CSF N-glycan profile reveals sialylation deficiency in a patient with GM2 gangliosidosis presenting as Autism Spectrum Disorder with regression

R. Barone[1,2°], L. Sturiale[2°], A. Fiumara[3], A. Palmigiano[2], R. O. Bua[2], R. Rizzo[1],

M. Zappia[4], D. Garozzo[2]

1) Child Neurology and Psychiatry, Department of Clinical and Experimental Medicine, University of Catania; 2) CNR- Institute for Polymers, Composites and Biomaterials IPCB, Catania; 3) Centre for Inherited Metabolic Diseases, Department of Clinical and Experimental Medicine; 4) Section of Neuroscience Department GF Ingrassia, University of Catania, Catania, Italy.

°These authors contributed equally to this work.

Text words:

Address correspondence to: Rita Barone, M.D. Child Neuropsychiatry Policlinico, Department of Clinical and Experimental Medicine - University of Catania- Via Santa Sofia, 78 95123 Catania- Italy [rbarone@unict.it] +39 095 3782467

ABSTRACT

Protein N-glycosylation consists in the synthesis and processing of the oligosaccharide moiety (N-glycan) linked to a protein and it serves for several functions for the proper central nervous system (CNS) development and function. Previous experimental and clinical studies have shown the importance of proper glycoprotein sialylation for the synaptic function and the occurrence of autism spectrum disorders (ASD) in the presence of sialylation deficiency in the CNS. Late-onset Tay Sachs disease (LOTSD) is a lysosomal disorder caused by mutations in the HEXA gene resulting in GM2-ganglioside storage in the CNS. It is characterized by progressive neurological impairment and high co-occurrence of psychiatric disturbances. We studied the N-glycome profile of the cerebrospinal fluid (CSF) in a 14 year old patient with GM2-gangliosidosis (LOTSD). At the age of 4, the patient presented regressive autism fulfilling criteria for childhood disintegrative disorder (CDD). A CSF sample was obtained in the course of diagnostic work-up for the suspicion of an underlying neurodegenerative disorder . We found definite changes of CSF N-glycans due to a dramatic decrease of sialylated biantennary and triantennary structures and an increase of asialo-core fucosylated bisected N-glycans. No changes of total plasma N-glycans were found. Herein results first suggest a global defect of cerebral protein sialylation in LOTSD. They also unveil further pathomechanisms of gangliosidosis disease. Finally, they support possible relationships between neuropsychiatric disorders and defective protein glycosylation in the CNS.

Key Words: Childhood Disintegrative Disorder; Autism Spectrum Disorders; CSF N-glycome; Tay Sachs Disease; GM2 gangliosidosis - MALDI-TOF MS.

Introduction

Childhood disintegrative disorder (CDD) is characterized by regression of developmental and behavioural functioning that occurs after at least two years of normal development (mean 3.4 years; Volkmar 1992). It is not clear to what extent this condition differs from autistic regression. According to DSM-5, it is placed under the autism spectrum disorders (ASD) (APA, 2013). CDD has been reported in patients with inborn metabolic diseases as neurolipidosis, metachromatic leukodystrophy and Schindler's disease or in acquired diseases including subacute sclerosing panencephalitis and Vit B12 deficiency (Malhotra and Gupta, 1999; Malhotra et al., 2013). However, in the large majority of patients molecular pathophysiology is unknown (Homan et al., 2011). Tay Sachs disease (MIM 272800) is a lysosomal disorder caused by mutations in the HEXA gene resulting in hexosaminidase A enzyme deficiency and chronic accumulation of GM2 ganglioside in neurons (Sandhoff and Harzer, 2013). Classic infantile Tay Sachs disease (TSD) affects children younger than one year old and rapidly progresses to sensory abnormalities, loss of motor function, epilepsy, swallowing difficulties and early mortality. Despite uniform presentation of infantile TSD, a wider clinical spectrum is encountered in the late-onset phenotypes. Late-onset TSD (LOTSD) has a more relentless clinical course with progressive mental impairment, psychiatric disturbances and motor deterioration with upper and lower motor neuron disease (Maegawa et al., 2006). Pathomechanism of GM2 gangliosidosis includes lipid storage in neurons with neuronal loss and dendritic changes, pointing to impairment of synaptic function and disturbed brain micro connectivity (McGlashan and Hoffman 2000). Protein N-glycosylation is based on the synthesis of carbohydrate moieties (glycans) covalently linked to specific asparagine residues. Glycans are ideally suited for signal functions and they serve as specific tags recognized by a spectrum of carbohydrate-binding proteins (lectins). Several glycoproteins involved in synaptic formation and plasticity as Neuroligins (NLs) strongly depend on glycosylation for normal functions (Scott and Panin, 2013). NLs mutations associated with ASD impair glycosylation and result in NLs abnormal folding, retention in the endoplasmic reticulum and defective expression in

the cell surface (Zhang et al., 2009). We analyzed the cerebrospinal fluid (CSF) protein N-glycans in a patient affected by LOTSD diagnosed with CDD at age 4. We found a deficiency of protein sialylation reinforcing current links between autism spectrum disorders and improper protein N-glycosylation in the brain. To our knowledge, this is the first study exploring the CSF N-glycome in LOTSD, a lysosomal disorder with high prevalence of psychiatric disturbances.

