Glycoblotting method allows for rapid and efficient glycome profiling of human Alzheimer's disease brain, serum and cerebrospinal fluid towards potential biomarker discovery

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ABSTRACT

Background: Understanding of the significance of posttranslational glycosylation in Alzheimer's disease (AD) is of growing importance for the investigation of the pathogenesis of AD as well as discovery research of the disease-specific serum biomarkers.

Methods: We designed a standard protocol for the glycoblotting—combined with MALDI-TOFMS to perform rapid and quantitative profiling of the glycan parts of glycoproteins (*N*-glycans) and glycosphingolipids (GSLs) using human AD's post-mortem samples such as brain tissues (dissected cerebral cortices such as frontal, parietal, occipital, and temporal domains), serum and cerebrospinal fluid (CSF).

Results: The structural profiles of the major *N*-glycans released from glycoproteins and the total expression levels of the glycans were found to be mostly similar between the brain tissues of the AD patients and those of the normal control group. In contrast, the expression levels of the serum and CSF protein *N*-glycans such as bisect-type and multiply branched glycoforms were increased significantly in AD patient group. In addition, the levels of some gangliosides such as GM1, GM2 and GM3 appeared to alter in the AD patient brain and serum samples when compared with the normal control groups.

Conclusion: Alteration of the expression levels of major *N*- and GSL-glycans in human brain tissues, serum and CSF of AD patients can be monitored quantitatively by means of the glycoblotting-based standard protocols.

General significance: The changes in the expression levels of the glycans derived from the human post-mortem samples uncovered by the standardized glycoblotting method provides potential serum biomarkers in central nervous system disorders and can contribute to the insight into the molecular mechanisms in the pathogenesis of neurodegenerative diseases and future

drug discovery. Most importantly, the present preliminary trials using human post-mortem samples of AD patients suggest that large-scale serum glycomics cohort by means of various-types of human AD patients as well as the normal control sera can facilitate the discovery research of highly sensitive and reliable serum biomarkers for an early diagnosis of AD.

Key words: *Alzheimer's disease, glycome, human brain, serum, cerebrospinal fluid, biomarker*

1. Introduction

Alzheimer's disease (AD), the most common devastating, late-onset neurodegenerative dementing disorder, is characterized by progressive neuronal loss accompanied by loss of memory, mood changes, problems with communication and reasoning. This devastating disorder can be early onset genetic cases (familial AD) and vast majority of late onset sporadic forms, which are caused by several factors (such as estrogen, anti-inflammatory drugs, cerebrovascular diseases, oxidants, and metals). However, the etiology and processes by which the factors may influence disease development in the sporadic cases remain poorly understood. Accumulating evidences clearly suggest that the etiology of AD might be multifactorial, including genetic predisposition, protein trafficking and turnover, glycosphingolipids (GSL) abnormalities, and impairment of neurotrophin signaling [1,2]. Moreover, in AD, morphological studies showed that selective neuronal degeneration in human brain is region specific [3].

The brains of patients with AD have an abundance of two abnormal structures – amyloid plaques and neurofibrillary tangles – that are made of misfolded proteins. This is especially true in certain regions of the brain that are important in memory. The other main feature of AD is the loss of connections between the neuronal cells. This leads to diminished cell function and cell death [4,5]. Amyloid beta ($A\beta$) is the major constituents of the fibrils composing senile plaques and vascular amyloid deposits. Scientists have been striving to clearly demonstrate the mechanism of $A\beta$ production from amyloid precursor proteins (APP) processed by the harmful pathway, in which APP is cleaved by a β -secretase yielding $sAPP\beta$ and amyloidogenic fragment A4CT (the soluble $sAPP\beta$ isoform, which extends from the $A\beta$ amino terminus to the carboxyl terminus of APP) and subsequent cleavage of A4CT within its transmembrane region by γ -secretase generating the $A\beta$ (1-40) and $A\beta$ (1-42/43). The clinically important $A\beta$ that deposits as

amyloid in AD brains is most likely to have been produced locally and also appears to occur in all cells and tissues that can be detected in plasma and cerebrospinal fluid (CSF) [2,6]. Recently there has been evidence that tau oligomer's, which form before paired helical filaments (PHFs) and neurofibrillary tangles (NFTs), could be the key structure mediating neurodegeneration even before the fibrillary tau is deposited [7]. Soluble aggregates (i.e., A β and α -synuclein) have also been implicated as the primary toxic species in many degenerative diseases [8].

The atrophy of the neurons due to pediatric and adulthood diseases of the central nervous system (CNS) may lead to the aberrant posttranslational glycosylation owing to the alteration in the biosynthesis of the glycans attached to various glycoconjugates. Understanding the glycosylation pattern of glycoproteins and glycosphingolipids which are affected by either genetic or environmental cellular stressors, can be a promising approach for the discovery of novel biomarkers to assist an easy prognosis. Posttranslational glycosylations in the proteins and sphingolipids serve as one of the most important bio-signals that are indispensable for the ordered social life of mammalian cells. They have a role in cell-cell communication, molecular signaling, the immune systems both in cellular and antibody-based immunology, pathogenicity (viral infection/bacterial adhesion), and the functions of hormones and growth factors. It is clear that a systematic approach for the glycome profiling by means of the different biological samples from the AD patients' post-mortem samples such as brain tissues (dissected domains of the cerebral cortices), serum and CSF would be inevitable in order to discover the novel biomarkers for the diagnosis and therapeutic treatments [9].

The glycomes – the spectrum of all glycan structures – expression levels of human brain tissues, serum and CSF of AD are now indispensable and demanding to acquire detailed knowledge of their total structures and functions of the glycans. However, most glycomics

studies have long been attempted on the basis of the chromatography-based methods involving many time-consuming and tedious procedures for the purification of the limited species of glycans released from various glycoconjugates. To overcome the bottlenecks in the glycome analysis, we established the glycoblotting method, an easy, speedy, and highly efficient glycan enrichment process to perfectly purify and label glycans released from common glycoproteins and GSLs [10-12]. We have reported that dynamic alterations in the human serum glycoform profile are promising diagnostic biomarkers not only in cancers such as hepatocellular carcinoma [13-15], pancreatic cancer [16], renal cell carcinoma [17], and prostate cancer [18], but also in various diseases such as osteoarthritis [19,20], ulcerative colitis [21], nonalcoholic steatohepatitis [22], and diabetic retinopathy [23,24]. Recently, we have demonstrated the feasibility of this unique technology for analyzing and quantifying major glycans of Huntington's disease (HD) transgenic mice, in which the trend in the major glycoforms and expression levels of total glycans were different significantly between the HD transgenic and control group mice [25]. In the present study, we assessed preliminarily the potentials of the glycoblotting-based protocols to characterize major *N*- and GSL-glycans of human AD's post-mortem brain, serum and cerebrospinal fluid (CSF) aiming to discover human AD-relevant biomarkers for an easy diagnosis and novel therapeutic targets in the CNS disorders.

