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24 **ABSTRACT**

25 The study of protein glycosylation can be regarded as an intricate but very important
26 task, making glycomics one of the most challenging and interesting, albeit under-
27 researched, type of “omics” science. Complexity escalates remarkably when
28 considering that carbohydrates can form severely branched structures with many
29 different constituents, which often leads to the formation of multiple isomers. In this
30 regard, ion mobility (IM) spectrometry has recently demonstrated its power for the
31 separation of isomeric compounds. In the present work, the potential of traveling wave
32 IM (TWIMS) for the separation of isomeric glycoconjugates was evaluated, using
33 mouse transferrin (mTf) as model glycoprotein. Particularly, we aim to assess the
34 performance of this platform for the separation of isomeric glycoconjugates due to the
35 type of sialic acid linkage, at the intact glycoprotein, glycopeptide and glycan level.
36 Straightforward separation of isomers was achieved with the analysis of released
37 glycans, as opposed to the glycopeptides which showed a more complex pattern.
38 Finally, the developed methodology was applied to serum samples of mice, to
39 investigate its robustness when analysing real complex samples.

40

41

42 **1. Introduction**

43 Glycosylation is by far one of the most common and complex posttranslational
44 modifications, with more than half of all secretory and cellular proteins being
45 glycosylated [1–3]. Carbohydrates enhance the functional diversity of proteins, but they
46 can also define their destination or elicit an immune response. The presence of glycans
47 in the surface of eukaryotic cells is vital, as they take part in important cellular events,
48 such as cell–cell interactions and receptor recognition [4]. Notwithstanding its
49 importance and the major role of glycosylation in a multitude of biological processes
50 [5–7], the analysis and characterization of carbohydrates is usually difficult due to their
51 inherent complexity - the main reason why advances in glycomics have been scarcer
52 compared to other “omics” sciences [8,9]. Very often, in contrast to more linearly
53 assembled biological molecules such as proteins or oligonucleotides, carbohydrates can
54 form complex structures, severely branched, with many monosaccharide constituents,
55 which usually results in a multitude of isomers [10].

56 Mass spectrometry (MS)-based techniques are the prime option for the characterization
57 of glycoproteins, as reliable structural information can be obtained [7,11]. MS is
58 frequently used in conjunction with chromatographic or electrophoretic separation
59 techniques, as this allows high sensitivity profiling and accurate characterisation of
60 heterogeneous glycan structures [12–14]. However, when analysing isomeric glycan
61 structures, MS often fails to separate them [8,15–17], as they have identical mass and
62 atomic composition. Some authors have suggested alternative strategies to separate
63 isomeric glycoconjugates based on their derivatization, the use of capillary
64 electrophoresis (CE) or hydrophilic interaction liquid chromatography (HILIC)[18–23].
65 But even then, derivatization protocols can be time-consuming, expensive or hinder the

66 ionization of some glycans, or, in the case of CE or HILIC, the unambiguous
67 identification is still impossible when different isomers coelute. Moreover, in the last
68 few years, several tandem mass spectrometry (MS/MS) methods have been reported
69 that allow the identification of glycan isomers and the characterization of their structure
70 [24–26]. However, few authors have studied the fragmentation of glycans with different
71 sialic acid linkages. Even then, distinguishing by MS/MS between isomeric glycans due
72 to sialic acid linkage is not trivial and, quite often, is based on differences in the relative
73 abundance of certain fragment ions [27,28]. Therefore, a straightforward technique that
74 helps to separate and differentiate those isomeric compounds is much needed.

75 In this regard, ion-mobility (IM) spectrometry coupled with MS has aroused some
76 interest in the last years, not only in the glycomics field but also in other omics sciences,
77 as a proficient analytical technique for the separation of isomeric compounds
78 [3,8,10,15–17,29–31]. Ion mobility provides an additional dimension for the separation
79 of compounds, where ions are not only separated due to their mass and charge, but also
80 on the basis of their shape and size - thereby resolving ions that would be otherwise
81 indistinguishable solely by MS, such as, for instance, isomers [32–36]. Particularly, IM
82 measures the time (drift time) that a particular ion takes to cross a cell filled with an
83 inert, neutral background gas (N_2 and He are most commonly used) at a controlled
84 pressure under the influence of a weak electric field. The drift time of a specific ion is
85 mainly due to ion-gas collisions; therefore, ions are separated due to their ion-neutral
86 collision cross-section (Ω), related to the overall shape and topology of the ion [32–36].
87 Small compact ions have the shortest drift times, i.e. they arrive first, as a result of their
88 smaller Ω . Moreover, the higher the charge of the ion, the greater the accelerating
89 electric force, and therefore the more quickly the ion will cross the chamber.

90 Consequently, the drift time of an ion is often described as being determined by the
91 collision cross-section-to-charge ratio (Ω/z) [35]. When coupled on-line with MS (IM-
92 MS), ion mobility provides three-dimensional analytical information for each detected
93 species, i.e. shape-to-charge, mass-to-charge and abundance, thus allowing reliable
94 analyte identification.

95 Nowadays, there are several IM methods next to the classical drift-time ion mobility
96 spectrometry (DTIMS), such as field asymmetric waveform ion mobility spectrometry
97 (FAIMS), but among them, traveling wave ion mobility spectrometry (TWIMS) is the
98 one that has seen a major growth in the last years [37,38]. In TWIMS, ions are propelled
99 thanks to a sequence of symmetric potential waves continually propagating through a
100 cell, each ion with its own velocity, thus different species transit the cell in different
101 times. One of the main advantages of TWIMS is that it disperses ion mixtures, allowing
102 the simultaneous measurement of multiple species. This, in conjunction with a high
103 sensitivity obtained when TWIMS is coupled to certain analyzers in MS, such as time-
104 of-flight (TOF), has made this platform an alluring option for structural analysis and
105 isomer separation [38–40]. This platform, along with other IM methods, have been
106 recently explored for the analysis of glycans or glycoconjugates by several authors
107 [8,15,16,41–47].

108 In this work, TWIMS combined with TOF-MS was used for the study of
109 glycoconjugate isomers which differ in the type of sialic acid linkage, with mouse
110 transferrin (mTf) as a model glycoprotein. Sialic acid, an important monosaccharide
111 residue of complex type N-glycans, may form primarily two types of linkages: α 2-3 or
112 α 2-6. We aim to assess the capacity of TWIMS-TOF-MS (from now on referred to as
113 IM-MS) as an analytical platform to separate α 2-3 and α 2-6 isomeric glycoconjugates at

114 the intact glycoprotein, glycopeptide and glycan level. The developed methodology was
115 also applied to serum samples of mice, to confirm its robustness when analysing real
116 complex samples.

117

118 **2. Materials and methods**

119 **2.1 Chemicals**

120 All chemicals used in the preparation of buffers and solutions were of analytical reagent
121 grade. Isopropanol (iPrOH), hydrochloric acid (HCl, 37% (w/v)), formic acid (FA, 98–
122 100%), ammonium acetate (NH₄Ac, ≥98.0%) and glycine (≥99.7%) were supplied by
123 Merck (Darmstadt, Germany). CNBr-activated-Sepharose 4B was provided by GE
124 Healthcare (Waukesha, WI, USA) and “NP-40 alternative” by Calbiochem (Darmstadt,
125 Germany). Sodium chloride (NaCl, ≥99.5%), DL-Dithiothreitol (DTT, ≥99%), sodium
126 cyanoborohydride (NaBH₃CN), 2-mercaptoethanol (β-ME), sodium dodecyl sulfate
127 (SDS), iodoacetamide (IAA), ammonium hydrogencarbonate, sodium azide (NaN₃,
128 ≥99.5%) water (LC-MS grade), acetonitrile (LC-MS grade) and mouse apotransferrin
129 (mTf, reference: T0523) were supplied by Sigma–Aldrich (St. Louis, MO, USA) and
130 Tris(hydroxymethyl) aminomethane (TRIS, ≥99.5%) by J.T. Baker (Deventer, Holland).
131 Trypsin (Sequencing grade modified) was provided by Promega (Madison, WI, USA).
132 RapiGest[®] from Waters (Bedford, MA, USA) was used to facilitate the enzymatic
133 digestion. Goat polyclonal antibody against human transferrin (hTf) (immunogen
134 affinity purified) was purchased from Abcam (Cambridge, UK). Human transferrin
135 (hTf) and human α-1-acid glycoprotein (hAGP) were used as additional examples of
136 other glycosylated glycoproteins and were also supplied by Sigma–Aldrich.

137 **2.2 Mice and induction of arthritis**

138 Wild-type (WT) mice were from Harlan Ibérica (Barcelona, Spain). All studies with live
139 animals were authorized by the Institute of Parasitology and Biomedicine "López-
140 Neyra" (IPBLN) and Universidad de Cantabria Institutional Laboratory Animal Care

141 and Use Committees. For the induction of collagen-induced arthritis (CIA), 8-12 weeks-
142 old male mice were immunized as described elsewhere [48,49].

143 **2.3 Purification of serotransferrin from mouse serum samples by immunoaffinity** 144 **chromatography (IAC)**

145 In order to isolate mTf from the rest of serum proteins, an immunoaffinity (IA)
146 purification was carried out using a cyanogen-bromide sepharose column where a
147 polyclonal antibody against human transferrin (hTf) was bound, as detailed previously
148 [50]. The IA procedure consisted of: first, a conditioning step with two washes of 10
149 mM Tris-HCl; second, approximately 25 μ L of serum were diluted 1:8 in 10 mM Tris-
150 HCl (pH 7.6-7.7) in order to improve antigen-antibody interaction, and passed through
151 the column ten times. After washing with 10 mM Tris-HCl and 0.5 M NaCl (pH 7.6-
152 7.7), retained mTf was eluted with 100 mM glycine-HCl (pH 2.5). Eluted mTf was
153 immediately neutralized with 0.5 M Tris. Afterwards, glycine-HCl buffer was
154 exchanged for water by ultracentrifugation, using Microcon YM-10 (MW cut-off 10
155 kDa, Millipore, Bedford, MA, USA). Then, samples were evaporated to dryness using a
156 SpeedVacTM concentrator (Thermo Fisher Scientific, Waltham, MA, USA) and stored at
157 -20°C until use. Finally, the IA column was washed and stored in 10 mM Tris-HCl and
158 0.01% (w/v) NaN₃ (pH 7.6-7.7).

159 **2.4 Analytical approaches for mTf glycosylation study**

160 2.4.1 Intact glycoprotein analysis

161 mTf standard (25 μ g) was desalted using three different procedures: dialysis, size
162 exclusion and ultracentrifugation. Briefly, in the first method, D-TubeTM dialyzers from
163 Merck-Millipore were left in contact with 100 mM NH₄Ac for 15 min. Afterwards, the

164 sample was placed into the dialyzer and left in contact with 500 mL of 100 mM NH₄Ac
165 for 2 h at 4°C. Later, the buffer was renewed with 500 mL and the dialysis was allowed
166 to continue for 2 more hours, repeating this process twice. Finally, the sample was
167 carefully recovered and stored at -20°C until analysis. Regarding the size exclusion
168 procedure, the sample was desalted and the buffer exchanged using Micro Bio-Spin™
169 columns from BioRad (Hercules, California, USA) following the manufacturer
170 instructions. Columns were centrifuged to remove the excess of packing buffer and
171 washed three times with 500 µL of 100 mM NH₄Ac. Finally, the sample was added and
172 collected in a new tube after centrifugation. Lastly, the ultracentrifugation procedure
173 was carried out using Microcon YM-10 (MW cut-off 10 kDa) to desalt and exchange
174 the buffer of the sample. Filters were washed with 100 mM NH₄Ac and centrifuged for
175 10 min at 10000 g. Afterwards, the sample was added and washed with 50 µL of 100
176 mM NH₄Ac a total of 4 times, centrifuging each time for 10 min at 10000 g. Finally, the
177 final volume was recovered in a new vial after centrifugation for 2 min at 1000 g, and
178 reconstituted to the initial volume (25 µL). Centrifugation procedures were carried out
179 in a MiniSpin® centrifuge (Eppendorf, Hamburg, Germany) at room temperature. In all
180 cases, experiments with the standard glycoprotein were carried out in triplicate and
181 intact mTf in 100 mM NH₄Ac was injected directly into the mass spectrometer under
182 non-denaturing conditions and detected in positive ion mode.

