Supplemental Information

Results:

The combination of Nano LC-MS$^2$ and exoglycosidase digestions enabled the elucidation of the linkage and conformation of the sialic acids. The additional sialic acid residues were confirmed to be linked (or α2,6) to the GlcNAc of one or both antennae. Analysis of the trisialylated fraction of the sample yielded 4 ions, with [M+2H] values of 1316.7 m/z, 1324.7 m/z, 1332.8 m/z, and 1340.8 m/z. These corresponded to A2G2S3, with 3 Neu5Ac, 2 Neu5Ac and 1 Neu5Gc, 1 Neu5Ac and 2 Neu5Gc, and 3 Neu5Gc. Isolation and fragmentation of the 948.0 m/z ion (from the doubly charged ammonium adduct of the 3 Neu5Ac variant with an m/z value of 1327.0) indicated the presence of an antennae with 2 Neu5Ac residues (Neu5Ac2HexNAc1Hexi). The MS$^2$ spectra resulted in 3 intense ions at 495.3 m/z, 366.3 m/z, and 454.2 m/z where the 495.3 ion corresponded to Neu5Ac-GlcNAc and the 454.2 m/z ion corresponded to Neu5Ac-Gal. Isolation of the 948 ion and fragmentation yielded a diagnostic ion with an m/z of 495.3, indicating that the Neu5Ac was linked to the GlcNAc. The presence of a Neu5Ac disaccharide (Neu5Acα2,8-Neu5Ac) would not result in the presence of the 495.3 m/z ion, and thus confirmed our hypothesis that the hypersialylation was due to the presence of sialic acids linked directly to GlcNAc.

In order to gain orthogonal evidence for this hypothesis, the trisialyalted fraction was digested with exoglycosidases NAN1 and BTG prior to analysis by nano LC-MS. The digested products included a structure which was detected at 1091.5 m/z (Figure S1), corresponding to a 2-AB labelled glycan containing 2 Neu5Ac, 1 Gal and 2 GlcNAc in addition to the trimannosyl chitibiose core. Fragmentation of this ion yielded 3 ions with m/z values of 495.2, 366.2 and 657.3, corresponding to Neu5Ac-GlcNAc, Gal-
GlcNAc, and Neu5Ac-Gal-GlcNAc or Gal-(Neu5Ac)GlcNAc, respectively. These results provide a very high degree of confidence that the undigested sample must have contained the structure shown in Figure S1, and therefore provide more evidence that the hypersialylation of the trisialylated and tetrasialylated glycans in the sample is due to sialic acid linked directly to GlcNAc.

By combining the HILIC and LC-MS² data, structural assignments were made. One benefit of HILIC combined with exoglycosidase digestions is the consistent shift in GU values as the enzymes remove terminal sugars. The ABS and NAN1 enzymes remove both N-acetyl neuraminic acid (Neu5Ac) and N-glycolyl neuraminic acid (Neu5Gc) from terminal GlcNAcs and Gals. The GU shifts resulting from the removal of sialic acids in different linkages from terminal Gal or GlcNAc by exoglycosidase enzymes are shown in Figure S2. The linkage of Neu5Gc to GlcNAc in an α2,3/6 linkage and Neu5Gc to Gal in an α2,3 linkage results in an 0.98 shift in GUs, whereas an α2,6 linkage of an Neu5Gc to Gal results in a shift of 1.24 GUs. The linkage of Neu5Ac to GlcNAc in an α2,3/6 linkage and Neu5Ac to Gal in an α2,3 linkage results in an 0.55 shift in GUs, whereas an α2,6 linkage of an Neu5Gc to Gal results in a shift of 0.88 GUs. This difference in shifts of sialic acids bound to Gals has been reported previously by Montensino et al (2010). The linkage of sialic acids to GlcNAc residues, in the presence of the sialic acids bound to the Gal residues, is defined as hypersialylation.