2. Materials and methods

2.1. Human samples

Human brain tissues/fluids specimens were obtained from the Human Brain and Spinal Fluid Resource Center (HBSFRC), VA West Los Angeles Healthcare Center, Los Angeles, CA 90073, which is sponsored by NINDS/NIMH, National Multiple Sclerosis Society, USA, and the study was performed by ethical standards of the declaration of Helsinki and approved by the Ethics Committee of Hokkaido University (Informed consent was obtained from all patients). The frontal, parietal, occipital, and temporal cortices were already dissected, serum and CSF were directly used without any prior treatment. The profile of all specimens such as brain sections, gender, autolysis time, age and weight of each brain tissues are summarized in Table 1. All samples were stored at -80°C from the day of received till use.

2.1.1. Gross description

High quality, high resolution colored digital images of the entire brain and 7 mm full coronal sections at all levels were examined in a frontal to occipital directions. Four 1 cm sections of brainstem and cerebellum are also examined. In both normal (non-AD) and AD cases, the coronal sections showed no evidence of metastatic cancer, no apparent stroke or infarction or any other softening, discoloration, hemorrhage, mass, or lesion. The junction between the cortex and white matters was well demarcated. The lateral cerebral ventricles were normal in shape and size. The striatum, lentiform nucleus, and thalamus were normal and there was no atrophy of cerebral folia. Specifically, in normal human brain tissues, there was no evidence of the metastatic carcinoma and no grossly identifiable evidence of the stroke. In addition, there was no cerebral edema or herniation of the unci or cerebellar tonsils. There was mild (in male) or no (in female) atrophy of frontal lobes. The basilar cerebral vasculature showed no atherosclerosis except

normal F2. However, in cases for AD samples, there was moderate-to-prominent atrophy of the frontal and temporal lobes. The basilar cerebral vasculature showed moderate-to-extensive atherosclerosis in male AD, while no atherosclerosis was observed in female AD cases.

2.1.2. Microscopic examination

Sections of frontal cortex, hippocampus, temporal cortex, and substantia nigra were examined with the Hematoxyline and Eosine (HE) stain. Sections of frontal cortex, temporal cortex, and the hippocampus were also tested with the modified Bielschowsky silver stain. In normal brain tissues, the neocortex showed normal neuronal cellularity and normal subcortical white matter. The neocortex, hippocampus, and substantia nigra exhibited no evidence of the neurodegeneration and hypoxia. None of the section showed evidence of hemorrhage, infarction, necrosis, inflammation, infection, or other pathology. The hippocampus was normal with no evidence of granulovacuolar degeneration, Hirano bodies, Pick bodies, Lewy bodies, or eosinophilic degeneration. The Bodian silver-stained sections failed to demonstrate neurotic plaque or neurofibrillary tangle formation in either the neocortex or hippocampus. The Bielschowsky silver-stained sections showed no evidence of neurotic plaque formation. A few scattered neurofibrillary tangles (NFTs) were found in the entorhinal cortex and even fewer in the hippocampus (only normal F2). There was no evidence of stroke or metastatic disease. As a result, these findings were concluded to be those of a neuro-pathologically normal brain. However, in cases of AD brain, the neocortex showed a moderate-to-marked decreased in neuronal cellularity, mild-to-extensive associated gliosis, and normal subcortical white matter. The Bielschowsky silver-stained sections showed frequent numbers of neurotic plaques and scattered NFTs. The hippocampus also contained plaques and NFTs as well as neuronal loss, gliosis, granulovacuolar degeneration, and Hirano body formation. These findings clearly

indicated the diagnosis of AD and were consistent with the diagnosis of “definite” AD according to CERAD (Consortium to Establish a Registry for Alzheimer’s Disease) and a Braak and Braak stage of 3-4 (in AD-M1) and 5 (in the rest AD samples) was also supported. These results and the background of the “normal” brain samples were summarized in Table 1.

2.2. Reagents and equipment

Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), tris(hydroxymethyl) aminomethane (Tris), trypsin, and dithiothreitol (DTT) were purchased from Sigma-Aldrich. Peptide *N*-glycosidase F (PNGase F) was purchased from New England Biolabs® Inc. (Ipswich, Massachusetts, USA). Chitooligosaccharide (GN4) mixture was from Seikagaku Biobusiness (Tokyo, Japan). BlotGlyco®H beads was purchased from Sumitomo Bakelite (Tokyo, Japan). Ammonium carbamate (99%) and 1-propansulfonic acid-2-hydroxyl-3-myristamido (PHM) were obtained from Tokyo Kasei (Chuo-ku, Tokyo, Japan). *O*-Benzylhydroxylamine hydrochloride (BOA), 3-methyl-1-*p*-tolyltriazene (MTT), and disialylated biantennary oligosaccharide [A2; (Hex)₂ (HexNAc)₂ (NeuAc)₂ + (Man)₃ (GlcNAc)₂] were from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Human IgG ELISA quantitation set (Cat. No. E80-1004) was purchased from Bethyl Laboratories, Inc., USA. Other reagents and solvents were obtained from Wako Pure Chemicals (Tokyo, Japan) and Kanto Chemical Co., Inc., unless otherwise stated. MultiScreen Solvinert filter plates and Mass PREP™ HILIC μElution plates were purchased from Millipore and Waters, respectively. The ozone generator used (NG81-NO3, T-153) was from Nigorikawakogyo, Japan. The automated glycan processing machine used for incubation (Sweetblot) was from Systems Instruments Corporation (SIC). All mass measurements were performed by using MALDI-TOF/MS (Ultraflex III, Bruker Daltonics, Germany).