183 2.4.2 Glycopeptide analysis

184 mTf standard (25 µg) was reduced, alkylated and immediately subjected to trypsin
185 digestion in the presence of RapiGest® as explained in previous work [51]. Briefly, a
186 solution of 0.5 M DL-dithiothreitol (DTT) in 50 mM NH₄HCO₃ was added to an aliquot
187 of mTf with 0.1% (w/v) RapiGest®. The mixture was incubated at 56°C for 30 min and

188 then alkylated with 50 mM iodoacetamide (IAA) for 30 min at room temperature in the
189 dark. Excess reagent was removed by ultracentrifugation with Microcon YM-10
190 columns (Millipore, Bedford, MA, USA). The final residue was recovered from the
191 upper reservoir and reconstituted with NH_4HCO_3 buffer with 0.1% RapiGest[®]. Trypsin
192 in a 1:40 ratio was added and the mixture was incubated overnight at 37°C. Afterwards,
193 the surfactant was hydrolyzed to avoid MS incompatibilities as follows: formic acid
194 (FA) was added to the digest to a final concentration of 5% (v/v) and the mixture was
195 incubated in the digester at 37°C for 30 min. Then, the solution was centrifuged to
196 separate RapiGest[®] residues. mTf tryptic digests were stored at -20°C until analysis. All
197 the experiments with the standard glycoprotein were carried out in triplicate.

198 For the analysis of glycopeptides, a Waters Nano ACQUITY UPLC[®] was used with a
199 double binary gradient pump, using a peptide BEH C18 column (1.7 μm particle
200 diameter, 130 Å pore, 100 x 0.1 mm length x ID; Waters). Experiments were performed
201 at room temperature with gradient elution at a flow rate of 400 nL min⁻¹. Eluting
202 solvents were A: water with 0.1% (v/v) of formic acid (FA), and B: acetonitrile with
203 0.1% (v/v) FA. Solvents were degassed by sonication (10 min) before use. The
204 optimum elution program was: solvent B from 10 to 60% (v/v) within 20 min as linear
205 gradient, followed by cleaning and re-equilibration steps of B: 60 to 100% (v/v) (5
206 min), 100% (v/v) (5 min), 100 to 10% (v/v) (5 min) and 10% (v/v) (5 min). Before
207 analysis, samples were filtered using a 0.22 μm polyvinylidene difluoride centrifugal
208 filter (Ultrafree-MC, Millipore, Bedford, MA, USA) centrifuging at 10,000 g for 4 min.
209 Sample injection (300 nL) was performed with an autosampler refrigerated at 4°C.
210 Control of the instrument was performed using MassLynx 4.1 (Waters).

211 2.4.3 Glycan analysis

212 IAC purified mTf or mTf standard (25 μ g) was reduced with 0.5% 2-mercaptoethanol
213 (β -ME) and 0.5% SDS in 50 mM NH_4HCO_3 (pH 7.6-7.7) and heated in a thermo block
214 at 100 $^\circ\text{C}$ for 30 min [19]. Once the sample was at room temperature, a volume of 50
215 mM NH_4HCO_3 (pH 7.6-7.7) with 1% (v/v) of NP-40 alternative was added to obtain a
216 final concentration of 0.1% SDS and β -ME in the sample. To release the N-glycans,
217 1 μL of PNGase F (1 U) solution was added to the mixture, which was afterwards
218 incubated at 37 $^\circ\text{C}$ for 18 h. Digestion was stopped by heating the sample in a thermo
219 block at 100 $^\circ\text{C}$ for 5 min. Then, released glycans were isolated by solid phase extraction
220 (SPE) using Hypercarb cartridges (25 mg, 1 mL volume, Thermo Fisher Scientific) and,
221 subsequently, purified by ice-cold acetone precipitation following the procedure
222 reported in [19] in both cases. Reduced glycans were diluted with 50:50 $\text{H}_2\text{O}/\text{ACN}$ with
223 0.1% FA and directly analyzed by IM-MS in negative ion mode. All the experiments
224 with the standard glycoprotein were carried out in triplicate. Mice derived transferrin
225 glycan analysis was carried out in duplicate, including purification and release of
226 glycans.

227 **2.5 Ion mobility-mass spectrometry**

228 For IM-MS analysis a Synapt G2 HDMS instrument from Waters was used. Samples
229 were directly introduced into the mass spectrometer using home-made nano-
230 electrospray ionization (nano-ESI) gold-coated borosilicate capillaries; unless when
231 analysed by nano-UPLC-IM-MS, in which case an in-line nano-ESI interface with
232 commercially available coated needles was used.

233 Spectra were acquired in positive mode for the analysis of intact glycoproteins and
234 glycopeptides, and in negative mode for glycans, and conditions were optimized in each
235 case. The voltages for spray capillary, sampling cone, trap collision energy (CE), trap

236 direct current (DC) bias and transfer CE were, respectively: intact glycoproteins, 1.4-1.6
237 kV, 30 V, 4 V, 40 V and 0 V; glycopeptides, 1.5-1.7 kV, 50 V, 4 V, 20 V and 0 V; and
238 glycans, 1.5 kV, 50 V, 4 V, 45 V and 0 V. The “trap CE” voltage was only increased to
239 60 V when fragmentation of the glycopeptides was the goal. Mass spectrometer control
240 and spectra processing were carried out using MassLynx 4.1 (Waters).

241 The software IMoS [52,53], available for free at *imospedia.com*, was used for the
242 theoretical calculations of the collision cross sections (CCS) of glycans, using their
243 minimum energy structures. The online tool *carbohydrate builder*, available at
244 *glycam.org* [54], was used to generate the required input for theoretical calculations.
245 The tool allows building different glycan isomers based on monosaccharide unit and
246 linkage type, and generates minimum energy structures.

247

248 3. Results and discussion

249 3.1 Intact glycoprotein analysis

250 It is well established that intact proteins can be analysed mainly in two different ways
251 when using electrospray ionization: under denaturing or non-denaturing (i.e., “native”)
252 conditions. In the first case, the protein appears highly charged with a broad charge state
253 distribution; thus, the separation of the different glycoforms is difficult. On the other
254 hand, an advantage of native MS is that it strongly reduces charging, hence the different
255 glycoforms can be more clearly resolved [55].

256 Using native MS in this work, the concentration of ammonium acetate and the nano-ESI
257 source parameters were optimized to improve and obtain the best possible sensitivity
258 and separation between mTf glycoforms in positive ion mode. Moreover, in order to
259 obtain an acceptable separation between the several glycoforms, mTf was washed
260 repeatedly with 100 mM NH₄Ac to eliminate excipients and salts, and injected directly
261 into the mass spectrometer without further separation. Without washing, no glycoform
262 separation was observed whatsoever. Three methods were evaluated to desalt the
263 glycoprotein: dialysis, size exclusion and ultracentrifugation, with the latter one giving
264 the best results (see supplementary Figure S1). Afterwards, the drift times of intact
265 glycoforms were determined by ion mobility. In Figure 1-a, the ion with charge +19 of
266 mTf is shown, as well as the arrival time distributions (ATD) of different sections of
267 this peak (Figure 1-a (i-v)), each corresponding to one intact glycoform of mTf). As can
268 be observed in the ATD profiles, only one, relatively wide peak is obtained for each
269 glycoform. Hence, these results imply that, at least for large glycoproteins with a low
270 degree of glycosylation, the glycan part has barely any influence on the overall size of
271 the whole molecule as the CCS appears virtually unaffected by glycosylation. hTf and

272 hAGP were also studied to see if the same behaviour was observed with other
273 glycoproteins. Figure 1-b and 1-c show the separation between intact glycoforms in the
274 MS spectra of hTf (for the ion with charge +19) and hAGP, respectively. As can be
275 observed, the peak was better resolved for hTf, but no separation was observed for
276 hAGP, as the number of glycoforms was considerably higher and the overlapping of
277 several ion distributions was unavoidable. Moreover, one wide peak was observed again
278 when the drift time of each section of the mass spectrum was measured for both
279 glycoproteins. Regarding hTf, each section corresponded to one glycoform (Figure 1-b
280 (i-iv)), whereas, in the case of hAGP, the resolution was not high enough to ensure that
281 a selected region of the peak corresponded to one defined glycoform (Figure 1-c (i-v)),
282 because of the high glycosylation degree of hAGP. The higher the m/z region of this
283 peak however, the bigger the carbohydrate fraction is (usually meaning more complex
284 and branched glycans). This is due to the fact that an increase of m/z within the same
285 charge state can only be due to an increase in the number of glycan subunits of the
286 carbohydrate fraction, as the polypeptide backbone remains unaltered. For the sake of
287 consistency, we will refer to each section of the peak as glycoform, even if, as in the
288 case of hAGP, they are not resolved. Interestingly, the drift time increased with
289 increasing carbohydrate fraction, i.e. complexity and branching of the glycoforms, in all
290 three cases (see Figure 1-a (i-v), 1-b (i-iv) and 1-c (i-v), type of glycoform indicated
291 where possible). The relative drift time differences between glycoforms were higher in
292 proteins with higher glycosylation content, as can be observed for hAGP (see Figure 1-
293 c). Moreover, if we compare the drift time between different glycoforms of mTf and
294 hTf, the increase in drift time was more noticeable in hTf, as the percentage of
295 glycosylation is slightly higher here in relation to the total protein mass. As Table 1
296 shows, for the same relative increase in m/z , the relative drift time increase is higher for

297 hAGP, which demonstrates that for glycoproteins with a high percentage of
298 glycosylation, differences in the glycosylation have a greater effect on the drift time of
299 the whole molecule (i.e., they significantly alter the CCS of the whole glycoprotein). It
300 could then also be conceivable to separate isomers due to the different sialic acid
301 linkage (of the same glycoform) at the intact protein level if the glycan:peptide ratio was
302 high enough. However, this option would not have been viable for all types of
303 glycoproteins, thus, other alternatives were studied.

304 **3.2 Glycopeptide analysis**

305 As mTf only shows one glycosite at asparagine 494, only one glycopeptide is expected
306 after tryptic digestion (N₄₉₄ glycopeptide, peptide: NSTLCDLCIGPLK). However, with
307 the direct injection of the digest, the N₄₉₄ glycopeptide was not detected, hence, an
308 additional separation before the MS was mandatory. In this regard, nano-UPLC was
309 used as a separation technique prior to IM-MS detection, in order to separate the
310 glycopeptide from the other peptides and simultaneously determine the drift time of the
311 different glycoforms. Injection volume, flow rate and gradient were optimized to obtain
312 the best sensitivity with a stable spray (see section 2.4, Materials and Methods). Table 2
313 shows the detected glycoforms at the peptide level, their theoretical and experimental
314 masses, mass error and observed charge states.

315 Regarding the determination of the drift time of the different glycopeptide glycoforms,
316 even though a range of different values for the wave velocity (WV) and wave height
317 (WH) of the TWIMS device were tested, only one drift time value was observed. This
318 suggests that no isomer separation was possible at this mobility resolution, which is
319 probably due to the fact that the different isomers had similar CCS despite the distinct
320 orientation of the sialic acid. Recently, Hinneburg et al. [15] and Guttman et al. [45]

321 also described this observation analysing glycopeptides directly by IM-MS. They
322 observed no isomeric separation, unless fragmentation of the glycopeptide was carried
323 out and one of the observed smaller fragments still contained the sialic acid. This
324 fragment (obtained before the IM cell) had different drift times depending on the sialic
325 acid linkage, because a change in the orientation of the sialic acid was more noticeable
326 (i.e. the CCS was more affected) in a smaller analyte. We also tested this approach, by
327 fragmenting the most intense glycopeptide glycoform (N(H5N4S2)STLCDLCIGPLK)
328 before the IM cell. Figure 2-a shows the collision induced dissociation (CID) MS/MS
329 spectrum for this glycoform. Several fragments were obtained, e.g., the
330 glycolylneuraminic acid (S1) and H1N1 and H2N1 fragments. Among all the fragments
331 however, H1N1S1 was the one that still retained the sialic acid linkage and had enough
332 intensity to yield a good and accurate drift time measurement. Isomer separation was
333 observed in the aforementioned glycan fragment. Specifically, as it is shown in Figure
334 2-c, two drift time peaks were clearly observed at this m/z, which are postulated to be
335 due to the sialic acid being α 2-6 and α 2-3 linked. To confirm that the sialic acid is the
336 monosaccharide that affects the CCS of the fragment the most, the ATD of the fragment
337 H1N1 is shown in supplementary Figure S2-c. In this case, we only observed one drift
338 time, which demonstrates that it is the sialic acid which causes observable CCS
339 differences seen as two different drift times of the fragment H1N1S1.

