviewed more extensively elsewhere [6,7,21,33]. The opportunities and challenges of the field and its obstacles for translation into clinical practice were discussed in detail in a special issue of BBA-General Subjects [34]. Although glycans were presented as promising biomarkers for prognosis, treatment monitoring, and response in various disease settings with follow-up data [10,12,35–38], the majority of publications in the field report on plasma *N*-glycome associations with one specific pathological condition as compared to controls in cross-sectional manner. However, the low comparability between studies due to the use of different glycomics methodologies does not allow to draw conclusions on whether there are plasma glycome signatures specific to a certain disease or disease group to be applied in future diagnostics approaches.

Only a few examples exist showing the potential of plasma glycans in differential diagnosis on larger sample sets, such as antennary fucose in maturity-onset diabetes of the young, arising from a mutation in the HNF1α gene [39], or bisection and galactosylation in liver cirrhosis [40]. Regarding protein-specific glycosylation, that is, after glycoprotein isolation from serum or plasma, core fucosylation of α-fetoprotein seems to be a promising marker for differential diagnosis of hepatocellular carcinoma [41]. Of note, diseases affecting the liver or adjacent tissues seem to be particularly interesting for the elucidation of total plasma *N*-glycome associations, since apolipoproteins and acute-phase proteins originating from the liver contribute a major portion of the glycans found in the human TPSNG [24]. In addition, glycans from IgA, G, and M are detected particularly among the fucosylated diantennary species (Fig. 1). A large body of evidence points at the involvement of IgG glycosylation in various diseases, as reviewed in [5]. Thus, in the following, we will mainly focus on non-IgG-related glycome changes reported for TPSNG by making first attempts to directly compare plasma glycan signatures across three different, major types of diseases, that is, type 2 diabetes (T2D) as a metabolic disease, inflammatory bowel diseases (IBD) as an immunological disorder, and colorectal cancer (CRC) (Fig. 2C). Moreover, we compared our results with the literature, whenever authors presented derived glycan traits reflecting general glycosylation features, such as glycan complexity, sialylation, bisection, galactosylation, and fucosylation, which substantially improves comparability between studies, even if different analytical techniques were used.

Association of *N*-glycome with type 2 diabetes

Glycosylation has been shown to be implicated in T2D pathogenesis and progression (diabetic complications), presenting great potential for T2D biomarker and medication development, as has been reviewed by Selak *et al.* [21] in this special issue. T2D-associated TPSNG signatures were recently described in a large case–control cohort, showing changes of all main glycosylation features, also after adjustment for age, sex, and risk factors for T2D [13] (excerpt in Fig. 2C; age- and sex-adjusted). The study relied on an automated matrix-assisted laser desorption/ionization time-of-flight mass spectrometry platform which enabled the quantification of 70 glycan structures, including sialic acid linkage isomers [42]. Other studies used techniques with lower resolution and without resolving sialic acid linkage isomers, or they removed sialic acids prior to analysis, resulting in the detection of six up to 39 individual structures [27,43,44].

Itoh *et al.* and Testa *et al.* [43,44] showed somewhat contradictory results on changes of two different core-fucosylated diantennary glycan species. Although both studies were similar with regard to the detection of glycan species after desialylation, the low resolution of both techniques bearing the risk of overlapping peaks and the low sample size used in Itoh *et al.*'s report may explain the contradictory findings. In contrast, in