2.3. N-glycans from glycoproteins of human brain tissue

Lysis buffer (2% SDS, 5 mM TCEP in 0.1 M Tris-acetate (pH 7.4), 100 mg/mL) was added to human brain tissue and homogenized by Polytron at 25,000 rpm (MAX) three times for 30 seconds on ice. We employed 500 μ L and 1.5 mL of tissue homogenates for *N*- and GSL-omics, respectively. For *N*-glycomics, the lysates were centrifuged at 20,400 g (MAX) for 20 mins at 4°C. Protein amount was quantified by using the Bicinchoninic acid (BCA) protein assay utilized by Pierce[®] BCA protein assay kit (prod# 23227, Thermo Scientific, USA). Ice-cold acetone (four times the volume of homogenates) was added and incubated overnight at -20°C to precipitate proteins. The precipitate was collected by centrifugation at 20,400 g for 20 mins at 4°C, and washed with 500 μ L ice-cold acetonitrile (ACN). The precipitate was collected by centrifugation at 20,400 g for 20 mins at 4°C and air dried. The dried sample was dissolved in freshly prepared ammonium bicarbonate (ABC, 50 μ L, 200 mM) containing 0.1% 1-propanesulfonic acid-2-hydroxyl-3-methylmercaptamido (PHM) in 10 mM ABC and the solution was incubated at 37°C for 10 mins in a rotary shaker. The solubilized proteins were reduced by 120 mM 1,4-dithiothreitol (DTT) at 60°C for 30 mins and were alkylated using 123 mM iodoacetamide (IAA) at room temperature in dark for 1 hr. The mixture was digested using trypsin (400 U/ μ L in 1 mM HCl) at 37°C overnight, followed by heat-inactivation of the enzyme (90°C, 10 mins). 10 U PNGase F was added and incubated at 37°C for 3 hrs. The sample was dried and stored at -30°C till the day of use. The dried sample was adjusted to a concentration of 200 μ g protein by using de-ionized water and applied to glycoblotting, as described below. In case of serum glycoproteins, 10 μ L was diluted six times in freshly prepared ABC (0.33 M) -1-PHM (0.4% in 10 mM ABC) - DTT mixture (120 mM) in 1:1.5:1 (v/v) ratio in de-ionized water. Internal standard (IS, A2amide, 110 pmole) was added according to the standard protocol [10-24]. The rest followed the same pretreatment as human brain tissue mentioned above. In case of

CSF, after BCA protein assay, 0.5 mL of CSF was used for acetone precipitation. The rest followed the same pretreatment as human brain tissue *N*-glycomics.

2.4. *Glycans from GSLs of human brain tissue*

1.5 mL of homogenates of human brain tissue were centrifuged at 100 g for 10 mins at 4°C and the supernatant was transferred into another 15 mL Falcon tube on ice. The precipitate was dissolved with 600 µL of phosphate buffer saline (PBS) and sonicated. The mixture was centrifuged at 800 g for 10 mins at 4°C, and the supernatant was mixed with the first homogenates. The whole homogenates were centrifuged at 13,000 g for 10 mins at 4°C, and the resulting precipitate was again dissolved with 500 µL PBS, and 5 µL of it was used for BCA protein assay. The remaining solution was allowed to precipitate by centrifugation at 13,000 g for 10 mins at 4°C. The precipitate was dissolved, successively, with a mixture of 3 mL chloroform:methanol to produce three solutions (1:2, 1:1 and 2:1 v/v) and chloroform:MeOH:water mixture (2:5:1 v/v), and it was sonicated for about 10 mins in each extraction followed by centrifugation at 800 g and 4°C. The supernatants of each step were collected in new 15 mL Falcon tube. The resulting supernatants were dried by using SpeedVac at room temperature. The total lipids were suspended in 2 mL C/MeOH (1:1 v/v) and sonicated. Ozone was generated from oxygen using a benchtop generator at a flow setting of 1 mL/min. The output from the ozone generator was bubbled through the solution for 5 mins. The reaction was quenched by passing N₂ gas in the total lipid suspension for a minute and the resulting solution was dried by SpeedVac. MeOH:0.5 M sodium methoxide in MeOH (4:1 v/v) was added to the dried sample and allowed to stand for 30 mins at room temperature for alkaline degradation. The reaction mixture was neutralized with 6 µL concentrated acetic acid (AcOH) and dried. The sample equivalent to 400 µg of protein adjusted with de-ionized water were used

for glycoblotting. In case of serum GSLs, a 100 μ L of freeze-dried serum was used to analyze serum glycan moieties released from GSLs according to the procedure reported previously [25]. Briefly, GSLs were extracted in 3 mL of chloroform:MeOH mixtures at 1:2, 1:1, 2:1 (v/v) and a chloroform:MeOH:water mixture at 2:5:1 (v/v). Each step was accompanied by sonication for 10 mins and centrifugation at 800 rpm for 10 mins. The resulting supernatants of each extraction were collected together in another 15 mL Falcon tube. The remaining steps were performed in accordance with the procedure of brain GSL-omics described above.

2.5. Glycoblotting

500 μ L of BlotGlyco[®] H beads (10 mg/mL) was added to the wells of MultiScreen Solvint filterplate and the water was removed by vacuum. Digested mixtures of brain tissue (20 μ L) worth 200-400 μ g protein (specified in each *N*- and GSL-glycan release protocols) and serum containing IS (A2amide for *N*-glycans, GN4 for GSL-glycans) were applied to each well together with 180 μ L of 2% AcOH/ACN. The plate was incubated at 80°C for 45 mins followed by two times successive washing using 200 μ L of 2 M guanidine-HCl in 16.6 mM ABC, water and 1% triethylamine in MeOH. After acetyl capped using 10% acetic anhydride in MeOH at room temperature for 30 mins, the remaining acetic anhydride was removed by vacuum. Each well was washed two times with 10 mM hydrochloric acid (HCl), MeOH and dioxane, consecutively. On bead methyl esterification was performed by adding 150 mM 3-methyl-1-p-tolyl triazene and incubated at 60°C for 90 mins. A consecutive one time wash was performed using 200 μ L of dioxane, water, MeOH and water. For an effective transamination reaction, 50 mM BOA was added accompanied by 2% AcOH/ACN and incubated at 80°C for 45 mins. Finally, labeled glycans were eluted with 100 μ L water.

2.6. Mass spectrometric analysis

The recovered glycans by glycoblotting method from human brain tissues, serum and CSF were analyzed after spotted on MTP 384 target plate (Polished steel TF, Bruker Daltonics) and crystallized together with matrices [DHB:DHB- Na^+ in 9:1]. MALDI-TOF/MS data were acquired on an Ultraflex III (Bruker Daltonics, Bremen, Germany) equipped with a reflector and controlled by the FlexControl 3.0 software package. Further fragment analysis was performed by MALDI-TOF/TOF analysis (Bruker Daltonics, Bremen, Germany). Annotation of the MS and TOF/TOF data were achieved with the FlexAnalysis 3.0 software package (Bruker Daltonics GmbSH, Bremen, Germany, S/N = 6 and quality factor threshold of 30). The experimental masses were used to predict the possible glycans composition reported on <http://web.expasy.org/glycomod/> and CFG (<http://www.functionalglycomics.org>). The data from Lipid Bank (<http://lipidbank.jp/>) and in-house database were used for the identification of glycans of GSLs.