340 Moreover, in order to confirm that the distinct drift times observed for the glycan
341 fragment could be due to different sialic acid linkages, the theoretical CCS of the
342 H1N1S1 isomers were calculated. The obtained CCS values can be found in Table 3.
343 The CCS for the two H1N1S1 were different enough to be separated by IM. Taking into
344 account the CCS values of Table 3, the more prominent, lower drift time peak (i.e.,

345 lower CCS) should correspond to the α 2-6 glycan, whereas the second peak represents
346 the α 2-3 glycan.

347 Our results are in accordance with those obtained by Hinneburg et al. [15], who, using
348 modelling, drew the same conclusions. This is also in agreement with Guttman et al.
349 [45], who found that biantennary glycoforms showed lower content of α 2-3 sialylation.
350 In our case, the percentage of α 2-3 glycan was approximately 14% (taking into account
351 the measured area of each drift time peak, see Figure 2-c). However, this ratio can vary
352 depending on the glycoprotein studied [45]. This glycopeptide approach, through the
353 analysis of the fragment H1N1S1, gave valuable information about the content of each
354 type of sialic acid as the fragment H1N1S1 came from both branches of the H5N4S2
355 glycopeptide (we did not detect any fragment with only one antenna remaining (H3N3
356 or H3N3S1), suggesting that both antennas might indeed be fragmented). This relevant
357 information could permit, in the future, to correlate changes in protein sialylation with
358 specific disease biomarkers which involve protein glycosylation, such as, e.g., in cancer
359 or inflammation.

360

361 **3.3 Glycan analysis**

362 Finally, we proceeded with the study of the enzymatically released glycans using
363 PNGase F. Different solvents were tested to obtain the best spray and ionization yield,
364 and a slightly superior glycan signal was obtained at 50:50 H₂O:ACN with 0.1% FA.
365 Table 4 shows the detected mTf glycans along with their theoretical and experimental
366 masses, mass error and observed charge states. In Figure 3 the ATD profile of H5N4S2
367 glycans at different values of WV and WH is shown. Two peaks are observed which are

368 believed to correspond to two different isomers of the glycan H5N4S2, in analogy to the
369 previously obtained results (Figure 2-c) with the fragment H1N1S1 of the glycopeptide
370 N(H5N4S2)STLCDLCIGPLK. Fine tuning of the IM parameters was mandatory in
371 order to resolve both drift time peaks. A WV value of 450 m s^{-1} and WH of 25 V was
372 selected as optimal for the analysis of all mTf glycans. It is worth mentioning that in
373 this case, glycans were analyzed in negative mode and, as can be observed in Figure 3,
374 the first isomer was clearly the less abundant one. This is in contrast to the glycopeptide
375 analysis which was done in positive mode, where the less abundant isomer was the
376 second one. This could be due to the fact that both molecules were, actually, quite
377 different - specifically, the glycan had eleven monosaccharide units, as opposed to the
378 fragment observed in the glycopeptide analysis which had only three. With regard to
379 other glycans, three different peaks, i.e., three drift times, were observed for H6N5S3
380 (see supplementary Figure S3-c), albeit separation was slightly worse than observed for
381 H5N4S2. H5N4S1 on the other hand only showed one wide peak (see supplementary
382 Figure S3-d), implying that only one glycan isomer exists or that the different isomers
383 have similar CCS, and IM is not able to distinguish them (as the absence of other drift
384 time peaks does not preclude the presence of other isomers). It is also worth pointing
385 out that when the glycan contained a fucose unit, the isomeric separation was somewhat
386 hampered, as observed in supplementary Figure S3-b. We reckon that the addition of
387 one extra monosaccharide unit might affect the global CCS of the glycan, and as the
388 whole glycan is bigger, a small variation in the orientation of the sialic acid is less
389 noticeable.

390 To confirm that separation of isomeric glycans due to the sialic acid linkage was also
391 possible with other glycoproteins, hAGP was also digested with PNGase F and the

392 released glycans analysed by IM-MS. Separation of isomeric glycans was achieved,
393 seemingly obtaining the same results as with mTf. As an example, the arrival time
394 distributions of the H5N4S2 and H6N5S2 glycans are shown in supplementary Figure
395 S4. As can be seen, two drift time peaks were obtained for the H5N4S2 glycan and
396 three peaks for H6N5S3, albeit separation was poorer in both cases when compared to
397 mTf.

398 Moreover, to confirm that ion mobility separation of these possible isomers is due to
399 different sialic acid linkages, Table 3 shows the theoretical CCS values of the four
400 possible isomers for the glycan H5N4S2. The differences between these calculated
401 values suggest that the observed ion mobility peaks could be due to isomeric glycans
402 with different types of sialic acid linkage. With the knowledge of theoretical CCS, and
403 the abundance of the α 2-3 H1N1S1 fragment being lower than α 2-6 (based on
404 fragmentation of the H5N4S2 glycopeptide, see above), some conclusions can be
405 drawn. We reckon that the presence of the isomer with two sialic acids α 2-3 can be
406 discarded and that the two peaks observed must correspond to the isomers with higher
407 content in α 2-6, that is, the isomer with two sialic acids α 2-6 and the isomer with one
408 sialic acid α 2-6 and one α 2-3 (although we cannot specify the branch where each sialic
409 acid is located). Alternative approaches for the study of isomeric glycoconjugates, for
410 instance using specific sialidases [18], would however allow to obtain complementary
411 information and reliably assign the different peaks to the corresponding isomers.

412 **3.4 Mouse serum sample**

413 To further assess the ability of the established method to separate isomeric
414 glycoconjugates in biological samples, we measured the drift time of mTf glycans
415 purified from serum samples. Only the analysis of the released glycans by IM-MS was

416 included in this study, as it was found to be the most sensitive and straightforward
417 approach to obtain information about isomeric forms. Two serum samples were
418 analysed: one healthy control and one sample with collagen-induced arthritis (CIA), an
419 autoimmune disease known to alter the glycosylation pattern of mTf [51,58]. As can be
420 seen in Figure 4, two peaks were observed for H5N4S2 glycan and three peaks for
421 H6N5S3 in both samples, with the same drift time and similar relative intensities as in
422 the mTf standard (compare Figure 4 with supplementary Figure S3). Although
423 additional samples are required to observe possible differences in the relative abundance
424 of glycan isomers between control and pathological samples, the presented
425 methodology shows great potential for the separation of isomeric glycans and the
426 discovery of novel biomarkers in glycomic studies.

427

428 **4. Concluding remarks**

429 In the present paper, the capability of IM-MS to separate isomeric glycoconjugates
430 which are due to different types of sialic acid linkage (i.e. α 2-3 and α 2-6) has been
431 evaluated at three different levels: intact glycoprotein, glycopeptides, and the released
432 glycans. Separation of isomeric glycoconjugates is an important task in the glycomics
433 field, because it has been reported that differences in the abundance of some glycan
434 isomers might be of great importance for the early diagnosis or control of, for instance,
435 inflammatory diseases and certain types of cancer.

436 With our current knowledge of glycosylation effects on molecule size, and the limited
437 ability of Synapt IM-MS instrumentation to resolve small CCS differences, isomeric
438 separation cannot be obtained at the intact glycoprotein and glycopeptide level.
439 Released glycans however can be separated after optimization of the IM parameters. As

440 stated before by others [15,45], and also demonstrated in this work, there is a
441 workaround to distinguish different types of sialic acid linkage in glycopeptides that
442 takes into account the mobility of an MS/MS fragment which still retains the sialic acid.
443 This method can however be time-consuming and rather difficult, as glycopeptides must
444 be separated from the rest of the digest and, besides, the obtained glycopeptide fragment
445 is, actually, a glycan.

446 The interest in using ion mobility for glycoconjugate separation and identification has
447 seen a major growth in the last years. Thus, it is likely that new technologies and
448 improvements will become available soon, including the advent of ion mobility
449 instrumentation with up to ten times higher resolution. Therefore, the separation of
450 sialic acid linkage isomers may also become possible at the level of glycopeptides and
451 intact proteins in the future.

452

453

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460

461 **5. References**

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656
657

658 **Figure legends**

659

660 **Figure 1:** Mass spectra showing the ion with charge +19 and the corresponding drift
661 time (arrival time) distributions, or ATD, of a) intact mTf and b) intact hTf; and also c)
662 ions with charge +13, +12 and +11 of intact hAGP and the corresponding ATD. The
663 value indicated corresponds to the approximate glycosylation percentage (w/w) of each
664 protein, calculated as the mass of the most abundant glycan per glycosylation site
665 divided by the mass of the glycoprotein. i)-v): indicates the glycoform or the region of
666 the mass spectrometric peak. H: hexose; N: N-acetylhexosamine; F: fucose; S: sialic
667 acid (in this case, all S are N-glycolylneuraminic acid). The voltages for spray capillary,
668 sampling cone, trap collision energy (CE), trap direct current (DC) bias and transfer CE
669 were, respectively: 1.4-1.6 kV, 30 V, 4 V, 40 V and 0 V.

670

671 **Figure 2:** a) MS/MS spectrum for the mTf glycopeptide glycoform
672 N(H5N4S2)STLCDLCIGPLK; b) mass spectra of a fragment that still keeps the sialic
673 acid (H1N1S1) and c) arrival time distribution of this fragment (m/z range: 673.3-
674 673.5). The symbols used for the representation of the glycoform H5N4S2 follow the
675 Symbol Nomenclature for Glycans (SNFG) rules [59]. H: hexose; N: N-
676 acetylhexosamine; F: fucose; S: sialic acid (in this case, all S are N-glycolylneuraminic
677 acid). The voltages for spray capillary, sampling cone, trap collision energy (CE), trap
678 direct current (DC) bias and transfer CE were, respectively: 1.5-1.7 kV, 50 V, 4 V, 20 V
679 and 60 V.

680

681 **Figure 3:** Arrival time distributions for the H5N4S2 glycan released from mTf at
682 different wave height (WH, in V) and wave velocity (WV, in m s^{-1}) combinations. The
683 symbols used for the representation of the H5N4S2 glycan follow the Symbol
684 Nomenclature for Glycans (SNFG) rules [59]. H: hexose; N: N-acetylhexosamine; F:
685 fucose; S: sialic acid (in this case, all S are N-glycolylneuraminic acid). The voltages for
686 spray capillary, sampling cone, trap collision energy (CE), trap direct current (DC) bias
687 and transfer CE were, respectively: 1.5 kV, 50 V, 4 V, 45 V and 0 V.

688

689 **Figure 4:** Arrival time distributions for the glycans a) H5N4S2 and b) H6N5S3 released
690 from mTf in a healthy mouse serum and a serum from a mouse with collagen-induced
691 arthritis (CIA). The symbols used for the glycan representation follow the Symbol
692 Nomenclature for Glycans (SNFG) rules [59]. H: hexose; N: N-acetylhexosamine; F:
693 fucose; S: sialic acid (in this case, all S are N-glycolylneuraminic acid). The voltages for
694 spray capillary, sampling cone, trap collision energy (CE), trap direct current (DC) bias
695 and transfer CE were, respectively: 1.5 kV, 50 V, 4 V, 45 V and 0 V.

1

2 **Evaluation of Ion mobility for the separation of glycoconjugate isomers due to**
3 **different types of sialic acid linkage, at the intact glycoprotein, glycopeptide and**
4 **glycan level**

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18 Abbreviations: ATD: arrival time distribution; CCS: collision cross section; CIA: collagen-
19 induced arthritis; hAGP: human α -1-acid glycoprotein; hTf: human transferrin; IM-MS: ion
20 mobility – mass spectrometry; mTf: mouse transferrin; nano-ESI: nano electrospray ionization;
21 nano-UPLC: nano ultra performance liquid chromatography; TOF: time-of-flight; TWIMS:
22 travelling wave ion mobility spectrometry.

23 **KEYWORDS:** glycosylation, ion mobility, isomers, mouse transferrin, sialic acid.

