N- AND O-LINKED GLYCAN PROFILES (UPDATED REPORT JANUARY 2016)

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DETERMINATION OF N- AND O-LINKED GLYCAN PROFILES

1. Samples

The samples, altogether eight cell pellets, were received at Glykos in good condition on October 15, 2015. There were two parallel Giantin cell samples (GIANTIN 1 and 2) and two Control samples (CON 1 and 2), with separate aliquots from both cell types for N-glycan (N) and O-glycan (O) analyses:

GIANTIN 1 N
GIANTIN 2 N
CON 1 N
CON 2 N
GIANTIN 1 O
GIANTIN 2 O
CON 1 O
CON 2 O

Giantin cells might have differential glycosylation compared to Control cells. This hypothesis was addressed by mass spectrometric profiling analysis of liberated total N-glycan and O-glycan fractions from both cell types.

2. Glycan analysis methodology

Glycoproteins were isolated from the cell pellets by precipitation with 90% ice-cold acetone and the supernatant was discarded. N-glycans were detached from the glycoproteins by PNGase F digestion (Prozyme, Hayward, CA) in 20mM sodium phosphate buffer (pH 7.3) overnight at +37°C. The detached glycans were then purified by micro-scale chromatography with Hypersep C_{18} and Hypercarb (Thermo Scientific) sorbents: 1) neutral glycans were eluted from the carbon column with 25% acetonitrile in water (v/v) and 2) acidic glycans with 0.05% trifluoroacetic acid and 25% acetonitrile in water (v/v). The acidic glycans were further purified by absorbing them to MassPREP™ HILIC µElution (Waters, Milford, MA) in 90% acetonitrile, and eluting by 20 mM NH_4HCO_3, passing in water through strong cation-exchange resin (Bio-Rad Laboratories, Hercules, CA) and C_{18} silica resin (Millipore, Billerica, MA). O-glycans were detached from the glycoproteins by alkaline β-elimination in 0.1 M NaOH and 1 M $NaBH_4$ at +60°C for 6 hours. The reaction solution was neutralized and the borohydride was destroyed by addition of dilute acetic acid, after which the solution was evaporated to dryness. The detached O-glycan alditols were then purified by micro-scale chromatography as described above and separated into neutral and acidic glycan fractions.

Matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry was performed with a Bruker Ultraflex III TOF/TOF instrument (Bruker Daltonics, Bremen, Germany). Neutral N- and O-glycans were detected in positive ion reflector mode as $[M+Na]^+$ ions, and acidic N- and O-glycans in negative ion mode as $[M-H]^-$ ions. Relative molar abundances of neutral and acidic glycan components were assigned based on their relative signal intensities in the mass spectra when analyzed separately as the neutral and acidic glycan fractions. The mass spectrometric raw data was converted into the present glycan profiles by removal of the effect of isotopic pattern overlapping, multiple alkali-metal adduct signals, products of elimination of water from reducing oligosaccharides, and other interfering mass spectrometric signals not arising from the sample similarly as previously described (Satomaa et al. 2009, BMC Cell Biol. 10:42). The identified glycan signals in the glycan profiles were normalized to 100% to allow relative quantitation between signals and reliable comparison of samples. The glycan signals were then assigned to biosynthetic groups based on their proposed monosaccharide composition.

3. Results

The **mass spectrometric profiles of neutral N-glycans** are presented in **Figure 1** along with the proposed monosaccharide compositions. Overall, the neutral N-glycan profiles were quite typical to human cells. The major neutral N-glycans in all samples, over two-thirds of the total neutral N-glycans, were typical high-mannose type glycans with compositions H5N2-H9N2 i.e. N-glycans with 5-9 hexose residues (mannose) and 2 N-acetylhexosamine residues (N-acetylglucosamine). High-mannose type N-glycans as well as the major differing glycan signals are shown as schematic structures based on known human N-glycosylation biosynthetic pathways. Other N-glycan structural classes shown in **Figure 1** that were identified based on the monosaccharide compositions were low-mannose, hybrid, monoantennary and complex type N-glycans. See **Table 1** for relative quantities of the different biosynthetic glycan class, as well as for the identified differing glycan biosynthetic groups between Giantin and Control cells.