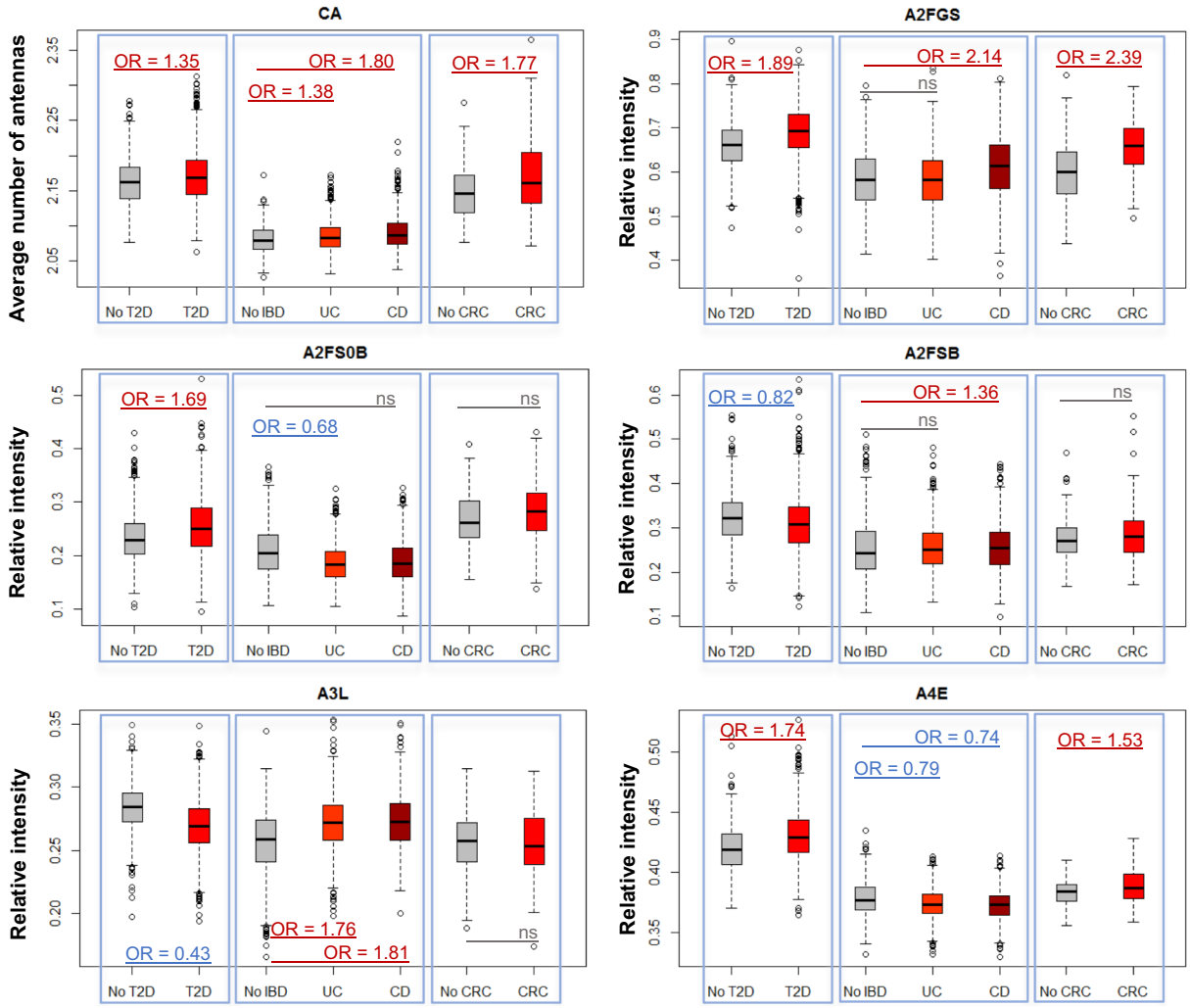

Fig. 3. Selected derived glycan traits in healthy controls (gray) vs. diseased individuals (red) with T2D, UC, CD, or CRC. The 25th, 50th, and 75th percentiles and whiskers at 1st quartile minus 1.5*interquartile range (Q1 − 1.5 * IQR) and Q3 + 1.5 * IQR of the relative intensities are shown. Boxplot data are not adjusted for covariates or for potential differences caused by sample collection, preparation, or measurement, whereas odds ratios shown are adjusted for age, sex, and the interaction thereof, and 'ns' stands for 'not significant' derived from logistic regression analysis as described in Fig. 2C.

various large cohort studies using higher-resolution techniques a reduced fucosylation of diantennary structures in T2D and metabolic syndrome was found, notably, in Caucasians as well as Chinese and Ghanaian populations, even after adjustment for possible confounders including age, sex, and BMI [13,27,45]. Consistent results were reported for IgG fucosylation in the same, large T2D case–control cohort as presented here [46].

Increased branching/antennarity as well as sialylation of both di- and triantennary glycans were consistently reported by our and other groups (Fig. 2C) [13,27] and were furthermore associated with a higher risk for T2D in a prospective cohort [36]. Importantly, our approach for assessing sialylation of different glycan-derived traits in linkage-specific manner has revealed that the sialylation increase in T2D was driven by a relative increase in α2,6-sialylation, while α2,3-sialylation in several glycan groups was decreased in T2D (A2L, A3L, A4L; Fig. 2C) [13]. To our knowledge, this was the first report on a disease-related (relative) decrease in α2,3-sialylation in TPSNG. A high-expression allele of the ST6GAL1 gene, encoding for the enzyme that attaches sialic acids to glycans in α2,6-linkage, has indeed recently been linked to T2D in a genome-wide association study [47].