24 **ABSTRACT**

25 The study of protein glycosylation can be regarded as an intricate but very important
26 task, making glycomics one of the most challenging and interesting, albeit under-
27 researched, type of “omics” science. Complexity escalates remarkably when
28 considering that carbohydrates can form severely branched structures with many
29 different constituents, which often leads to the formation of multiple isomers. In this
30 regard, ion mobility (IM) spectrometry has recently demonstrated its power for the
31 separation of isomeric compounds. In the present work, the potential of traveling wave
32 IM (TWIMS) for the separation of isomeric glycoconjugates was evaluated, using
33 mouse transferrin (mTf) as model glycoprotein. Particularly, we aim to assess the
34 performance of this platform for the separation of isomeric glycoconjugates due to the
35 type of sialic acid linkage, at the intact glycoprotein, glycopeptide and glycan level.
36 Straightforward separation of isomers was achieved with the analysis of released
37 glycans, as opposed to the glycopeptides which showed a more complex pattern.
38 Finally, the developed methodology was applied to serum samples of mice, to
39 investigate its robustness when analysing real complex samples.

40

41

42 1. Introduction

43 Glycosylation is by far one of the most common and complex posttranslational
44 modifications, with more than half of all secretory and cellular proteins being
45 glycosylated [1–3]. Carbohydrates enhance the functional diversity of proteins, but they
46 can also define their destination or elicit an immune response. The presence of glycans
47 in the surface of eukaryotic cells is vital, as they take part in important cellular events,
48 such as cell–cell interactions and receptor recognition [4]. Notwithstanding its
49 importance and the major role of glycosylation in a multitude of biological processes
50 [5–7], the analysis and characterization of carbohydrates is usually difficult due to their
51 inherent complexity - the main reason why advances in glycomics have been scarcer
52 compared to other “omics” sciences [8,9]. Very often, in contrast to more linearly
53 assembled biological molecules such as proteins or oligonucleotides, carbohydrates can
54 form complex structures, severely branched, with many monosaccharide constituents,
55 which usually results in a multitude of isomers [10].

56 Mass spectrometry (MS)-based techniques are the prime option for the characterization
57 of glycoproteins, as reliable structural information can be obtained [7,11]. MS is
58 frequently used in conjunction with chromatographic or electrophoretic separation
59 techniques, as this allows high sensitivity profiling and accurate characterisation of
60 heterogeneous glycan structures [12–14]. However, when analysing isomeric glycan
61 structures, MS often fails to separate them [8,15–17], as they have identical mass and
62 atomic composition. Some authors have suggested alternative strategies to separate
63 isomeric glycoconjugates based on their derivatization, ~~or~~ the use of capillary
64 electrophoresis (CE) or hydrophilic interaction liquid chromatography (HILIC)—thus
65 they remain undistinguishable unless derivatization or less common stationary phases in

66 ~~liquid chromatography (LC) are used~~ [18–23]. But even then, derivatization protocols
67 can be time-consuming, expensive or hinder the ionization of some glycans, or, in the
68 case of ~~LCCE~~ or HILIC, the unambiguous identification is still impossible when
69 different isomers coelute. Moreover, in the last few years, several tandem mass
70 spectrometry (MS/MS) methods have been reported that allow the identification of
71 glycan isomers and the characterization of their structure [24–26]. However, few
72 authors have studied the fragmentation of glycans with different sialic acid linkages.
73 Even then, distinguishing by MS/MS between isomeric glycans due to sialic acid
74 linkage is not trivial and, quite often, is based on differences in the relative abundance
75 of certain fragment ions [27,28]. Therefore, a straightforward technique that helps to
76 separate and differentiate those isomeric compounds is much needed.

77 In this regard, ion-mobility (IM) spectrometry coupled with MS has aroused some
78 interest in the last years, not only in the glycomics field but also in other omics sciences,
79 as a proficient analytical technique for the separation of isomeric compounds
80 [3,8,10,15–17,29–31]. Ion mobility provides an additional dimension for the separation
81 of compounds, where ions are not only separated due to their mass and charge, but also
82 on the basis of their shape and size - thereby resolving ions that would be otherwise
83 indistinguishable solely by MS, such as, for instance, isomers [32–36]. Particularly, IM
84 measures the time (drift time) that a particular ion takes to cross a cell filled with an
85 inert, neutral background gas (N₂ and He are most commonly used) at a controlled
86 pressure under the influence of a weak electric field. The drift time of a specific ion is
87 mainly due to ion-gas collisions; therefore, ions are separated due to their ion-neutral
88 collision cross-section (Ω), related to the overall shape and topology of the ion [32–36].
89 Small compact ions have the shortest drift times, i.e. they arrive first, as a result of their

90 smaller Ω . Moreover, the higher the charge of the ion, the greater the accelerating
91 electric force, and therefore the more quickly the ion will cross the chamber.
92 Consequently, the drift time of an ion is often described as being determined by the
93 collision cross-section-to-charge ratio (Ω/z) [35]. When coupled on-line with MS (IM-
94 MS), ion mobility provides three-dimensional analytical information for each detected
95 species, i.e. shape-to-charge, mass-to-charge and abundance, thus allowing reliable
96 analyte identification.

97 Nowadays, there are several IM methods next to the classical drift-time ion mobility
98 spectrometry (DTIMS), such as field asymmetric waveform ion mobility spectrometry
99 (FAIMS), but among them, traveling wave ion mobility spectrometry (TWIMS) is the
100 one that has seen a major growth in the last years [37,38]. In TWIMS, ions are propelled
101 thanks to a sequence of symmetric potential waves continually propagating through a
102 cell, each ion with its own velocity, thus different species transit the cell in different
103 times. One of the main advantages of TWIMS is that it disperses ion mixtures, allowing
104 the simultaneous measurement of multiple species. This, in conjunction with a high
105 sensitivity obtained when TWIMS is coupled to certain analyzers in MS, such as time-
106 of-flight (TOF), has made this platform an alluring option for structural analysis and
107 isomer separation [38–40]. This platform, along with other IM methods, have been
108 recently explored for the analysis of glycans or glycoconjugates by several authors
109 [8,15,16,41–47].

110 In this work, TWIMS combined with TOF-MS was used for the study of
111 glycoconjugate isomers which differ in the type of sialic acid linkage, with mouse
112 transferrin (mTf) as a model glycoprotein. Sialic acid, an important monosaccharide
113 residue of complex type N-glycans, may form primarily two types of linkages: α -2→3

114 or α -2→6. We aim to assess the capacity of TWIMS-TOF-MS (from now on referred
115 to as IM-MS) as an analytical platform to separate α -2→3 and α -2→6 isomeric
116 glycoconjugates at the intact glycoprotein, glycopeptide and glycan level. The
117 developed methodology was also applied to serum samples of mice, to confirm its
118 robustness when analysing real complex samples.

119

120 **2. Materials and methods**

121 **2.1 Chemicals**

122 All chemicals used in the preparation of buffers and solutions were of analytical reagent
123 grade. Isopropanol (iPrOH), hydrochloric acid (HCl, 37% (w/v)), formic acid (FA, 98–
124 100%), ammonium acetate (NH₄Ac, ≥98.0%) and glycine (≥99.7%) were supplied by
125 Merck (Darmstadt, Germany). CNBr-activated-Sepharose 4B was provided by GE
126 Healthcare (Waukesha, WI, USA) and “NP-40 alternative” by Calbiochem (Darmstadt,
127 Germany). Sodium chloride (NaCl, ≥99.5%), DL-Dithiothreitol (DTT, ≥99%), sodium
128 cyanoborohydride (NaBH₃CN), 2-mercaptoethanol (β-ME), sodium dodecyl sulfate
129 (SDS), iodoacetamide (IAA), ammonium hydrogencarbonate, sodium azide (NaN₃,
130 ≥99.5%) water (LC-MS grade), acetonitrile (LC-MS grade) and mouse apotransferrin
131 (mTf, reference: T0523) were supplied by Sigma–Aldrich (St. Louis, MO, USA) and
132 Tris(hydroxymethyl) aminomethane (TRIS, ≥99.5%) by J.T. Baker (Deventer, Holland).
133 Trypsin (Sequencing grade modified) was provided by Promega (Madison, WI, USA).
134 RapiGest[®] from Waters (Bedford, MA, USA) was used to facilitate the enzymatic
135 digestion. Goat polyclonal antibody against human transferrin (hTf) (immunogen
136 affinity purified) was purchased from Abcam (Cambridge, UK). Human transferrin
137 (hTf) and human α-1-acid glycoprotein (hAGP) were used as additional examples of
138 other glycosylated glycoproteins and were also supplied by Sigma–Aldrich.

139 **2.2 Mice and induction of arthritis**

140 Wild-type (WT) mice were from Harlan Ibérica (Barcelona, Spain). All studies with live
141 animals were authorized by the Institute of Parasitology and Biomedicine "López-
142 Neyra" (IPBLN) and Universidad de Cantabria Institutional Laboratory Animal Care

143 and Use Committees. For the induction of collagen-induced arthritis (CIA), 8-12 weeks-
144 old male mice were immunized as described elsewhere [48,49].

145 **2.3 Purification of serotransferrin from mouse serum samples by immunoaffinity** 146 **chromatography (IAC)**

147 In order to isolate mTf from the rest of serum proteins, an immunoaffinity (IA)
148 purification was carried out using a cyanogen-bromide sepharose column where a
149 polyclonal antibody against human transferrin (hTf) was bound, as detailed previously
150 [50]. The IA procedure consisted of: first, a conditioning step with two washes of 10
151 mM Tris-HCl; second, approximately 25 μ L of serum were diluted 1:8 in 10 mM Tris-
152 HCl (pH 7.6-7.7) in order to improve antigen-antibody interaction, and passed through
153 the column ten times. After washing with 10 mM Tris-HCl and 0.5 M NaCl (pH 7.6-
154 7.7), retained mTf was eluted with 100 mM glycine-HCl (pH 2.5). Eluted mTf was
155 immediately neutralized with 0.5 M Tris. Afterwards, glycine-HCl buffer was
156 exchanged for water by ultracentrifugation, using Microcon YM-10 (MW cut-off 10
157 kDa, Millipore, Bedford, MA, USA). Then, samples were evaporated to dryness using a
158 SpeedVacTM concentrator (Thermo Fisher Scientific, Waltham, MA, USA) and stored at
159 -20°C until use. Finally, the IA column was washed and stored in 10 mM Tris-HCl and
160 0.01% (w/v) NaN₃ (pH 7.6-7.7).

161 **2.4 Analytical approaches for mTf glycosylation study**

162 2.4.1 Intact glycoprotein analysis

163 mTf standard (25 μ g) was desalted using three different procedures: dialysis, size
164 exclusion and ultracentrifugation. Briefly, in the first method, D-TubeTM dialyzers from
165 Merck-Millipore were left in contact with 100 mM NH₄Ac for 15 min. Afterwards, the

166 sample was placed into the dialyzer and left in contact with 500 mL of 100 mM NH₄Ac
167 for 2 h at 4°C. Later, the buffer was renewed with 500 mL and the dialysis was allowed
168 to continue for 2 more hours, repeating this process twice. Finally, the sample was
169 carefully recovered and stored at -20°C until analysis. Regarding the size exclusion
170 procedure, the sample was desalted and the buffer exchanged using Micro Bio-Spin™
171 columns from BioRad (Hercules, California, USA) following the manufacturer
172 instructions. Columns were centrifuged to remove the excess of packing buffer and
173 washed three times with 500 µL of 100 mM NH₄Ac. Finally, the sample was added and
174 collected in a new tube after centrifugation. Lastly, the ultracentrifugation procedure
175 was carried out using Microcon YM-10 (MW cut-off 10 kDa) to desalt and exchange
176 the buffer of the sample. Filters were washed with 100 mM NH₄Ac and centrifuged for
177 10 min at 10000 g. Afterwards, the sample was added and washed with 50 µL of 100
178 mM NH₄Ac a total of 4 times, centrifuging each time for 10 min at 10000 g. Finally, the
179 final volume was recovered in a new vial after centrifugation for 2 min at 1000 g, and
180 reconstituted to the initial volume (25 µL). Centrifugation procedures were carried out
181 in a MiniSpin® centrifuge (Eppendorf, Hamburg, Germany) at room temperature. In all
182 cases, experiments with the standard glycoprotein were carried out in triplicate and
183 intact mTf in 100 mM NH₄Ac was injected directly into the mass spectrometer under
184 non-denaturing conditions and detected in positive ion mode.

