Clustering is a highly glycosylated secreted protein implicated in the pathogenesis of Alzheimer’s disease (AD). Expression of the clusterin gene is significantly elevated in AD brain (May et al., 1990) and levels of plasma clusterin correlate with AD progression (Thambisetty et al., 2010). We previously identified a plasma clusterin isoform as a candidate biomarker for AD using 2-dimensional gel electrophoresis (2DE). Immunoblotting and use of unmodified peptides in selected reaction monitoring fail to replicate the regulation seen in AD, as they are blind to post-translational events. Since glycosylation plays an important role in physiological functions of clusterin (Shibahara et al., 2007), we hypothesised that detailed glycoprofiling of plasma clusterin may reveal more potent biomarker isoforms.

**Methods**

Human clusterin was enriched by immunoprecipitation (IP) from albumin/triglyceride-depleted clinical plasma using a monoclonal anti-clusterin antibody (Millipore). IP proteins were first analysed by Western blotting as a quality control, then separated by either 2DE or SDS-PAGE. The spots and single band of interest respectively were excised, reduced, alkylated and digested in-gel with trypsin prior to analysis by mass spectrometry (MS).

Samples were analysed via LC-MS/MS using nanoflow reverse phase chromatography and a Top20 collision induced dissociation (CID) method (Orbitrap Velos, ThermoFinnigan Scientific). Glycopeptides were manually identified by the presence of glycan-specific oxonium ion fragments, m/z 204.08 for N-acetylgalactosamine, [HexNAc]+, m/z 366.14 for hexose-N-acetylgalactosamine, [Hex-HexNAc]+, and m/z 657.24 for N-acetylgalactosamine and N-acetylgalactosamine [NeuAc-Hex-HexNAc]+ in the MS/MS spectra.

**Results**

After separation by 2DE, 16 distinct spots (previously confirmed as clusterin positive by Western blotting, Thambisetty et al., 2010), were isolated and analysed (Figure 1). The shift in pI observed for different gel spots on the 2DE gel is most likely driven by glycosylation via alterations in number of antennae and/or sialic acids. A basic vector was devised to illustrate this phenomena (Figure 2).

Using IP enrichment and Gel-LCMS/MS, we have identified and characterised 41 glycopeptides covering 5 of 6 anticipated N-linked glycosylation sites in plasma clusterin.

Glycopeptide HNN*STGCLR (site II*) exhibited the greatest glycosylation diversity with 12 types of oligosaccharides identified. An example of the MSMS spectrum for HNN*STGCLR is shown in Figure 3.

Upon analysis, it became apparent that the most abundant covalent modification of each of the 2DE spots was always fully sialylated bi- or bi-antennary glycosylations of clusterin (Table 1 for an example) and it transpires that the detected clusterin glycopeptides provide evidence to support both the removal of sialic acids alone (291Da green to blue spot in figure 2), as well as removal of sialylated antennae (-450Da green to red spot in figure 2), suggesting distinct neuroimimidase and β- acetylgalactosaminidase activity respectively.

The LC-MS/MS results indicated a trend towards lower number of sialylated antennae, suggesting the successive removal of whole antennae which has a pronounced effect on the location of the 2DE spots.

Next we considered the analysis of the IP clusterin population in plasma from low atrophy and high atrophy patients by LC-MS/MS. Differences in the glycosylation profile of clusterin were seen when comparing low and high atrophy subjects. We were only able to detect bi-antennary structures in the low atrophy group. The loss of whole antennae therefore appears to correlate with higher atrophy (n=4).

An example of this loss of whole antennae can be seen when we look at the sialylated forms of the bi-antennary glycopeptides presented as highly charged molecular ions at m/z 1284.17 and m/z 1391.54, but only in individuals with low atrophy (Figure 5). These molecular ions are therefore potential biomarkers concordant with the extent of hippocampal atrophy.

**References**


**Conclusions**

- Analysis of immunoprecipitated clusterin creates a unique glycopeptide reference resource for clusterin.
- Site specific glycosylation of plasma clusterin may represent a novel biomarker for Alzheimer’s disease.
- Plasma biomarker potential becomes apparent by considering the relative abundance of all glycosylated forms identified at distinct glycosylation sites within the amino acid sequence of clusterin.