3. Results

3.1. Human AD brain tissue *N*-glycomics

Firstly, we needed to test whether the standard glycoblotting protocol designed for the glycomics of HD transgenic mice [25] is feasible for the dissected human brain tissue samples such as frontal, parietal, occipital, and temporal in the cerebral cortices. It was demonstrated that the basic procedure established for the pretreatment of HD transgenic mice brain samples can be applied directly for the glycome profiling of protein *N*-glycans and GSL-glycans of the dissected human brain tissues with a slight modification (Figure 1). Lysis using 0.1 M Tris-acetate buffer (pH7.4) in the presence of 2% SDS and 5 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) facilitated further procedures of the glycoblotting protocol and made an ideal MALDI-TOFMS-based glycan analysis possible. We have profiled and assigned 40 *N*-glycans that can be classified as known high mannose-, hybrid- and complex-type *N*-glycans (Table 2 and Figure S1 A-D in the Supporting Information). Quantitative glycan analyses revealed that the expression levels of whole *N*-glycans in the frontal cortices of AD patients was decreased in comparison with the normal control groups of both sex (Table 3, $p < 0.05$). Except in the male temporal cortex of AD patients, which was found increased in the expression levels of *N*-glycans, no significant difference in most *N*-glycan profiles was observed in other cerebral cortices of AD patient and the normal control subjects. From the glycotyping analysis [26] of the results shown in Table 2 and Table S1 (Supporting Information), although high mannose type *N*-glycans seemed to increase slightly, the proximal (core-fucose) and bisecting-GlcNAc type of *N*-glycans were decreased in AD patient brain tissues when compared with those of the normal control groups. In addition, no significant differences were found in the expression levels of tri- and tetra-antennary *N*-glycans carrying sialic acids between AD patient brain tissues and the normal control groups.

Taken all together, it could be summarized that there is no significant difference in the major protein *N*-glycan expression levels between the AD patient brain tissues and those of normal control groups.

3.2. Human AD serum *N*-glycomics

By applying the general glycoblotting protocol for the human serum glycomics [13-24], we identified 52 *N*-glycans that can be used for further comparative study between AD patient and normal control group (Table 2). The total expression levels of the serum *N*-glycans seemed to increase significantly in AD patients when compared with the normal control groups ($p < 0.001$ in male; $p < 0.05$ in female, Table 4). As shown in Figure 2, it is likely that the total expression levels of whole *N*-glycans of the AD patient serum were higher than those of the normal control groups. Comparing with the male AD patient serum, it is interesting to note that the specific *N*-glycan expression levels appeared to increase more pronouncedly in the female AD serum than those of the male AD patient serum (Figure S2 in the Supporting Information). The major *N*-glycans with higher expression levels in female AD patient serum in comparison to the normal control serum can be identified as the glycoforms involving biantennary, bisecting-GlcNAc, and proximal fucose residues. For example, the mass intensity of the signal at m/z 2117.9 corresponding to one of the typical bisect-type *N*-glycoforms was found to be 17 times higher in female AD patient serum than that of the normal female control serum (Figure 3 and Figure S2 in the Supporting Information). Furthermore, the glycotyping analysis of the serum *N*-glycans profiles also suggested that the proximal fucose, bisecting-GlcNAc and tri- and tetra-antennary type *N*-glycans were significantly increased while di-sialylated *N*-glycans were decreased in AD patient serum when compared with the normal control sera. It is not surprising

that there was no direct evidence in the whole MALDI-TOFMS results for the existence of the *N*-glycolylneuraminic acid (Neu5Gc) in human serum of this study.

3.3. Human CSF *N*-glycomics

It was demonstrated that the glycoblotting protocol established for the dissected human brain tissue *N*-glycomics can also be used for human CSF samples after the acetone precipitation. We profiled 50 *N*-glycans in human CSF, in which asialo- and agalacto-type *N*-glycans containing the proximal fucose and bisecting-GlcNAc residues seemed to be CSF characteristic glycoforms (Table 2). Total *N*-glycans expression levels from 100 µg protein equivalent of CSF were found to be increased significantly in AD patients of both sexes when compared with those of the normal control group (Figure 4A). In addition, human CSF *N*-glycans showed a clear difference between male and female subjects. Importantly, the expression levels of the major human CSF relevant *N*-glycans profiled in Table 2 were increased markedly in AD patient serum compared with the normal control groups (Figure 4B and 4C). Those *N*-glycans which are, in particular, the glycoforms involving the bisecting-GlcNAc, proximal fucose, and tri- and tetra-antennary motifs are increased more pronouncedly in AD patients than those of the normal control groups. The most highly expressed glycoform in AD patients' CSF was found to be the GlcNAc-terminated biantennary *N*-glycan with a bisecting GlcNAc and core fucose residues identified as the signal at m/z 1793.67. The expression level of this *N*-glycan seemed to increase 10 and 7 times higher in the male and female AD patient serum than those of the normal control groups, respectively (Figure 4B and C).

Aiming at the target proteins that could be a source for the up-regulation of the specific *N*-glycans in the AD patient serum and CSF, we have quantified the concentration of the IgG, which is one of the most abundant human serum glycoprotein carrying above mentioned CSF

relevant glycoforms, by using human IgG ELISA quantitation kit based on the manufacturer's protocol. It was revealed clearly that the concentration of human serum *N*-glycans in both AD and normal subjects showed highly positive correlation ($r = 0.85$) with the concentration of serum IgG while there was no significant correlation in case for the CSF ($r = 0.30$) as indicated in Figure 5.

3.4. Human brain and serum glycosphingolipidomics

The human brain is rich in GSLs, especially gangliosides – sialylated versions of the complex GSLs are known to be abundant in brain and the peripheral nervous system. We have profiled abundant gangliosides in human brain of both disease and the normal subjects according to the protocols described in the previous reports [25,27]. Although the use of endoglycoceramidases can be alternative method to release GSL-glycans, it is important to note that feasibility in the large-scale glycomics appears to be unclear due to the substrate specificity as well as commercial availability. On the contrary, the ozonolysis-based protocol showed the benefits of indiscriminate glycan release of intact oligosaccharides independent from the structures of the glycosphingolipids. Total expression levels of gangliosides were found to be decreased specifically in the frontal cortex of AD brain tissue ($p < 0.05$, Table 3 and Figure 6C) while it seems that there was no significant difference in other cerebral cortices. Interestingly, GM1, one of the important products derived biosynthetically from GM3/GM2 as shown in Figure 6A, appeared to decrease in AD brain tissues when compared with the normal subject (Figure 6B, Table 6 and Figure S3 A-D in the Supporting Information). In the contrary, it was revealed that no significant difference in the total amount of the human serum gangliosides between AD patients and normal control groups was observed (Table 4), while GM3 and GM2

appeared to increase in AD patients comparing to the normal control serum (Figure 6D and Table 5).