185 2.4.2 Glycopeptide analysis

186 mTf standard (25 µg) was reduced, alkylated and immediately subjected to trypsin
187 digestion in the presence of RapiGest® as explained in ~~a~~ previous work [51]. Briefly, a
188 solution of 0.5 M DL-dithiothreitol (DTT) in 50 mM NH₄HCO₃ was added to an aliquot
189 of mTf with 0.1% (w/v) RapiGest®. The mixture was incubated at 56°C for 30 min and

190 then alkylated with 50 mM iodoacetamide (IAA) for 30 min at room temperature in the
191 dark. Excess reagent was removed by ultracentrifugation with Microcon YM-10
192 columns (Millipore, Bedford, MA, USA). The final residue was recovered from the
193 upper reservoir and reconstituted with NH₄HCO₃ buffer with 0.1% RapiGest[®]. Trypsin
194 in a 1:40 ratio was added and the mixture was incubated overnight at 37°C. Afterwards,
195 the surfactant was hydrolyzed to avoid MS incompatibilities as follows: formic acid
196 (FA) was added to the digest to a final concentration of 5% (v/v) and the mixture was
197 incubated in the digester at 37°C for 30 min. Then, the solution was centrifuged to
198 separate RapiGest[®] residues. mTf tryptic digests were stored at -20°C until analysis. All
199 the experiments with the standard glycoprotein were carried out in triplicate.

200 For the analysis of glycopeptides, a Waters Nano ACQUITY UPLC[®] was used with a
201 double binary gradient pump, using a peptide BEH C18 column (1.7 μm particle
202 diameter, 130 Å pore, 100 x 0.1 mm length x ID; Waters). Experiments were performed
203 at room temperature with gradient elution at a flow rate of 400 nL min⁻¹. Eluting
204 solvents were A: water with 0.1% (v/v) of formic acid (FA), and B: acetonitrile with
205 0.1% (v/v) FA. Solvents were degassed by sonication (10 min) before use. The
206 optimum elution program was: solvent B from 10 to 60% (v/v) within 20 min as linear
207 gradient, followed by cleaning and re-equilibration steps of B: 60 to 100% (v/v) (5
208 min), 100% (v/v) (5 min), 100 to 10% (v/v) (5 min) and 10% (v/v) (5 min). Before
209 analysis, samples were filtered using a 0.22 μm polyvinylidene difluoride centrifugal
210 filter (Ultrafree-MC, Millipore, Bedford, MA, USA) centrifuging at 10,000 g for 4 min.
211 Sample injection (300 nL) was performed with an autosampler refrigerated at 4°C.
212 Control of the instrument was performed using MassLynx 4.1 (Waters).

213 2.4.3 Glycan analysis

214 ~~IAC purified mTf or mTf standard (25 µg) was reduced with 0.5% 2-mercaptoethanol~~
215 ~~(β-ME) and 0.5% SDS and subjected to enzymatic digestion with PNGase F as~~
216 ~~described in.~~ IAC purified mTf or mTf standard (25 µg) was reduced with 0.5% 2-
217 mercaptoethanol (β-ME) and 0.5% SDS in 50 mM NH₄HCO₃ (pH 7.6-7.7) and heated
218 in a thermo -block at 100 °C for 30 min [19]. Once the sample was at room temperature,
219 a volume of 50 mM NH₄HCO₃ (pH 7.6-7.7) with 1% (v/v) of NP-40 alternative was
220 added to obtain a final concentration of 0.1% SDS and β-ME in the sample. To release
221 the N-glycans, 1µL of PNGase F (1 U) solution was added to the mixture, which was
222 afterwards incubated at 37°C for 18 h. Digestion was stopped by heating the sample in a
223 thermo block at 100°C for 5 min. Afterwards Then, released glycans were isolated by
224 solid phase extraction (SPE) using Hypercarb cartridges (25 mg, 1 mL volume, Thermo
225 Fisher Scientific) and, subsequently, purified by ice-cold acetone precipitation
226 following the procedure reported in [19] in both cases. Reduced glycans were diluted
227 with 50:50 H₂O/ACN with 0.1% FA and directly analyzed by IM-MS in negative ion
228 mode. All the experiments with the standard glycoprotein were carried out in triplicate.
229 Mice derived transferrin glycan analysis was carried out in duplicate, including
230 purification and release of glycans.

231 **2.5 Ion mobility-mass spectrometry**

232 For IM-MS analysis a Synapt G2 HDMS instrument from Waters was used. Samples
233 were directly introduced into the ~~vacuum of the~~ mass spectrometer using home-made
234 nano-electrospray ionization (nano-ESI) gold-coated borosilicate capillaries; unless
235 when analysed by nano-UPLC-IM-MS, in which case an in-line nano-ESI interface with
236 commercially available coated needles was used.

237 Spectra were acquired in positive mode for the analysis of intact glycoproteins and
238 glycopeptides, and in negative mode for glycans, and conditions were optimized in each
239 case. The voltages for spray capillary, sampling cone, trap collision energy (CE), trap
240 direct current (DC) bias and transfer CE were, respectively: intact glycoproteins, 1.4-1.6
241 kV, 30 V, 4 V, 40 V and 0 V; glycopeptides, 1.5-1.7 kV, 50 V, 4 V, 20 V and 0 V; and
242 glycans, 1.5 kV, 50 V, 4 V, 45 V and 0 V. The “trap CE” voltage was only increased to
243 60 V when fragmentation of the glycopeptides was the goal. Mass spectrometer control
244 and spectra processing were carried out using MassLynx 4.1 (Waters).

245 The software IMoS [52,53], available for free at *imospedia.com*, was used for the
246 theoretical calculations of the collision cross sections (CCS) of glycans, using their
247 minimum energy structures. The online tool *carbohydrate builder*, available at
248 *glycam.org* [54], was used to generate the required input for theoretical calculations.
249 The tool allows building different glycan isomers based on monosaccharide unit and
250 linkage type, and generates minimum energy structures.

251

252 **3. Results and discussion**

253 **3.1 Intact glycoprotein analysis**

254 It is well established that intact proteins can be analysed mainly in two different ways
255 when using electrospray ionization: under denaturing or non-denaturing (i.e., “native”)
256 conditions. In the first case, the protein appears highly charged with a broad charge state
257 distribution; thus, the separation of the different glycoforms is difficult. On the other
258 hand, an advantage of native MS is that it strongly reduces charging, hence the different
259 glycoforms can be more clearly resolved [55].

260 Using native MS in this work, the concentration of ammonium acetate and the nano-ESI
261 source parameters were optimized to improve and obtain the best possible sensitivity
262 and separation between mTf glycoforms in positive ion mode. Moreover, in order to
263 obtain an acceptable separation between the several glycoforms, mTf was washed
264 repeatedly with 100 mM NH₄Ac to eliminate excipients and salts, and injected directly
265 into the mass spectrometer without further separation. Without washing, no glycoform
266 separation was observed whatsoever. Three methods were evaluated to desalt the
267 glycoprotein: dialysis, size exclusion and ultracentrifugation, with the latter one giving
268 the best results (see supplementary Figure S1). Afterwards, the drift times of intact
269 glycoforms were determined by ion mobility. In Figure 1-a, the ion with charge +19 of
270 mTf is shown, as well as the arrival time distributions (ATD) of different sections of
271 this peak (Figure 1-a (i-v)), each corresponding to one intact glycoform of mTf). As can
272 be observed in the ATD profiles, only one, relatively wide peak is obtained for each
273 glycoform. Hence, these results imply that, at least for large glycoproteins with a low
274 degree of glycosylation, the glycan part has barely any influence on the overall size of
275 the whole molecule as the CCS appears virtually unaffected by glycosylation. hTf and

276 hAGP were also studied to see if the same behaviour was observed with other
277 glycoproteins. Figure 1-b and 1-c show the separation between intact glycoforms in the
278 MS spectra of hTf (for the ion with charge +19) and hAGP, respectively. As can be
279 observed, the peak was better resolved for hTf, but no separation was observed for
280 hAGP, as the number of glycoforms was considerably higher and the overlapping of
281 several ion distributions was unavoidable. Moreover, one wide peak was observed again
282 when the drift time of each section of the mass spectrum was measured for both
283 glycoproteins. Regarding hTf, each section corresponded to one glycoform (Figure 1-b
284 (i-iv)), whereas, in the case of hAGP, the resolution was not high enough to ensure that
285 a selected region of the peak corresponded to one defined glycoform (Figure 1-c (i-v)),
286 because of the high glycosylation degree of hAGP. The higher the m/z region of this
287 peak however, the bigger the carbohydrate fraction is (usually meaning more complex
288 and branched glycans). This is due to the fact that an increase of m/z within the same
289 charge state can only be due to an increase in the number of glycan subunits of the
290 carbohydrate fraction, as the polypeptide backbone remains unaltered. For the sake of
291 consistency, we will refer to each section of the peak as glycoform, even if, as in the
292 case of hAGP, they are not resolved. Interestingly, the drift time increased with
293 increasing carbohydrate fraction, i.e. complexity and branching of the glycoforms, in all
294 three cases (see Figure 1-a (i-v), 1-b (i-iv) and 1-c (i-v), type of glycoform indicated
295 where possible). The relative drift time differences between glycoforms were higher in
296 proteins with higher glycosylation content, as can be observed for hAGP (see Figure 1-
297 c). Moreover, if we compare the drift time between different glycoforms of mTf and
298 hTf, the increase in drift time was more noticeable in hTf, as the percentage of
299 glycosylation is slightly higher here in relation to the total protein mass. As Table 1
300 shows, for the same relative increase in m/z , the relative drift time increase is higher for

301 hAGP, which demonstrates that for glycoproteins with a high percentage of
302 glycosylation, differences in the glycosylation have a greater effect on the drift time of
303 the whole molecule (i.e., they significantly alter the CCS of the whole glycoprotein). It
304 could then also be conceivable to separate isomers due to the different sialic acid
305 linkage (of the same glycoform) at the intact protein level if the glycan:peptide ratio was
306 high enough. However, this option would not have been viable for all types of
307 glycoproteins, thus, other alternatives were studied.

308 **3.2 Glycopeptide analysis**

309 As mTf only shows one glycosite at asparagine 494, only one glycopeptide is expected
310 after tryptic digestion (N₄₉₄ glycopeptide, peptide: NSTLCDLCIGPLK). However, with
311 the direct injection of the digest, the N₄₉₄ glycopeptide was not detected, hence, an
312 additional separation before the MS was mandatory. In this regard, nano-UPLC was
313 used as a separation technique prior to IM-MS detection, in order to separate the
314 glycopeptide from the other peptides and simultaneously determine the drift time of the
315 different glycoforms. Injection volume, flow rate and gradient were optimized to obtain
316 the best sensitivity with a stable spray (see section 2.4, Materials and Methods). Table 2
317 shows the detected glycoforms at the peptide level, their theoretical and experimental
318 masses, mass error and observed charge states.

319 Regarding the determination of the drift time of the different glycopeptide glycoforms,
320 even though a range of different values for the wave velocity (WV) and wave height
321 (WH) of the TWIMS device were tested, only one drift time value was observed. This
322 suggests that no isomer separation was possible at this mobility resolution, which is
323 probably due to the fact that the different isomers had similar CCS despite the distinct
324 orientation of the sialic acid. Recently, Hinneburg et al. [15] and Guttman et al. [45]

325 also described this observation analysing glycopeptides directly by IM-MS. They
326 observed no isomeric separation, unless fragmentation of the glycopeptide was carried
327 out and one of the observed smaller fragments still contained the sialic acid. This
328 fragment (obtained before the IM cell) had different drift times depending on the sialic
329 acid linkage, because a change in the orientation of the sialic acid was more noticeable
330 (i.e. the CCS was more affected) in a smaller analyte. We also tested this approach, by
331 fragmenting the most intense glycopeptide glycoform (N(H5N4S2)STLCDLCIGPLK)
332 before the IM cell. Figure 2-a shows the collision induced dissociation (CID) MS/MS
333 spectrum for this glycoform. Several fragments were obtained, e.g., the
334 glycolylneuraminic acid (S1) and H1N1 and H2N1 fragments. Among all the fragments
335 however, H1N1S1 was the one that still retained the sialic acid linkage and had enough
336 intensity to yield a good and accurate drift time measurement. Isomer separation was
337 observed in the aforementioned glycan fragment. Specifically, as it is shown in Figure
338 2-c, two drift time peaks were clearly observed at this m/z, which are postulated to be
339 due to the sialic acid being α -2 \rightarrow 6 and α -2 \rightarrow 3 linked. To confirm that the sialic acid is
340 the monosaccharide that affects the CCS of the fragment the most, the ATD of the
341 fragment H1N1 is shown in supplementary Figure S2-c. In this case, we only observed
342 one drift time, which demonstrates that it is the sialic acid which causes observable CCS
343 differences seen as two different drift times of the fragment H1N1S1.
344 Moreover, in order to confirm that the distinct drift times observed for the glycan
345 fragment could be due to different sialic acid linkages, the theoretical CCS of the
346 H1N1S1 isomers were calculated. The obtained CCS values can be found in Table 3.
347 were: 233.4 Å² for the α -2 \rightarrow 6 linkage and 243.7 Å² for the α -2 \rightarrow 3 glycan. The CCS for
348 the two H1N1S1 were different enough to be separated by IM. Taking into account the

349 CCS values of Table 3. ~~This suggests that~~ the more prominent, lower drift time peak
350 (i.e., lower CCS) should corresponds to the α -2 \rightarrow 6 glycan, whereas the second peak
351 represents the α -2 \rightarrow 3 glycan.