Regarding bisection, our results point out that a more differentiated view on derived traits is crucial for a detailed *N*-glycome data interpretation. Importantly, IgG- vs. IgA/M-related glycans seem to have divergent trends in the T2D-associated TPSNG, as reflected in an increase of bisection in fucosylated, asialylated species (A2FS0B; mainly IgG-Fc-derived [24]) and a decrease of bisection in the sialylated variant (A2FSB; mainly IgA/M-derived [24]) (Fig. 2C and Fig. 3 middle panel) [13]. When simply assessing the composite-derived trait of bisection in diantennary glycans, one may miss out relevant associations, since the effects in the two different glycan classes might cancel each other out, as might have happened in Adua *et al*. [27]. Similarly, the galactosylation of diantennary species showed a positive association with T2D in nonfucosylated glycans (A2F0SG), in contrast to a negative association in fucosylated, nonsialylated variants that are, for a large part, likely derived from IgG-Fc (A2FS0G; Fig. 2C).

Association of *N*-glycome with inflammatory bowel diseases

Multiple TPSNG features are associated with ulcerative colitis (UC) and Crohn's disease (CD), disease severity, and common markers of inflammation, such as C-reactive protein and erythrocyte sedimentation rate [10,48,49]. A high ratio between multibranched sialylated and galactosylated diantennary glycans in UC patients showed higher prognostic value than the two commonly used markers [48]. In inflammatory diseases including IBD, the altered fucosylation and galactosylation of diantennary glycans can be attributed to changes in relative levels and the glycosylation of Igs, especially IgG [5,50], while the increased branching and sialylation were mostly caused by glycans derived from α1-acid glycoprotein and other acute-phase proteins released mainly by the liver [24,51,52] (Fig. 1). Interestingly, IgG-Fc fucosylation changes differed between UC and CD [50]. Similarly, the bisection of fucosylated, sialylated glycans (A2FSB), which is mostly attributable to IgA, IgM, and possibly also IgG-Fab glycans, was increased in CD, but not significantly affected in UC [10]. A previous study on captured IgA1 was not able to find *N*-glycan associations with UC or CD in contrast to IgA *O*-glycosylation. However, the authors focused on IgA galactosylation and did not mention bisection [53].

When looking at IgG-Fc glycans, bisection in both TPSNG (A2FS0B) and isolated-IgG data was decreased in UC. However, the latter trait showed contradicting trends between discovery and replication cohort [10,50]. Different medication regimens might have affected the disease associations in this case and should be considered in future studies on TPSNG disease markers. Similarly, the fucosylation of triantennary glycans, especially in glycans carrying two fucoses which are indicative of antennary fucosylation (A3F, A3Fa), differed in its association patterns with IBD between the discovery and replication cohorts [10].

Strikingly, a detailed analysis of derived traits of sialylation revealed divergent associations with IBD: While α2,3-sialylation was increased in di-, tri-, and tetraantennary structures, α2,6-sialylation of tetraantennary glycans showed negative associations with disease [10] (Fig. 2C).

Association of *N*-glycome with colorectal cancer

One of the commonly used CRC markers, carcinoembryonic antigen (CEA), is a heavily glycosylated protein. Its protein levels, disregarding its glycosylation, are being used in different clinical CRC settings, especially prognosis. However, CEA testing lacks both sensitivity and specificity in diagnosis of asymptomatic patients [54]. Several glycomics marker sets have shown an improved diagnostic performance compared to CEA or other clinical parameters such as C-reactive protein. For example, compared to healthy controls, a decreased core fucosylation on nonsialylated total

serum *N*-glycans was reportedly found in CRC [12,38,55]. This mostly affects diantennary glycans. In contrast, the fucosylation of triantennary structures, including doubly fucosylated ones (CFa in Fig. 2C), mainly reflects antennary fucosylation, which was increased in CRC [12]. Furthermore, galactosylated diantennary glycans decreased and, at the same time, tri- and tetragalactosylated glycans increased, along with an increase in highly branched sialylated glycans [38]. With regard to sialic acid linkage, both α2,3- and α2,6-sialylation were increased in CRC (Fig. 2C). Several TPSNG glycan features were also associated with surgical therapy success and patient survival [12]. Possible mechanisms behind these changes were previously reviewed in [33,56].