4. Discussion

The present study clearly demonstrated that the glycome profiling protocol established by using HD transgenic mice samples [25] can be applied basically to the glycoblotting-based sample preparation combined with MALDI-TOFMS analysis of human postmortem brain tissues, CSF, and sera toward discovery research for the diagnosis and therapeutic treatments. In case for the human samples, it was demonstrated that a simple BOA-labeling of the all released glycans through the imine exchanging reaction of the captured glycans with the benzyloxyamine (BOA) enables highly sensitive and quantitative analysis using MALDI-TOFMS while mice glycome analysis often needed to label with an aminooxy-Trp-Arg derivative (aoWR) showing higher ionization potential than BOA tagging [25]. It was revealed for the first time that total expression levels of the major *N*-glycans released from glycoproteins and GSLs were mostly similar in the human brain cerebral cortices while the decrease was observed only in the frontal cortex among the human AD dissected brain tissues compared with the normal control groups. The lower expression levels of *N*-glycans with bisecting-GlcNAc and core-fucose residues in the frontal cortex of AD patients can be explained by the down regulations of glycosyltransferases GnTIII and FUT8 in the brain. In contrast, serum and CSF protein *N*-glycans and the gangliosides such as GM3 and GM2 were increased markedly in AD patients compared with the normal control groups. These results might be due to an early onset of progressive and severe β -amyloid deposits, the selective vulnerability of the frontal cortex and then the spreading of the protein aggregates, as the moving aggregates, towards the other parts of the brain. Molecular mechanisms for selective vulnerability remain mostly to be elucidated while the neuropathological lesions involved deposition of abnormal proteins, which can be intranuclear, cytoplasmic and extracellular, might contribute to the reduction of *N*-glycans in

neurodegenerative diseases [29]. Those abnormal protein aggregates may lead to the degeneration of the neurons which come up with not only the loss of the neurons but also cause astrogliosis of the non-neuronal cells. As a result, the loss and functional abnormalities of both of neuronal and non-neuronal cells regulate and/or affect the activities of glycosyltransferases towards alteration in glycosylation. The pathogenesis of neurodegeneration, which are caused by either genetic or environmental cellular stressors, evoke biochemical changes in the brain cells from the cerebral cortex. It is important to note that there is an association between the perturbation of protein/sphingolipid glycosylation machinery and pathology of the number of diseases [9, 30].

Human serum *N*-glycomics showed an increase of the bisecting-GlcNAc, core-fucose, tri- and tetra-antennary structures of *N*-glycans in AD patients compared to the normal control groups. Beta A4 amyloid deposition in the brain, which is characteristic of AD, may result from either over expression of the amyloid protein precursor (APP) or failure of APP to be correctly processed. AD related abnormalities of APP metabolism (which can increase the A β concentration and result in A β deposition) in the CNS might be reflected in the circulation and A β peptides which are secreted by cells can be detected in plasma and CSF. Study showed that 50% increase in the proportion of 130-kDa APP species in patients with AD. In addition, point mutation in the β -APP gene found in familial Alzheimer's disease (FAD) kindred results directly in increased generation of the β -amyloid accompanied by a decrease P3 peptide [31]. Mutant APP contained higher contents of bisecting-GlcNAc and core-fucose residues compared to the wild type APP [32] and tri- and tetra-antennary *N*-glycans are increased in pathogenic prion protein isoforms (PrP^{Sc}) compared to the normal (PrP^C) prion proteins [33]. It seems likely that the elevation of the bisecting-GlcNAc, core fucose and tri- and tetra-antennary *N*-glycans in AD

patient serum might be due to an elevation of mutant APP in the circulation which corresponds to increase in A β and decrease in P3 peptide – the neuropathological blueprints of human AD.

CSF proteomes are produced from the brain, choroid plexuses in the brain ventricles originating from the blood, following active and passive transport through the blood brain barrier, and from drainage of interstitial liquid of the nervous system [34]. Since the CSF compartment is in close anatomical contact with the brain interstitial fluids, where biochemical changes related to chronic neurological diseases (CND) are reflected, CSF is a promising source of biomarkers [35]. The total amount of *N*-glycans expression levels were increased in AD CSF compared to the normal. We have also found high amounts of proximal fucose, bisecting–GlcNAc and terminal galactose types of *N*-glycans in AD compared to the normal subjects. Our result further strengthen the high presence of “brain type” neutral *N*-glycans in CSF [36,37-40]. The *N*-glycans difference between genders could be explained by the different sex hormones in which many of them are glycosylated [36].

Two major *N*-glycans (m/z 2117.75 in serum and m/z 1793.67 in CSF) showed a clear-cut difference between AD and control. Futakawa *et al.* [38] reported that Tf-2 and Tf-1 have unique *N*-glycans (m/z 2117.75 and 1793.67, respectively) and Tf-2/Tf-1 ratio was also used as diagnostic index for idiopathic normal pressure hydrocephalus (iNPH) and AD with their respective controls. In our CSF *N*-glycomics we have found that the ratio of Tf-2/Tf-1 is significantly lower (data not shown) in AD compared to the normal. Since reports declare there were no significant differences in AD CSF transferrin and A β 1-42 levels compared to the nondemented control [41] and such unique glycan (m/z 1793. 67) cannot persist in blood due to the existence of specific hepatic clearance mechanism [42], those glycans are the most promising glyco-biomarkers in CSF that clearly differentiate AD and the normal subjects. The stronger

correlation between the concentration of serum IgG and *N*-glycans; moreover, similar major IgG bound *N*-glycans [43] with gender difference [44], the present preliminary results suggest that serum IgG concentration is elevated in AD patient sera compared with the normal control group. Therefore, it seems likely that *N*-glycoform of the human serum IgG becomes a promising candidate for the biomarker of AD.

From human brain GSL-omics profiling, we have found smaller amounts of GD1a-/GD1b-lactone gangliosides, which are human brain specific. The GD1b-lactone (GD1b-L) is not artifact-originated but generated as an authentic product of brain metabolism [45]. Significant amount of gangliosides loss were also detected in the frontal cortex of AD compared to the control [46]. Gangliosides are enriched in neurons and the decrease of gangliosides, particularly a-series gangliosides, resulted from the loss of neurons and brain shrinkage, which are the pathological hallmarks of AD. The variation is most probably related to different progress of disease reflecting by different ratio of astrogliosis and/or neuronal degeneration. Biochemical findings on gangliosides in AD brains are in agreement with neuroanatomic finding of most concentrated neuritic plaques in these areas and that the number of neurons in frontal and temporal cortex decreased [47,48]. The reduction of GM1 in AD compared to the normal may suggest that membrane-bound A β 1-42 tightly binds to GM1 (GAB), which acts as a seed for amyloid, and exhibits early pathological changes of AD [49]. Simpler and small gangliosides such as GM3 and GM2 were observed abundantly in the serum of both AD patients and normal subjects when compared with the larger gangliosides such as GM1 and GD1a (Figure 6D). The serum levels of GM3 and GM2 appeared to be elevated in the AD patients, especially in the female AD group, compared with the corresponding normal control subjects. These phenomena might be due to the acceleration of lysosomal degradation of gangliosides, enhanced activities of

acidic β -D-galactosidase and the reduced sialyltransferase activity; all these changes are considered to be associated with Alzheimer's disease [49].