352 Our results are in accordance with those obtained by Hinneburg et al. [15], who, using
353 modelling, drew the same conclusions. This is also in agreement with Guttman et al.
354 [45], who found that biantennary glycoforms showed lower content of α -2 \rightarrow 3
355 sialylation. In our case, the percentage of α -2 \rightarrow 3 glycan was approximately 14%
356 (taking into account the measured area of each drift time peak, see Figure 2-c).

357 However, this ratio can vary depending on the glycoprotein studied [45]. ~~With this~~
358 ~~method, using nano-UPLC-IM-MS and drift time measurement of the MS/MS~~
359 ~~fragments, separation of glycopeptide isomers was achieved.~~ This glycopeptide
360 approach, through the analysis of the fragment H1N1S1, gave valuable information
361 about the content of each type of sialic acid as the fragment H1N1S1 came from both
362 branches of the H5N4S2 glycopeptide (we did not detect any fragment with only one
363 antenna remaining (H3N3 or H3N3S1), suggesting that both antennas might indeed be
364 fragmented). This relevant information could permit, in the future, to correlate changes
365 in protein sialylation with specific disease biomarkers which involve protein
366 glycosylation, such as, e.g., in cancer or inflammation.

367

368 **3.3 Glycan analysis**

369 Finally, we proceeded with the study of the enzymatically released glycans using
370 PNGase F. Different solvents were tested to obtain the best spray and ionization yield,
371 and a slightly superior glycan signal was obtained at 50:50 H₂O:ACN with 0.1% FA.

372 Table [3-4](#) shows the detected mTf glycans along with their theoretical and experimental
373 masses, mass error and observed charge states. In Figure 3 the ATD profile of H5N4S2
374 glycans at different values of WV and WH is shown. Two peaks are observed which are
375 believed to correspond to two different isomers of the glycan H5N4S2, in analogy to the
376 previously obtained results ([Figure 2-c](#)) with the [fragment H1N1S1 of the](#) glycopeptide
377 N(H5N4S2)STLCDLCIGPLK. Fine tuning of the IM parameters was mandatory in
378 order to resolve both drift time peaks. A WV value of 450 m s⁻¹ and WH of 25 V was
379 selected as optimal for the analysis of all mTf glycans. It is worth mentioning that in
380 this case, glycans were analyzed in negative mode and, as can be observed in Figure 3,
381 the first isomer was clearly the less abundant one. This is in contrast to the glycopeptide
382 analysis which was done in positive mode, where the less abundant isomer was the
383 second one. This could be due to the fact that both molecules were, actually, quite
384 different - specifically, the glycan had eleven monosaccharide units, as opposed to the
385 fragment observed in the glycopeptide analysis which had only three. With regard to
386 other glycans, three different peaks, i.e., three drift times, were observed for H6N5S3
387 (see supplementary Figure [2S3-c](#)), albeit separation was slightly worse than observed
388 for H5N4S2. H5N4S1 on the other hand only showed one wide peak (see
389 supplementary Figure [2S3-d](#)), ~~most probably~~ implying that only one glycan isomer
390 exists or that the different isomers have similar CCS, and IM is not able to distinguish
391 them (as-However, the absence of other drift time peaks does not preclude the presence
392 of other isomers). ~~, as they can have similar CCS.~~ It is also worth pointing out that when
393 the glycan contained a fucose unit, the isomeric separation was somewhat hampered, as
394 observed in supplementary Figure [2S3-b](#). We reckon that the addition of one extra
395 monosaccharide unit might affect the global CCS of the glycan, and as the whole glycan
396 is bigger, a small variation in the orientation of the sialic acid is less noticeable.

397 To confirm that separation of isomeric glycans due to the sialic acid linkage was also
398 possible with other glycoproteins, hAGP was also digested with PNGase F and the
399 released glycans analysed by IM-MS. Separation of isomeric glycans was achieved,
400 seemingly obtaining the same results as with mTf. As an example, the arrival time
401 distributions of the H5N4S2 and H6N5S2 glycans are shown in supplementary Figure
402 [3S4](#). As can be seen, two drift time peaks were obtained for the H5N4S2 glycan and
403 three peaks for H6N5S3, albeit separation was poorer in both cases when compared to
404 mTf.

405 Moreover, to confirm that ion mobility separation of these possible isomers is due to
406 different sialic acid linkages, [Table 3 shows](#) the theoretical CCS values of [the four](#)
407 [possible isomers for the glycan H5N4S2 with \$\(\alpha-2 \rightarrow 3\)_2\$, \$\(\alpha-2 \rightarrow 6\)\$ and \$\(\alpha-2 \rightarrow 3\)_1\$, \$\(\alpha-2-6\)\$](#)
408 [and \$\(\alpha-2 \rightarrow 3\)\$ and \$\(\alpha-2 \rightarrow 6\)_2\$ sialic acids were calculated. The obtained CCS were,](#)
409 [following the same order: \$652.3 \text{ \AA}^2\$ for the \$\(\alpha-2 \rightarrow 3\)_2\$ glycan, \$635.4 \text{ \AA}^2\$ for \$\(\alpha-2 \rightarrow 6\)\(\alpha-\$](#)
410 [2 \$\rightarrow 3\)\$, \$xxx \text{ \AA}^2\$ and \$623.9 \text{ \AA}^2\$ for the \$\(\alpha-2 \rightarrow 6\)_2\$ form.](#) The differences between these
411 calculated values suggest that the observed ion mobility peaks could be due to isomeric
412 glycans with different types of sialic acid linkage. With the knowledge of theoretical
413 CCS, and the abundance of the $\alpha-2 \rightarrow 3$ H1N1S1 fragment being lower than ~~the $\alpha-2 \rightarrow 6$~~
414 (based on fragmentation of the H5N4S2 glycopeptide, see above), ~~the two peaks~~
415 ~~observed for the H5N4S2 glycan were tentatively assigned~~ [some conclusions can be](#)
416 [drawn. We reckon that the presence of the isomer with two sialic acids \$\alpha-2-3\$ can be](#)
417 [discarded and that the two peaks observed must correspond to the isomers with higher](#)
418 [content in \$\alpha-2-6\$, that is, the isomer with two sialic acids \$\alpha-2-6\$ and the isomer with one](#)
419 [sialic acid \$\alpha-2-6\$ and one \$\alpha-2-3\$ \(although we cannot specify the branch where each sialic](#)
420 [acid is located\).](#) ~~We suggest that the peak with the highest drift time, and highest~~

421 ~~abundance, could be the glycan (α 2 \rightarrow 6)(α 2 \rightarrow 3) linkage, however, we cannot specify~~
422 ~~the branch where each sialic acid is located bound. Whereas, the one with the lowest~~
423 ~~drift time (and lowest abundance) could be the (α 2 \rightarrow 6)₂-linked glycan. However,~~
424 A alternative approaches for the study of isomeric glycoconjugates, for instance using
425 specific sialidases [18], ~~could would however be allow to useful to~~ obtain
426 complementary information and reliably assign the different isomers peaks to the
427 corresponding isomers.

428 **3.4 Mousiee serum sample**

429 To further asses the ability of the established method to separate isomeric
430 glycoconjugates in biological samples, we measured the drift time of mTf glycans
431 purified from serum samples. Only the analysis of the released glycans by IM-MS was
432 included in this study, as it was found to be the most sensitive and straightforward
433 approach to obtain information about isomeric forms. Two serum samples were
434 analysed: one healthy control and one sample with collagen-induced arthritis (CIA), an
435 autoimmune disease known to alter the glycosylation pattern of mTf [51,58]. As can be
436 seen in Figure 4, two peaks were observed for H5N4S2 glycan and three peaks for
437 H6N5S3 in both samples, with the same drift time and similar relative intensities as in
438 the mTf standard (compare Figure 4 with supplementary Figure [2S3](#)). Although
439 additional samples are required to observe possible differences in the relative abundance
440 of glycan isomers between control and pathological samples, the presented
441 methodology shows great potential for the separation of isomeric glycans and the
442 discovery of novel biomarkers in glycomic studies.

443

444 4. Concluding remarks

445 In the present paper, the capability of IM-MS to separate isomeric glycoconjugates
446 which are due to different types of sialic acid linkage (i.e. α -2 \rightarrow 3 and α -2 \rightarrow 6) has
447 been evaluated at three different levels: intact glycoprotein, glycopeptides, and the
448 released glycans. Separation of isomeric glycoconjugates is an important task in the
449 glycomics field, because it has been reported that differences in the abundance of some
450 glycan isomers might be of great importance for the early diagnosis or control of, for
451 instance, inflammatory diseases and certain types of cancer.

452 With ~~our~~the current knowledge of glycosylation effects on molecule size, and the
453 limited abilitycapabilities of Synapt IM-MS instrumentation to resolve small CCS
454 differences, isomeric separation cannot be obtained at the intact glycoprotein and
455 glycopeptide level. Released glycans however can be separated after optimization of the
456 IM parameters. As stated before by others [15,45], and also demonstrated in this work,
457 there is a workaround to distinguish different types of sialic acid linkage in
458 glycopeptides that takes into account the mobility of an MS/MS fragment which still
459 retains the sialic acid. This method can however be time-consuming and rather difficult,
460 as glycopeptides must be separated from the rest of the digest and, besides, the obtained
461 glycopeptide fragment is, actually, a glycan.

462 The interest in using ion mobility for glycoconjugate separation and identification has
463 seen a major growth in the last years. Thus, it is likely that new technologies and
464 improvements will become available soon, including the advent of ion mobility
465 instrumentation with up to ten times higher resolution. Therefore, the separation of
466 sialic acid linkage isomers may also become possible at the level of glycopeptides and
467 intact proteins in the future.

468

469

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476

477 **5. References**

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672

673

674 **Figure legends**

675

676 **Figure 1:** Mass spectra showing the ion with charge +19 and the corresponding drift
677 time (arrival time) distributions, or ATD, of a) intact mTf and b) intact hTf; and also c)
678 ions with charge +13, +12 and +11 of intact hAGP and the corresponding ATD. The
679 value indicated corresponds to the approximate glycosylation percentage (w/w) of each
680 protein, calculated as the mass of the most abundant glycan per glycosylation site
681 divided by the mass of the glycoprotein. i)-v): indicates the glycoform or the region of
682 the mass spectrometric peak. H: hexose; N: N-acetylhexosamine; F: fucose; S: sialic
683 acid (in this case, all S are N-glycolylneuraminic acid). The voltages for spray capillary,
684 sampling cone, trap collision energy (CE), trap direct current (DC) bias and transfer CE
685 were, respectively: 1.4-1.6 kV, 30 V, 4 V, 40 V and 0 V.

686 **Figure 2:** a) MS/MS spectrum for the mTf glycopeptide glycoform
687 N(H5N4S2)STLCDLCIGPLK; b) mass spectra of a fragment that still keeps the sialic
688 acid (H1N1S1) and c) arrival time distribution of this fragment (m/z range: 673.3-
689 673.5). The symbols used for the representation of the glycoform H5N4S2 follow the
690 Consortium for Functional Glycomics Symbol Nomenclature for Glycans (SNFG)
691 (CFG) rules [59]. H: hexose; N: N-acetylhexosamine; F: fucose; S: sialic acid (in this
692 case, all S are N-glycolylneuraminic acid). The voltages for spray capillary, sampling
693 cone, trap collision energy (CE), trap direct current (DC) bias and transfer CE were,
694 respectively: 1.5-1.7 kV, 50 V, 4 V, 20 V and 60 V.