Conclusions and perspectives

Comparing the disease associations of TPSNG reported in the literature by others and our own studies (summarized in Fig. 2C), we can conclude that the following glycan changes are shared between different diseases and probably reflect chronic inflammation: decreased IgG-Fc galactosylation (A2FS0G), increased branching/antennarity, and increased sialylation per galactose in fucosylated diantennary structures (CA and A2FGS; Fig. 3 upper panel). A2FGS and IgG-Fc galactosylation, moreover, seem to be strong indicators of aging in healthy individuals, supporting the inflammaging concept. Fucosylation on the antenna and on larger glycans (CFa, A34F, A3EF, A3LF) showed positive associations with age, male sex, and CRC. Interestingly, the fucosylation of diantennary structures was increased with age, while it was generally decreased with the here presented diseases.

Of note, bisection was positively associated with age in different glycan types, but showed differential patterns of disease associations, especially when comparing IgG-Fc- vs. IgA-related bisection (A2FS0B and A2FSB in Fig. 3 middle panel). Of note, IgA-related bisection (A2FSB) showed a negative association with T2D only, in contrast to its positive association with CD and age (Fig. 2). The glycosylation of both Igs is thus worthwhile to investigate further, especially since IgA is a heavily glycosylated Ig which is highly abundant in plasma and secretions and for which the functional effects of, in particular, *N*-glycosylation are not well explored [7,57].

Considering that T2D is regarded as an aging disease, it is remarkable that TPSNG association patterns of age, BMI, and T2D show a large overlap, especially in the generally increased α2,6-sialylation and otherwise mostly decreased α2,3-sialylation. In contrast, α2,3-sialylation was increased in both IBD and CRC. Possibly, both higher α2,6- and α2,3-sialylation are general inflammatory markers shared by many diseases. However, in T2D specifically, overexpressed α2,6-sialylation may also have a role in pathogenesis [21], leading to a relatively higher increase of α2,6-sialylation over α2,3-sialylation, as observed in our large T2D study (Fig. 3 lower panel). Of note, α2,6-sialylation of tetraantennary glycans was the only sialylation trait that was negatively associated with UC and CD (Fig. 3 lower panel).

Our knowledge on the utility of IgG-Fc glycosylation as promising biomarker for disease severity and therapy monitoring has rapidly increased over the last decade due to technological advances enabling a high throughput, broad applicability, high standardization, and, thus, comparability of results between different centers and clinical settings. However, in case of the glycosylation of other plasma proteins which are less abundant but whose glycosylation is more complex, such as IgA or AGP, or for the entire TPSNG, further adjustments are needed to make substantial contributions to both biomarker research and the elucidation of disease mechanisms.

Although clinical translation of most glycomic markers is still pending, the recently launched Glyco Liver Profile of Helena Biosciences is an excellent first example for serum glycomics in the clinics. This test relies on the analysis of a limited set of serum glycans for assessing liver health and detecting markers of inflammation, fibrosis, cirrhosis, and hepatocellular carcinoma (http://www.helena-biosciences.com/en/clinical-electrophoresis/v8-nexus/tests/glyco-liver-profile/). The test does not cover many of the disease-associated TPSNG features highlighted in this review, for example, sialylation. Nevertheless, it shows a roadmap for clinical translation of glycomic markers for a range of inflammatory and malignant diseases.

Here, we presented direct comparisons of glycosylation features between different clinical cohorts which were only possible, since we applied comparable techniques and robust data processing pathways for all three cohorts. Employing linkage-specific derivatization of sialic acids has revealed potential differences between disease signatures, which have not been reported previously. As explained in more detail in a recent review, there is a need for analytical technologies with quantitation of glycan structural isomers which can exert differential biological effects [58]. Although techniques such as porous graphitized carbon liquid chromatography, capillary electrophoresis, ion mobility, or fragmentation-based mass spectrometry approaches are very powerful in isomer separation [58–60], their applicability for larger clinical studies is

to date hampered due to the resulting complexity of the data and the concurrent lack of efficient bioinformatic approaches for data processing to tackle this complexity. Moreover, as demonstrated by our comparative data re-analysis here, having consensus approaches in data analysis comes with additional advantages, especially since a large body of glycomic data from studies with many different disease contexts and clinical parameters are available and can be re-used for further comparative analyses. Hereby, we would like to stress again the importance of calculating derived traits that reveal patterns within glycans of similar physicochemical properties reflecting specific steps in their biosynthetic pathway. This can improve the robustness of data analysis and can further enhance the interpretation of biological relevance of the observed glycan signatures.

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