In conclusion, we demonstrated that the present glycoblotting-based glycome analysis is feasible for the human postmortem brain tissues, CSF, and sera toward discovery research for the diagnosis and therapeutic treatments. Herein we hypothesized that the alteration in the pattern of glycosylation and regulation of glycan expression levels can be influenced by the accumulation of A β in AD patient brain and circulation. It is obvious that the blood brain barrier (BBB) permeability is significantly increased in AD [50]. In AD, the receptor for the advanced glycation end products (RAGE) levels at the BBB are increased and low-density lipoprotein receptor related protein-1 (LRP1) levels at the BBB and the capacity of a soluble form of LRP1 (sLRP1) binding of peripheral A β are reduced, favoring A β accumulation in the brain [51], and resulting in the aggravation of neuronal death and subsequent perturbation of sugar metabolism which in particular may reduce the concentration of brain specific *N*-glycans and gangliosides. Aberrant or failure of A β glycosylation in those specimens can elevate the *N*-glycans with proximal fucose, bisecting-GlcNAc, tri- and tetra-antennary types, and smaller gangliosides (GM3, GM2) in serum of AD patients as preliminarily shown in the present results, while the molecular mechanisms remain unclear. It should be emphasized that the large-scale serum glycomics of a variety stages of AD patients will accelerate the discovery research of novel class of biomarkers and molecular targets towards the development of the diagnostic and therapeutic reagents.

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Figure captions

Figure 1. A general protocol of the glycoblotting-based analysis for protein *N*-glycans and GSL-glycans enriched from human brain tissues.

Figure 2. MALDI-TOF/MS profiles for human serum *N*-glycans of AD patients and normal control groups. M, male; F, Female; and IS, internal standard. Glycan cartoons were constructed using the standard Consortium for Functional nomenclature, i.e., blue square, *N*-acetyl-D-glucosamine; red triangle, L-fucose; green circle, D-mannose; yellow circle, D-galactose; and purple diamond, sialic acid (*N*-acetyl neuraminic acid).

Figure 3. Comparison of the serum expression levels of one of the AD patient specific *N*-glycans between the female AD patients and the normal control subjects (F1 and F2 indicate Female 1 and 2, respectively). The serum concentration of this *N*-glycan was estimated by integrating the mass intensity of the signal at m/z 2117.9.

Figure 4. A) Total amount of human CSF *N*-glycans in AD patients and normal control subjects, B and C) MALDI-TOF/MS profile of human CSF *N*-glycans that indicate the ratio of the specific CSF *N*-glycans of AD patients to the normal control groups in male and female, respectively. M, male; F, Female; and IS, internal standard. The numbers indicating 2x, 3x, 4x, etc. represent the ratio of the intensities for the mass signals of the specific *N*-glycans in AD patients compared with the normal control groups.

Figure 5. Comparison of IgG and *N*-glycan concentrations in the serum (A) and CSF (B) ($r = 85$ for the serum and $r = 0.30$ for the CSF).

Figure 6. Human brain GSL-glycans. A) Focused gangliosides biosynthetic pathway. B) GM1 concentration in the human brain frontal cortex of the AD patients and normal control subjects.

C) MALDI-TOFMS of the human brain tissue (frontal cortex) GSL-glycans of the AD patients and normal control subjects. D) MALDI-TOFMS of the human serum GSL-glycans of the AD female patients and normal control subjects. Glycans cartoons were constructed using the standard Consortium for Functional nomenclature, i.e., blue circle, D-glucose; yellow circle, D-galactose; yellow square, *N*-acetyl-D-galactosamine; purple diamond, sialic acid (*N*-acetylneuraminic acid). M, male; F, female; and f, frontal cortices.

Table 1. Profiles of human samples used in this study

	Sex (age)	Weight (g)					Autolysis time (hr)	Clinical diagnosis	Neuropathological diagnosis
		Total brain	Frontal	Temporal	Parietal	Occipital			
Normal	M1 (57)	1350	1.8	1.8	2.4	2.3	12.6	Colon cancer Liver cancer Stroke	Essentially normal brain History of recent stroke (verbal only)
	M2 (76)	1150	1.5	1.8	2.8	1.8	22.2	Recent history of dementia Dementia	Brain with minimal aging changes
	M3 (67)	1300	1.5	1.9	2.4	2.2	12.3	Prostate cancer Depression	Essentially normal brain
	F1 (73)	1040	2.1	2.0	2.0	1.7	18.5	Pancreatic cancer Diabetes Hypertension	Essentially normal brain
	F2 (55)	970	1.3	1.8	1.6	1.7	19.7	AD (early onset) Seizure disorder Dementia	Minimal changes consistent with aging
	F3*(75)	1050	2.6	1.8	2.0	2.2	15.4	Possible stroke Liver cancer	Essentially normal brain
AD	M1 (76)	1190	1.4	2.4	2.4	1.8	9.8	AD	AD, “definite” Atherosclerosis, (extensive)
	M2 (76)	1225	1.2	1.0	2.4	1.5	13.8	AD	AD, “definite” Atherosclerosis (moderate)
	M3 (81)	1360	1.4	1.8	1.3	1.5	15.8	AD Dementia	AD, “definite”
	F1 (78)	900	2.4	2.3	2.4	1.5	19.3	Probable AD Vascular dementia	AD, “definite”
	F2*(45)	1000	2.1	1.0	1.6	1.3	16.2	AD Dementia	AD, “definite”
	F3*(66)	950	2.2	1.5	1.1	1.5	15.7	AD Vascular dementia	AD, “definite”

M1, M2, M3 are male sample 1, 2, and 3, respectively; F1, F2, F3 are female samples 1, 2, and 3, respectively. F2* only tissue and serum; F3*, only tissue and CSF. Further clinical information, see also experimental section.