In addition to N-glycans, also **soluble glycans** were detected in the neutral glycan samples, characterized by the presence of only one N-acetylhexosamine residue in the monosaccharide composition (these glycan signals are not shown in the N-glycan profile of **Figure 1**, but they are shown in **Table 1**). These soluble glycans may arise from catabolism of inproperly folded and degraded glycoproteins. Giantin cells contained more soluble glycans than Control cells.

Acidic N-glycan profiles are shown in **Figure 2** and the corresponding biosynthetic group analysis in **Table 2**. Acidic N-glycan profiles of the present samples were again typical to human cells. Most acidic glycans were sialylated i.e. they carried a charged sialic acid (N-acetylneuraminic adic). Minor acidic N-glycans had a typical composition corresponding

to phosphorylated glycans. There was a lower level of multisialylated glycan species in Giantin cells compared to Control cells, indicating lower level of overall sialylation in Giantin cells (**Figure 2**).

Relative proportions of neutral and acidic N-glycans cannot be determined in the present analyses. Typically, about about 50% of N-glycans in cultured human cells are neutral and about 50% are acidic.

Regarding potential differences in **polylactosamine glycans** between Control and Giantin samples, the present analyses do not directly measure polylactosamines. However, they should be included in the *Large N-glycans* glycan biosynthetic group (expressed as % of complex-type N-glycans) in **Tables 1 and 2**. However, the mass spectrometric profiling analysis as performed here does not differentiate between different isomeric structures classified as Large N-glycans, namely 1) branched structures, such as tri- or tetra-antennary N-glycans, and 2) polylactosamines. Further structural analyses such as endo-β-galactosidase digestions and MS/MS fragmentation experiments could be done in a follow-on analysis project. Alternatively, Western blotting could be performed with cell lysates with specific anti-glycan antibodies or lectins such as tomato lectin (LEA) or L-PHA to detect polylactosamines and branched N-glycans, respectively.

O-glycan analysis results are shown for acidic O-glycans in **Table 3**. Neutral O-glycans were not detected in reasonably abundant amounts and therefore only acidic O-glycans could be analysed in the present work (data not shown). Perhaps the amount of cells in the present sample was not enough for analyzing the more scarce neutral O-glycans; it is possible that from a larger scale preparation, better signals could be obtained from neutral O-glycans, too. However, it was clear that similarly as in acidic N-glycans, also **acidic O-glycans detected in Giantin cells showed lower sialylation level than in Control cells (Table 3)**.

Neutral O-glycans are of interest regarding the present samples since previous results have suggested a reduction in **O-glycosylation of LAMP1**. LAMP1 is a large glycoprotein that carries numerous N-glycans and also O-glycans (Carlsson et al. 1993, Arch. Biochem. Biophys. 304:65-73). LAMP glycosylation has been studied in iPSC-derived cardiomyocytes (Raval et al. 2015, J. Biol. Chem. 290:3121-36). However, the present analyses show O-glycans recovered from total cellular glycoproteins, which are not necessarily representative of O-glycans of LAMP glycoproteins. In the present O-glycan profiles polylactosamine-modified O-glycans are not among the detected glycans – in contrast, only small sialylated O-glycans were detected. Also, the few signals that were observed from the neutral O-glycans of the present samples had too small molecular weight to carry polylactosamine chains. It is possible that the potential polylactosamines are restricted to LAMPs and cannot be detected in the total cellular O-glycan profiles of the present samples due to more abundant smaller structures and too little sample amount. In a potential in a follow-on study, it should be feasible to purify LAMP1 (Carlsson et al. 1993) or at least enrich it by WGA lectin affinity chromatography (Carlsson et al. 1988, J. Biol. Chem. 263:18911-9), since previous reports have shown that LAMPs are major glycoproteins in some cancer cells (e.g. 1/1000 of total protein; Carlsson et al. 1988). Protein bands cut from SDS-PAGE gels blotted to PVDF membranes could be analyzed, for example by direct mass spectrometric analysis (Satomaa et al. 2002, Blood 99:2609-11) or by structure-specific anti-glycan antibodies, glycosidase enzymes or lectins.