695 **Figure 3:** Arrival time distributions for the H5N4S2 glycan released from mTf at
696 different wave height (WH, in V) and wave velocity (WV, in m s⁻¹) combinations. The
697 symbols used for the representation of the H5N4S2 glycan follow the Symbol
698 Nomenclature for Glycans (SNFG) Consortium for Functional Glycomics (CFG) rules

699 [59]. H: hexose; N: N-acetylhexosamine; F: fucose; S: sialic acid (in this case, all S are
700 N-glycolylneuraminic acid). The voltages for spray capillary, sampling cone, trap
701 collision energy (CE), trap direct current (DC) bias and transfer CE were, respectively:
702 1.5 kV, 50 V, 4 V, 45 V and 0 V.

703 **Figure 4:** Arrival time distributions for the glycans a) H5N4S2 and b) H6N5S3 released
704 from mTf in a healthy mouse serum and a serum from a mouse with collagen-induced
705 arthritis (CIA). The symbols used for the glycan representation follow the Symbol
706 Nomenclature for Glycans (SNFG) Consortium for Functional Glycomics (CFG) rules
707 [59]. H: hexose; N: N-acetylhexosamine; F: fucose; S: sialic acid (in this case, all S are
708 N-glycolylneuraminic acid). The voltages for spray capillary, sampling cone, trap
709 collision energy (CE), trap direct current (DC) bias and transfer CE were, respectively:
710 1.5 kV, 50 V, 4 V, 45 V and 0 V.

711
712 **Figure S1:** Mass spectra of the ion with charge +19 of intact mTf after desalting with
713 three different methods: a) dialysis, b) size exclusion columns, and c)
714 ultracentrifugation. The voltages for spray capillary, sampling cone, trap collision
715 energy (CE), trap direct current (DC) bias and transfer CE were, respectively: 1.4-1.6
716 kV, 30 V, 4 V, 40 V and 0 V.

717 **Figure S2:** a) MS/MS spectrum for the mTf glycopeptide glycoform
718 N(H5N4S2)STLCDLCIGPLK; b) mass spectra of a fragment that still keeps the sialic
719 acid (H1N1) and c) arrival time distribution of this fragment (m/z range: 366.2-366.4).
720 The symbols used for the representation of the glycoform H5N4S2 follow the Symbol
721 Nomenclature for Glycans (SNFG) rules [59]. H: hexose; N: N-acetylhexosamine; F:

722 fucose; S: sialic acid (in this case, all S are N-glycolylneuraminic acid). The voltages for
723 spray capillary, sampling cone, trap collision energy (CE), trap direct current (DC) bias
724 and transfer CE were, respectively: 1.5-1.7 kV, 50 V, 4 V, 20 V and 60 V.

725
726 **Figure 2S3:** Arrival time distributions for several glycans of mTf standard: a) H5N4S2,
727 b) H5N4F1S2, c) H6N5S3 and d) H5N4S1. The symbols used for the glycan
728 representation follow the Symbol Nomenclature for Glycans (SNFG) Consortium for
729 Functional Glycomics (CFG) rules [59]. H: hexose; N: N-acetylhexosamine; F: fucose;
730 S: sialic acid (in this case, all S are N-glycolylneuraminic acid). The voltages for spray
731 capillary, sampling cone, trap collision energy (CE), trap direct current (DC) bias and
732 transfer CE were, respectively: 1.5 kV, 50 V, 4 V, 45 V and 0 V.

733
734 **Figure 3S4:** Arrival time distributions for a) H5N4S2 glycan and b) H6N5S3 glycan of
735 hAGP. The symbols used for the glycan representation follow the Symbol
736 Nomenclature for Glycans (SNFG) rules [59] ~~Consortium for Functional Glycomics~~
737 ~~(CFG) rules~~. H: hexose; N: N-acetylhexosamine; F: fucose; S: sialic acid (in this case,
738 all S are N-acetylneuraminic acid). The voltages for spray capillary, sampling cone, trap
739 collision energy (CE), trap direct current (DC) bias and transfer CE were, respectively:
740 1.5 kV, 50 V, 4 V, 45 V and 0 V.

743 **Table 1:** Relative increase in m/z and drift time between glycoforms i) and ii) (Figure
744 1) of mTf, hTf and hAGP.

	mTf	hTf	hAGP
Relative m/z difference (%)	0.247	0.239	0.746
Relative drift time difference (%)	0.149	0.290	1.324
Normalization*	Relative m/z (%)	0.247	0.247
	Relative drift time (%)	0.149	0.299

745

746 * The relative m/z for hTf and hAGP was changed to have the same value as mTf
747 (0.247 %) and, therefore, the relative drift time was proportionally modified.

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749

750 **Table 2:** Theoretical and experimental molecular mass (M_r), mass error and detected
 751 charge states of the 10 glycoforms for the N_{494} glycopeptide of mTf detected by nano-
 752 UPLC-IM-MS.

Glycopeptide	Glycoform*	Theoretical M_r	Experimental M_r	Mass error (ppm)	Observed charge state
N_{494}	H3N3S1	2892.2138	2892.2840	24	+2
	H3N3F1S1	3038.2717	3038.2920	7	+2
	H5N4S1	3419.3988	3419.3908	2	+2, +3
	H5N4F1S1	3565.4568	3565.4656	2	+3
	H5N4S2	3726.4892	3726.4993	3	+2, +3
	H5N4F1S2	3872.5471	3872.5600	3	+2, +3
	H5N4S3	4033.579	4033.6210	10	+2, +3
	H5N4F1S3	4179.6374	4179.6958	14	+2, +3
	H6N5S3	4398.7117	4398.7813	16	+3
	H6N5F1S3	4544.7696	4544.8321	14	+3

753
 754 * H: hexose; N: N-acetylhexosamine; F: fucose; S: sialic acid (in this case, all S are N-
 755 glycolylneuraminic acid)

756

757 **Table 3:** CCS values of the two isomers for the fragment H1N1S1 and the four isomers
 758 for the H5N4S2 glycan.

		Isomer		CCS (Å ²)
Glycopeptide N ₄₉₄ -H5N4S2	Fragment	H1N1S1	α2-6	233.4
		H1N1S1	α2-3	243.7
Glycan H5N4S2		3-antenna	α2-6	584.7
		6-antenna	α2-3	
		2 x α2-6		623.9
		3-antenna	α2-3	635.4
		6-antenna	α2-6	
2 x α2-3		652.3		

759

760 **Table 34:** Theoretical and experimental molecular mass (Mr), mass error and detected
 761 charge states of the 10 mTf glycans detected by IM-MS.

Glycan*	Theoretical Mr	Experimental Mr	Mass error (ppm)	Observed charge state
H3N3S1	1420.4975	1420.4659	22	-1
H3N3F1S1	1566.5554	1566.5692	9	-1
H5N4S1	1947.6825	1947.7031	11	-1
H5N4F1S1	2093.7404	2093.7891	23	-1
H5N4S2	2254.7729	2254.7650	4	-2
H5N4F1S2	2400.8308	2400.8240	3	-2
H5N4S3	2561.8632	2561.9150	20	-2
H5N4F1S3	2707.9211	2707.9470	10	-2
H6N5S3	2926.9954	2926.9334	21	-2, -3
H6N5F1S3	3073.0533	3073.0036	16	-3

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763

764 * H: hexose; N: N-acetylhexosamine; F: fucose; S: sialic acid (in this case, all S are N-

765 glycolylneuraminic acid)

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Table 1: Relative increase in m/z and drift time between glycoforms i) and ii) (Figure 1) of mTf, hTf and hAGP.

	mTf	hTf	hAGP
Relative m/z difference (%)	0.247	0.239	0.746
Relative drift time difference (%)	0.149	0.290	1.324
Normalization*	Relative m/z (%)	0.247	0.247
	Relative drift time (%)	0.149	0.299

* The relative m/z for hTf and hAGP was changed to have the same value as mTf (0.247 %) and, therefore, the relative drift time was proportionally modified.

Table 2: Theoretical and experimental molecular mass (M_r), mass error and detected charge states of the 10 glycoforms for the N_{494} glycopeptide of mTf detected by nano-UPLC-IM-MS.

Glycopeptide	Glycoform*	Theoretical M_r	Experimental M_r	Mass error (ppm)	Observed charge state
N_{494}	H3N3S1	2892.2138	2892.2840	24	+2
	H3N3F1S1	3038.2717	3038.2920	7	+2
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	H6N5S3	4398.7117	4398.7813	16	+3
	H6N5F1S3	4544.7696	4544.8321	14	+3

* H: hexose; N: N-acetylhexosamine; F: fucose; S: sialic acid (in this case, all S are N-glycolylneuraminic acid)

Table 3: CCS values of the two isomers for the fragment H1N1S1 and the four isomers for the H5N4S2 glycan.

		Isomer		CCS (Å ²)
Glycopeptide N ₄₉₄ -H5N4S2	Fragment	H1N1S1	α2-6	233.4
		H1N1S1	α2-3	243.7
Glycan H5N4S2		3-antenna	α2-6	584.7
		6-antenna	α2-3	
		2 x α2-6		623.9
		3-antenna	α2-3	635.4
		6-antenna	α2-6	
2 x α2-3		652.3		

Table 4: Theoretical and experimental molecular mass (M_r), mass error and detected charge states of the 10 mTf glycans detected by IM-MS.

Glycan*	Theoretical M_r	Experimental M_r	Mass error (ppm)	Observed charge state
H3N3S1	1420.4975	1420.4659	22	-1
H3N3F1S1	1566.5554	1566.5692	9	-1
H5N4S1	1947.6825	1947.7031	11	-1
H5N4F1S1	2093.7404	2093.7891	23	-1
H5N4S2	2254.7729	2254.7650	4	-2
H5N4F1S2	2400.8308	2400.8240	3	-2
H5N4S3	2561.8632	2561.9150	20	-2
H5N4F1S3	2707.9211	2707.9470	10	-2
H6N5S3	2926.9954	2926.9334	21	-2, -3
H6N5F1S3	3073.0533	3073.0036	16	-3

* H: hexose; N: N-acetylhexosamine; F: fucose; S: sialic acid (in this case, all S are N-glycolylneuraminic acid)