Table 2. Estimated composition of *N*-glycans from human brain tissues, serum, and CSF. These peaks were common to both AD patient and normal control groups in this study. The blank space corresponding to each samples shows that particular *N*-glycans not found or negligibly low intensity in the peaks detected by MALDI-TOFMS analysis using FlexAnalysis.

Composition	<i>m/z</i>	Glycan ID (peak #)		
		Brain	Serum	CSF
(Hex)2 + (Man)3 (GlcNAc)2	1362.47	1	1	1
(HexNAc)2 + (Man)3 (GlcNAc)2	1444.54		2	
(Hex)3 + (Man)3 (GlcNAc)2	1524.53	2	3	2
(Hex)1 (HexNAc)1 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	1549.60		4	
(HexNAc)2 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	1590.60	3	5	3
(Hex)1 (HexNAc)2 + (Man)3 (GlcNAc)2	1606.59	4	6	4
(HexNAc)3 + (Man)3 (GlcNAc)2	1647.61	5		5
(Hex)4 + (Man)3 (GlcNAc)2	1686.59	6	7	6
(Hex)1 (HexNAc)1 (Neu5Ac)1 + (Man)3 (GlcNAc)2	1708.63		8	7
(HexNAc)2 (Deoxyhexose)2 + (Man)3 (GlcNAc)2	1736.63		9	
(Hex)1 (HexNAc)2 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	1752.65	7	10	8
(Hex)2 (HexNAc)2 + (Man)3 (GlcNAc)2	1768.63	8	11	9
(HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	1793.67	9	12	10
(Hex)1 (HexNAc)3 + (Man)3 (GlcNAc)2	1809.66	10		11
(Hex)5 + (Man)3 (GlcNAc)2	1848.64	11	13	
(Hex)1 (HexNAc)1 (Deoxyhexose)1 (Neu5Ac)1 + (Man)3 (GlcNAc)2	1854.69		14	
(Hex)2 (HexNAc)1 (Neu5Ac)1 + (Man)3 (GlcNAc)2	1870.68		15	
(Hex)1 (HexNAc)2 (Deoxyhexose)2 + (Man)3 (GlcNAc)2	1898.70	12		12
(Hex)1 (HexNAc)2 (Neu5Ac)1 + (Man)3 (GlcNAc)2	1911.70		16	13
(Hex)2 (HexNAc)2 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	1914.70	13	17	14
(HexNAc)3 (Deoxyhexose)2 + (Man)3 (GlcNAc)2	1939.69		18	
(Hex)1 (HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	1955.73	14	19	15
(Hex)2 (HexNAc)3 + (Man)3 (GlcNAc)2	1971.66	15		16
(HexNAc)4 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	1996.93		20	
(Hex)6 + (Man)3 (GlcNAc)2	2010.69	16	21	17
(Hex)3 (HexNAc)1 (Neu5Ac)1 + (Man)3 (GlcNAc)2	2032.72		22	
(Hex)1 (HexNAc)2 (Deoxyhexose)1 (Neu5Ac)1 + (Man)3 (GlcNAc)2	2057.73		23	18
(Hex)2 (HexNAc)2 (Deoxyhexose)2 + (Man)3 (GlcNAc)2	2060.76	17		
(Hex)2 (HexNAc)2 (Neu5Ac)1 + (Man)3 (GlcNAc)2	2073.74	18		
(Hex)3 (HexNAc)2 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	2076.75	19		
(Hex)1 (HexNAc)3 (Deoxyhexose)2 + (Man)3 (GlcNAc)2	2101.77	20	25	20
(Hex)2 (HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	2117.75	21	26	21
(HexNAc)4 (Deoxyhexose)2 + (Man)3 (GlcNAc)2	2142.75		27	
(Hex)1 (HexNAc)4 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	2158.78	22		22

(Hex)7 + (Man)3 (GlcNAc)2	2172.71		28	
(Hex)1 (HexNAc)2 (Deoxyhexose)2 (Neu5Ac)1 + (Man)3 (GlcNAc)2	2203.94			23
(Hex)2 (HexNAc)2 (Deoxyhexose)3 + (Man)3 (GlcNAc)2	2206.81	23		24
(Hex)2 (HexNAc)2 (Deoxyhexose)1 (Neu5Ac)1 + (Man)3 (GlcNAc)2	2219.79	24	29	25
(Hex)3 (HexNAc)2 (Deoxyhexose)2 + (Man)3 (GlcNAc)2	2222.83	25		
(Hex)3 (HexNAc)2 (Neu5Ac)1 + (Man)3 (GlcNAc)2	2235.80			26
(Hex)1 (HexNAc)3 (Deoxyhexose)3 + (Man)3 (GlcNAc)2	2247.78	26	30	27
(Hex)1 (HexNAc)3 (Deoxyhexose)1 (Neu5Ac)1 + (Man)3 (GlcNAc)2	2260.79	27	31	28
(Hex)2 (HexNAc)3 (Deoxyhexose)2 + (Man)3 (GlcNAc)2	2263.83	28	32	
(Hex)2 (HexNAc)3 (Neu5Ac)1 + (Man)3 (GlcNAc)2	2276.79	29	33	29
(HexNAc)4 (Deoxyhexose)3 + (Man)3 (GlcNAc)2	2288.77		34	30
(Hex)1 (HexNAc)4 (Deoxyhexose)2 + (Man)3 (GlcNAc)2	2304.84	30		31
(Hex)2 (HexNAc)4 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	2320.82	31		32
(Hex)3 (HexNAc)4 + (Man)3 (GlcNAc)2	2336.81		35	33
(Hex)2 (HexNAc)2 (Neu5Ac)2 + (Man)3 (GlcNAc)2	2378.86	32	36	34
(Hex)1 (HexNAc)3 (Deoxyhexose)2 (Neu5Ac)1 + (Man)3 (GlcNAc)2	2406.87	33		
(Hex)2 (HexNAc)3 (Deoxyhexose)3 + (Man)3 (GlcNAc)2	2409.90	34		35
(Hex)2 (HexNAc)3 (Deoxyhexose)1 (Neu5Ac)1 + (Man)3 (GlcNAc)2	2422.84	35	37	36
(Hex)3 (HexNAc)3 (Neu5Ac)1 + (Man)3 (GlcNAc)2	2438.93		38	37
(Hex)1 (HexNAc)4 (Deoxyhexose)1 (Neu5Ac)1 + (Man)3 (GlcNAc)2	2463.88	36		38
(Hex)2 (HexNAc)4 (Deoxyhexose)2 + (Man)3 (GlcNAc)2	2466.90	37		39
(Hex)3 (HexNAc)4 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	2482.91		39	40
(Hex)2 (HexNAc)2 (Deoxyhexose)1 (Neu5Ac)2 + (Man)3 (GlcNAc)2	2524.90	38	40	41
(Hex)3 (HexNAc)5 + (Man)3 (GlcNAc)2	2539.98			42
(Hex)2 (HexNAc)3 (Neu5Ac)2 + (Man)3 (GlcNAc)2	2581.95		41	
(Hex)3 (HexNAc)3 (Deoxyhexose)1 (Neu5Ac)1 + (Man)3 (GlcNAc)2	2584.92		42	43
(Hex)2 (HexNAc)4 (Deoxyhexose)3 + (Man)3 (GlcNAc)2	2612.97	39		
(Hex)2 (HexNAc)3 (Deoxyhexose)1 (Neu5Ac)2 + (Man)3 (GlcNAc)2	2728.00		43	44
(Hex)3 (HexNAc)3 (Neu5Ac)2 + (Man)3 (GlcNAc)2	2744.01		44	45
(Hex)2 (HexNAc)4 (Deoxyhexose)2 (Neu5Ac)1 + (Man)3 (GlcNAc)2	2772.01	40		46
(Hex)3 (HexNAc)3 (Deoxyhexose)1 (Neu5Ac)2 + (Man)3 (GlcNAc)2	2890.06		45	47
(Hex)3 (HexNAc)3 (Neu5Ac)3 + (Man)3 (GlcNAc)2	3049.11		46	48
(Hex)4 (HexNAc)4 (Neu5Ac)2 + (Man)3 (GlcNAc)2	3109.19		47	
(Hex)3 (HexNAc)3 (Deoxyhexose)1 (Neu5Ac)3 + (Man)3 (GlcNAc)2	3195.19		48	49
(Hex)4 (HexNAc)4 (Neu5Ac)3 + (Man)3 (GlcNAc)2	3414.31		49	
(Hex)4 (HexNAc)4 (Deoxyhexose)1 (Neu5Ac)3 + (Man)3 (GlcNAc)2	3560.41		50	
(Hex)4 (HexNAc)4 (Neu5Ac)4 + (Man)3 (GlcNAc)2	3719.45		51	50
(Hex)4 (HexNAc)4 (Deoxyhexose)1 (Neu5Ac)4 + (Man)3 (GlcNAc)2	3865.56		52	