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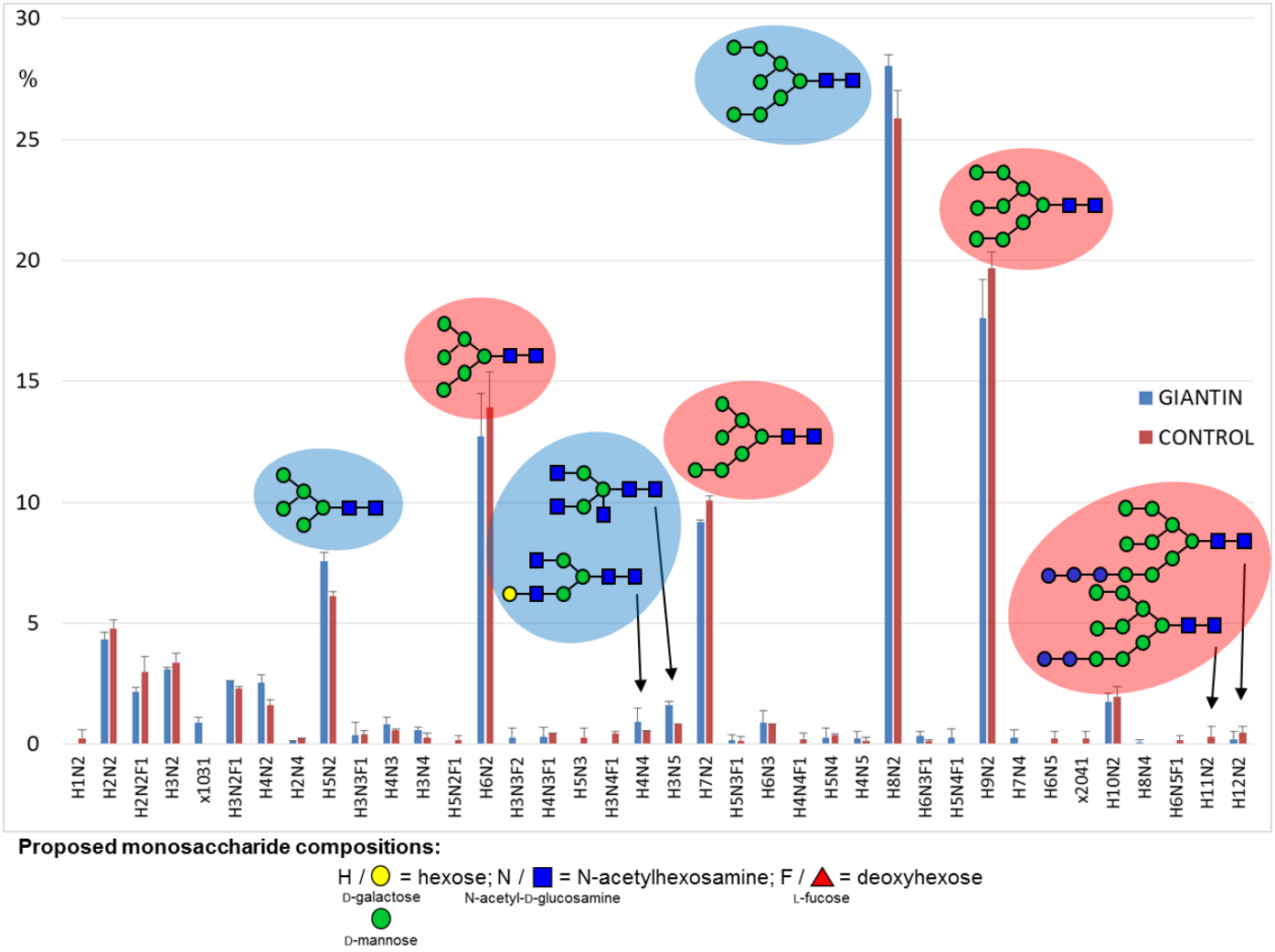


Figure 1. MALDI-TOF mass spectrometric profiles of neutral N-glycans liberated from Giantin (blue columns) and Control (red columns) samples. Y-axis shows relative abundance of the glycans as percent of total detected glycan signals. The columns show average values of two samples. Error bars show standard deviation. 38 most abundant glycan signals are shown. Glycans are detected as as $[M+Na]^+$ ions. Proposed structures of glycan signals differing between the Giantin and Control samples are indicated with blue and red highlighting, respectively (statistical significance was not evaluated). Signals marked with *x* could not be assigned to a monosaccharide composition.