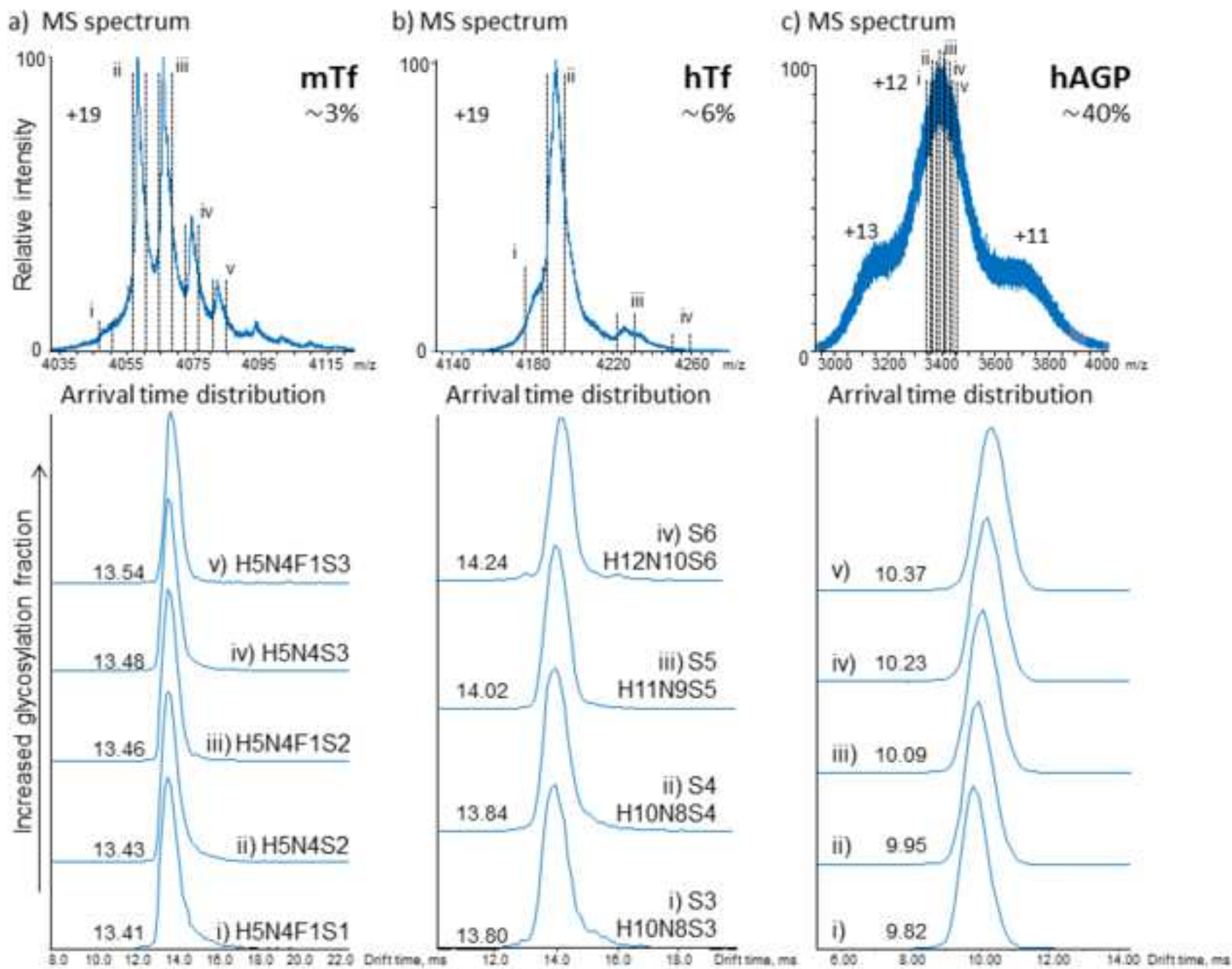


Figure 1

Figure 2
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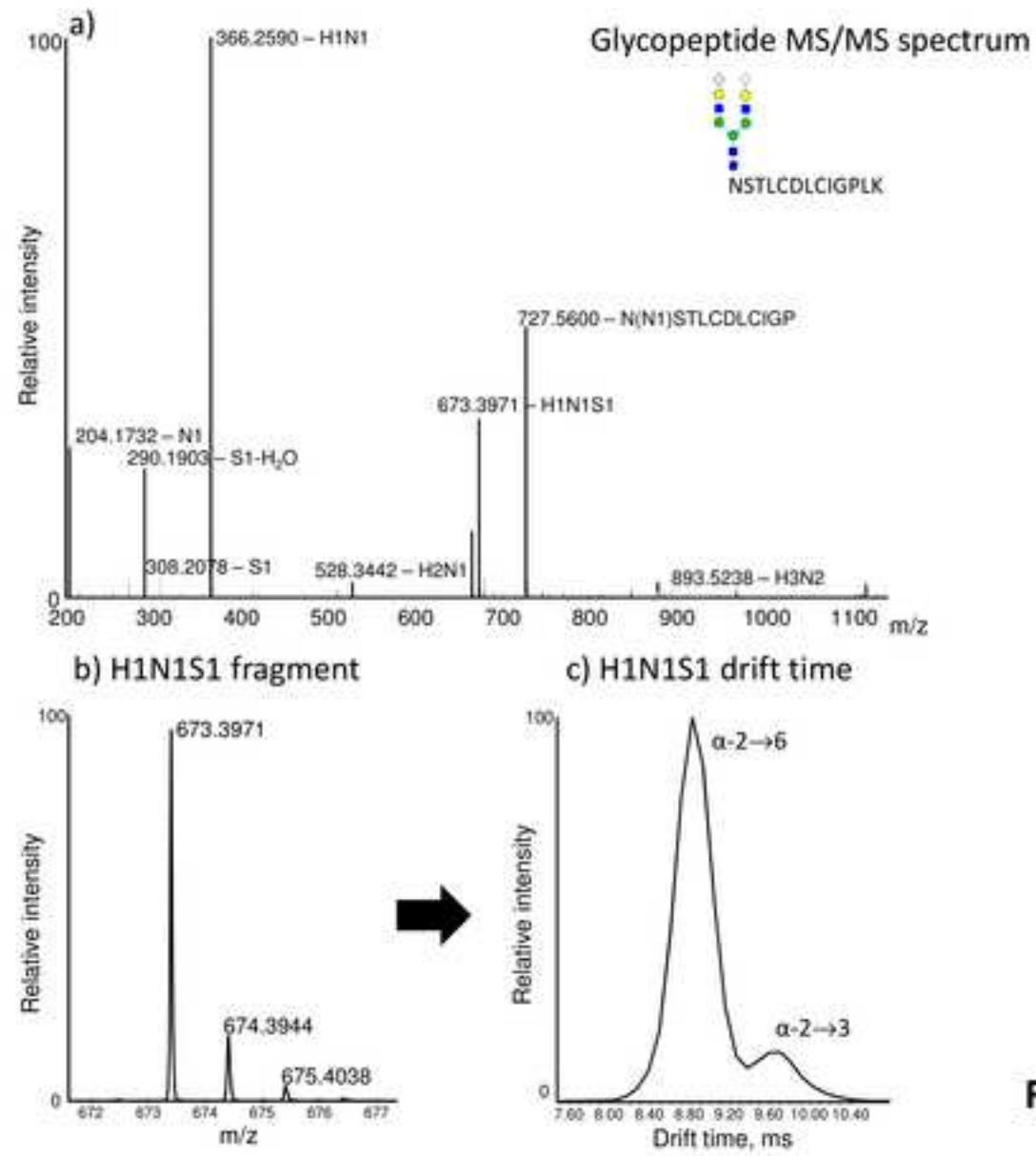


Figure 2

Figure 3
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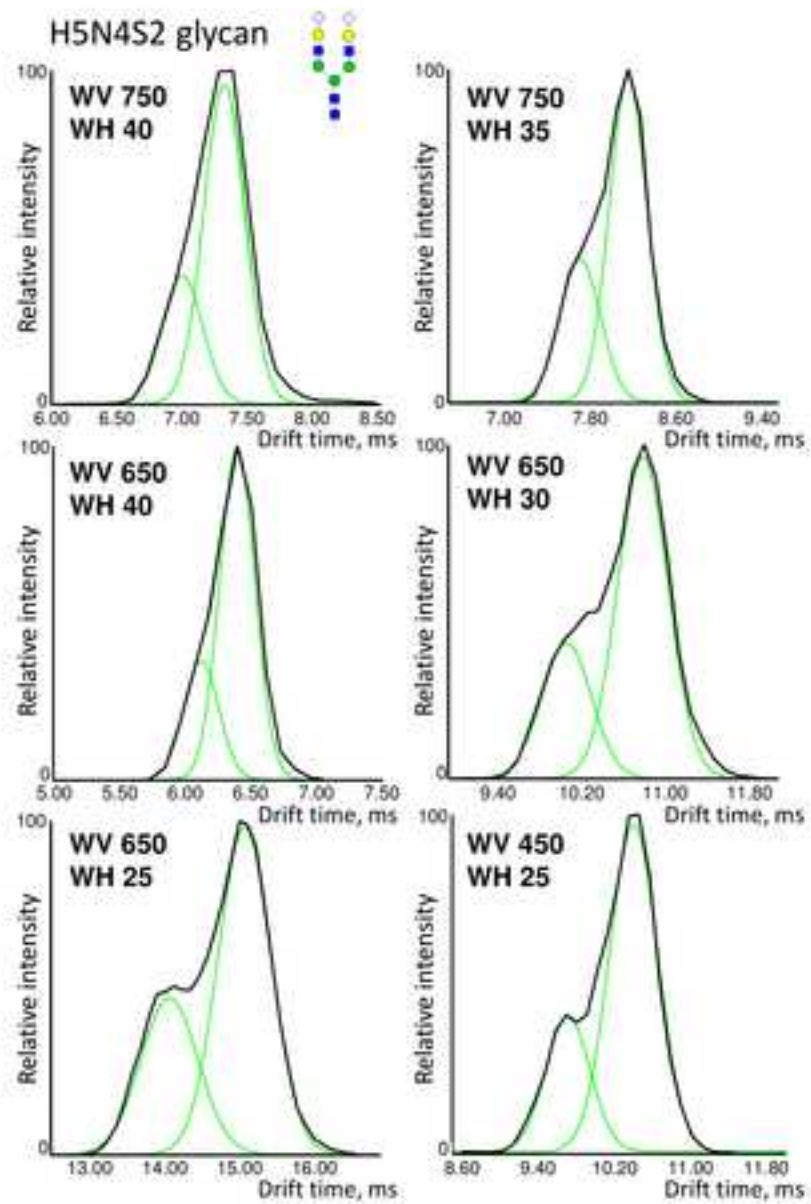


Figure 3

Figure 4
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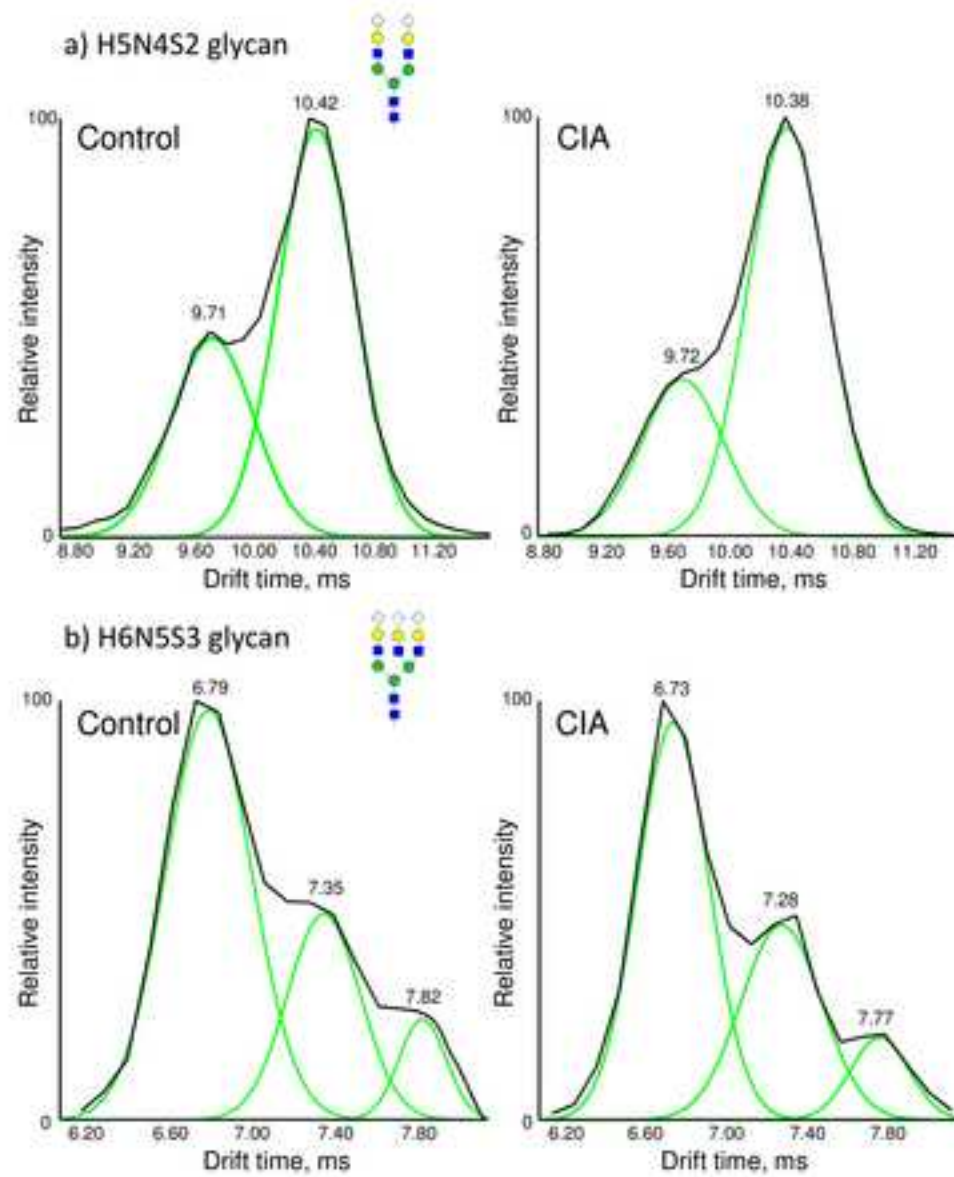


Figure 4

Supplementary material

[Click here to download Supplementary material: Supplementary_Material_revised.docx](#)

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