Table 3. Human brain tissue total *N*- and GSL-glycans (pmole $\pm$ SD*) of AD patient and normal control groups

<i>N</i> -glycans	Cerebral cortices	Normal_M	Normal_F	AD_M	AD_F
	Frontal	437.03 $\pm$ 143.78	449.76 $\pm$ 288.39	387.88 $\pm$ 156.11	306.36 $\pm$ 116.45
	Parietal	501.89 $\pm$ 117.92	607.29 $\pm$ 136.49	689.96 $\pm$ 266.98	671.48 $\pm$ 182.88
	Occipital	194.38 $\pm$ 78.78	146.02 $\pm$ 43.38	239.32 $\pm$ 112.73	137.54 $\pm$ 52.89
	Temporal	276.32 $\pm$ 160.97	387.52 $\pm$ 79.82	562.80 $\pm$ 295.28	478.85 $\pm$ 187.99
GSL-glycans					
	Frontal	4058.98 $\pm$ 826.07	3318.73 $\pm$ 595.33	2549.96 $\pm$ 377.87	2624.74 $\pm$ 369.12
	Parietal	2268.61 $\pm$ 613.93	2469.42 $\pm$ 662.37	2490.60 $\pm$ 186.33	2246.24 $\pm$ 606.58
	Occipital	4964.92 $\pm$ 1817.01	4109.25 $\pm$ 1275.13	5370.86 $\pm$ 1914.80	6062.46 $\pm$ 1437.21
	Temporal	27494.25 $\pm$ 4324.08	12426.84 $\pm$ 7607.46	14323.63 $\pm$ 5326.37	15728.95 $\pm$ 5799.87

SD*, standard deviation

Table 4. Total amount of serum *N*- and GSL-glycans (pmole) of human AD patient and normal control groups

	Sample	M1	M2	M3
<i>N</i> -glycans	Normal	39.12 ± 4.53	404.56 ± 18.55	944.98 ± 264.73
	AD	896.39 ± 19.59	731.60 ± 58.45	76.65 ± 6.84
GSL-glycans	Normal	14.38 ± 2.40	52.50 ± 5.57	57.31 ± 13.50
	AD	93.30 ± 17.20	30.14 ± 13.59	19.95 ± 9.94
		F1	F2	F3*
<i>N</i> -glycans	Normal	532.33 ± 26.65	145.82 ± 12.71	–
	AD	997.02 ± 132.97	979.16 ± 22.83	–
GSL-glycans	Normal	37.60 ± 13.40	17.01 ± 10.80	–
	AD	49.57 ± 7.20	70.01 ± 44.42	–

*No third serum sample is specified which is paired with female AD brain tissue. M1, M2, and M3 are male 1, 2, and 3, respectively; F1 and F2 are female 1 and 2, respectively.

Table 5. Focused serum gangliosides concentration (in pmole/400 µg serum proteins) in human AD patients and normal subjects.

Gangliosides	Normal_F1	Normal_F2	AD_F1	AD_F2
GM3	20.49	9.33	30.66	30.35
GM2	11.63	2.23	12.12	28.07
GM1	4.13	2.13	4.31	7.05
GD1a	1.03	0.37	0.00	1.39

F1 and F2 mean female sample 1 and sample 2, respectively.

Table 6. Focused GM1 concentration (pmole $\pm$ SD) of the frontal cortex in human AD and normal subjects.

Sample	GM1 concentration		
	M1	M2	M3
Normal	1273.71 $\pm$ 95.79	1662.48 $\pm$ 236.81	1914.20 $\pm$ 102.77
AD	1075.51 $\pm$ 42.64	731.60 $\pm$ 58.45	1061.10 $\pm$ 40.93
Sample	F1	F2	F3*
Normal	1454.88 $\pm$ 386.61	1173.34 $\pm$ 174.06	1460.54 $\pm$ 146.62
AD	920.84 $\pm$ 66.44	1099.42 $\pm$ 150.57	1193.67 $\pm$ 21.79

M1, M2, and M3 are male 1, 2, and 3, respectively; F1, F2, and F3 are female 1, 2, and 3, respectively. Student t-test showed $p < 0.001$ for male and $p < 0.01$ for female frontal cortices of AD compared to the control samples.

Figure 1

Figure 2

Figure 3

Figure 4

A

B

C

Figure 5

A

B

Figure 6

D

Graphical Abstract

Glycoblotting method allows for rapid and efficient glycome profiling of human Alzheimer's disease brain, serum and cerebrospinal fluid towards potential biomarker discovery

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