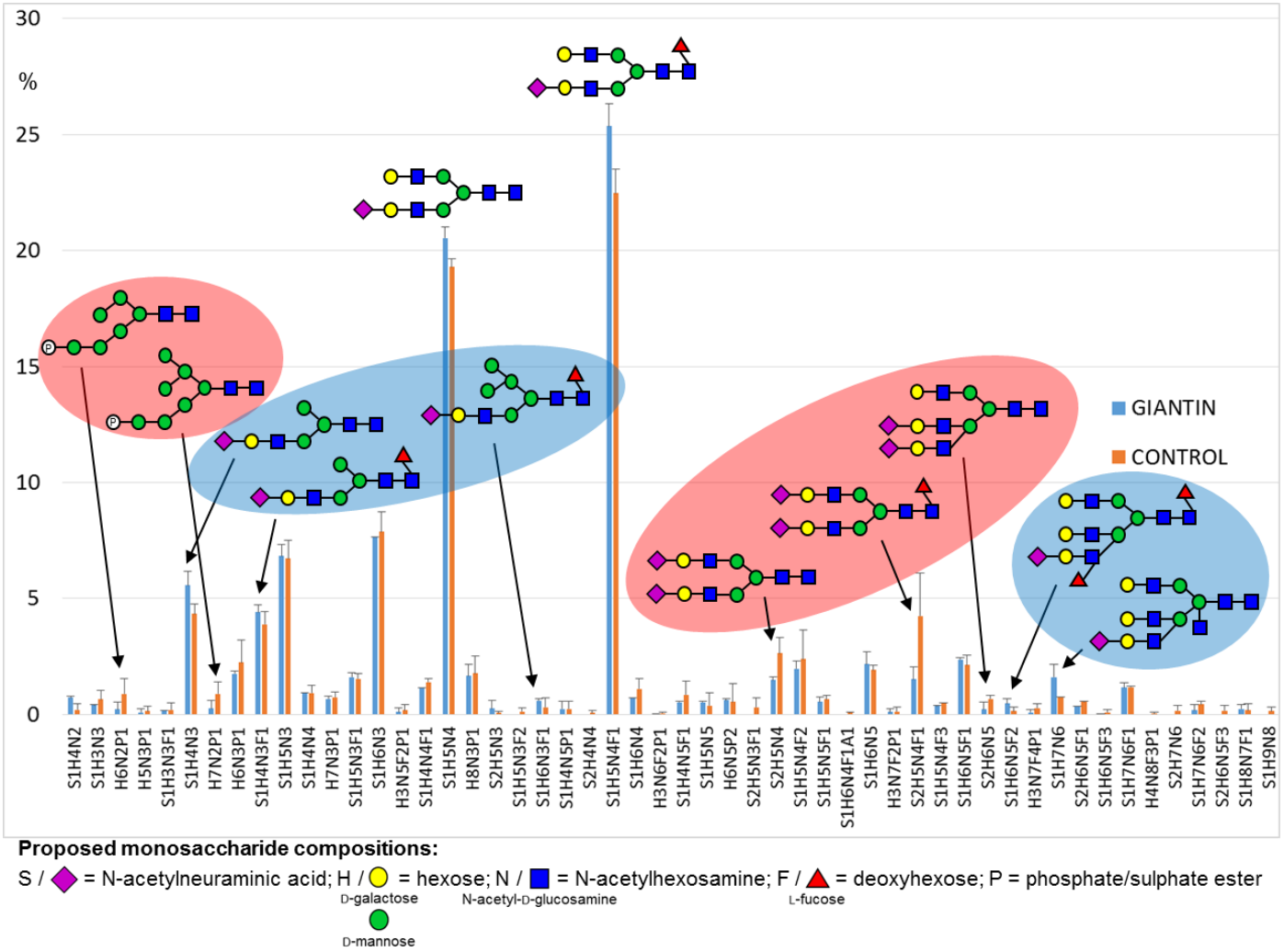


Figure 2. MALDI-TOF mass spectrometric profiles of acidic N-glycans liberated from Giantin (blue columns) and Control (red columns) samples. Y-axis shows relative abundance of the glycans as percent of total detected glycan signals. The columns show average values of two samples. Error bars show standard deviation. 52 most abundant glycan signals are shown. Glycans are detected as as $[M-H]^-$ ions. Proposed structures of glycan signals differing between the Giantin and Control samples are indicated with blue and red highlighting, respectively (statistical significance was not evaluated). Major difference between samples was **lowered sialylation level in Giantin cells**, as shown by higher amount of monosialylated glycan species (e.g. S1H5N4, S1H5N4F1, S1H6N5F2 and S1H7N6) and lower amount of disialylated glycans (e.g. S2H5N4, S2H5N4F1 and S2H6N5) compared to Control samples.

Table 1. Neutral N-glycan structural grouping. Glycan biosynthetic groups differing between the Giantin and Control samples are indicated with blue and red highlighting, respectively (statistical significance was not evaluated).

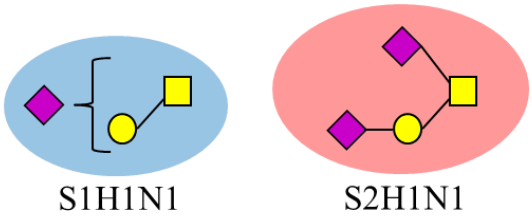
		Averages	
		GIANTIN	**CONTROL**
N-glycan biosynthetic group		**%**	**%**
High-mannose type	% of N-glycans	77,1	78,5
Low-mannose type	% of N-glycans	14,7	15,2
Hybrid-type	% of N-glycans	3,1	2,7
Complex-type	% of N-glycans	4,1	3,1
Other	% of N-glycans	1,0	0,4
Soluble (HexNAc 1)	% of total glycan signals	28,6	23,7
Large-N-glycans	% of complex-type N-glycans	0,0	10,7
Biantennary-size	% of complex-type N-glycans	12,9	12,6
Monoantennary	% of hybrid-type N-glycans	14,8	15,1
Glucosylated	% of high-mannose type N-glycans	2,6	3,4
Small high-mannose type	% of high-mannose type N-glycans	9,8	8,0
Fucosylation	% of N-glycans	6,4	7,3
Fucosylation in low-mannose type	% of low-mannose type N-glycans	32,5	34,5
Fucosylation in hybrid-type	% of hybrid-type N-glycans	43,8	40,4
Fucosylation in complex-type	% of complex-type N-glycans	6,9	24,0
Complex fucosylation	% of N-glycans	0,3	0,0
Terminal HexNAc	% of N-glycans	2,5	1,9

Table 2. Acidic N-glycan biosynthetic structural grouping. Biosynthetic groups differing between the Giantin and Control samples are indicated with blue and red highlighting, respectively (statistical significance was not evaluated).

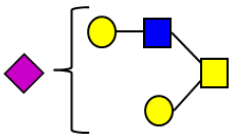
		Averages	
		GIANTIN	**CONTROL**
N-glycan biosynthetic group		**%**	**%**
High-mannose type	% of N-glycans	0,80	1,78
Hybrid-type	% of N-glycans	31,78	30,97
Complex-type	% of N-glycans	66,56	67,05
Large-N-glycans	% of complex-type N-glycans	16,54	15,06
Biantennary-size	% of complex-type N-glycans	77,29	77,00
Monoantennary	% of hybrid-type N-glycans	33,28	29,33
Fucosylation	% of N-glycans	43,60	44,52
Complex fucosylation	% of N-glycans	3,50	4,48
Terminal HexNAc	% of N-glycans	1,16	1,75
Terminal HexNAc (bisecting-size)	% of complex-type N-glycans	1,62	1,57
Proportion of sialylated glycans	% of N-glycans	93,99	92,11
Proportion of sulfated/phosphorylated glycans	% of N-glycans	6,24	8,13
Acetylation	% of N-glycans	0,00	0,07

Table 3. MALDI-TOF mass spectrometric profiles of acidic O-glycans. Glycan alditols were detected as $[M-H]^-$ ions. Proposed compositions of the detected glycan signals are indicated. Glycan signals differing between the Giantin and Control samples are indicated with blue and red highlighting, respectively (statistical significance was not evaluated). Major difference between samples was **lowered sialylation level in Giantin cells**, as shown by higher amount of monosialylated glycan species (S1H1N1) and lower amount of disialylated glycan (S2H1N1) compared to Control samples. Proposed structures of the detected O-glycans are shown below.

		%				Average	
Composition	**m/z**	GIANTIN 1O	GIANTIN 2O	CON 1O	CON 2O	**GIANTIN**	**CONTROL**
S1H1N1	675,2	88	92	80	84	90	82
S2H1N1	966,3	9	6	18	13	7,5	15,5
S1H2N2	1040,4	3	2	2	3	2,5	2,5



S1H1N1 S2H1N1



S1H2N2

Proposed monosaccharide compositions:

S / ◆ = N-acetylneuraminic acid; H / ○ = hexose; N / □ = N-acetylhexosamine

D-galactose N-acetyl-D-galactosamine