Case Report

The child was born to healthy, unrelated parents after an uneventful pregnancy and delivery with birth-weight 3.8 Kg. Family history was positive for schizophrenia. Early developmental milestones were achieved at appropriate times: she sat unsupported at 8 months and walked unaided at 15 months. Her first words appeared at 10 months and at 24 months she used meaningful phrases pointing to objects of interest. Meanwhile, she was said responsive to social initiations, showing clear interest in people. She was toilet trained at 30 months. After the age of 36 months she had an increasing pattern of behavioral changes with unmotivated cry, fearfulness , temper tantrums and restlessness. Sleep disturbances with attacks of severe *pavor nocturnes* (night terror) and nocturnal enuresis occurred by the age 48 months, when she appeared to have a regression in social behaviour and verbal communication. Thus, she first came to our observation. Physical and neurological examinations were unremarkable. Clinical assessment was positive for gaze-avoiding, impaired use of facial expressions and social reciprocity, repetitive behaviours such as head nodding and hand motor stereotyped movements. There was immediate echolalia for two-word sentences and comprehension of one-step command. Psychoeducational Profile (PEP-3) composite scores for communication and motor abilities corresponded to 25 and 31 months respectively (Schopler et al., 2005). By interview, her parents and her pediatrician failed to recognize regression signs in social and communicative skills in the first 36 months of age although they reported behavioral changes featuring anxiety and sleep disturbances that preceded regression between 36 and 48 months. At the time of the first visit (54 months) the child met criteria for autism in ADI-R subscales [Lord,

Rutter, & Le Couteur, 1994] (Qualitative Abnormalities in Reciprocal Social Interaction, Qualitative Abnormalities in Communication, and Restricted, Repetitive and Stereotyped Patterns of Behavior) as reported by her mother. ADOS module 1 was used because the spontaneous functional language was almost absent and limited to single words. Total scores for Social Affect and Restricted Behaviors domains were 14 and 3 respectively consistent with a classification of autism in the areas of communication, reciprocal social interactions and repetitive behaviours. Based on the apparent normalcy of social-communicative development in the first 3 years of life and the dramatic loss of skills after 48 months of age, with impairment of communication and social interaction and presence of repetitive behavior the child was considered affected by CDD. Brain magnetic resonance imaging (MRI) showed mild cortical cerebral atrophy. Fundus oculi showed no macular degeneration. She became progressively clumsy and at 7 years of age, she had gait difficulties and frequent falls for feet malposition. Neurological examination was remarkable for left foot dystonia and increased deep tendon reflexes. Electromyography disclosed fair fibrillations and sporadic sharp waves in the brachial triceps and anterior tibialis muscles. Motor unit recruitment was reduced. Serial MRI at age 11, 14 and 17 documented progressive cortical and sub-cortical atrophy, thinning of the corpus callosum and slight, bilateral changes of posterior deep white matter. The clinical course run parallel with loss of ambulation, progressive dysarthria and loss of verbal language and severe intellectual disability. In addition she had associated chronic movement disorder with orofacial dyskinesia, focal and generalized dystonia, and sub-continuous chorea movements of the neck and upper limbs. A therapeutic trial with the anticholinergic drug trihexyphenidyl (12 mg/day) lead to a relief of dystonia with reduction of abnormal posturing. At age 14, plasma lactic acid, ammonia, Vitamin B12, urinary organic acids and glycosaminoglycans and array CGH molecular analyses were normal. Cerebrospinal fluid (CSF) analyses for oligoclonal bands, IgG index and cell count yielded normal results. A deficiency of lysosomal enzyme hexosaminidase A in leukocytes was found . Molecular analyses of *HEXA* gene showed she was homozygous for c.533G>A (p.R178H) mutation.

Materials and Methods

The study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 1983. Patient CSF sample (300 microliters) was obtained before the diagnosis of TSD by spinal lumbar puncture at age 14 in the context of diagnostic workup while the patient was under general anesthesia for MRI execution and after parental informed consent. For comparison, CSF samples (300 microliters each) were obtained from 8 sex and age matched subjects without neurological disorders that underwent spinal tap in the course of anesthetic procedure for surgery. Sample collection of healthy individuals was performed within an ongoing project at University Hospital of Catania approved by the local ethics committee and aimed to decipher CSF N-glycome in healthy people and patients with neuropsychiatric disorders. Informed consent was obtained by control subjects and/or their relatives.

N-glycan profiling by mass spectrometry.

Two hundred µl of CSF and 15 µl of serum were used for N-glycan profiling. Proteins were denaturated with RapiGest^{TM}, reduced and alkylated with DTT and IAA respectively and deglycosylated using PNGase F at 37°C. Released N-glycans were permethylated, as previously reported (Edvardson et al., 2013), according to Ciucanu and Kerek protocol (Ciucanu & Kerek, 1984) and re-suspended in methanol prior to mass spectrometry analysis. MALDI-TOF MS (matrix-assisted laser desorption ionization-time of flight mass spectrometry) and MS/MS experiments were acquired, in positive reflector mode, on a 4800 Proteomic Analyzer (Applied Biosystems, Framingham, MA).

Data evaluation

Assignment of N-glycan structures corresponding to monoisotopic masses was performed by tools from Expasy GlycoMod (http://web.expasy.org/glycomod/), Expasy GlycanMass, the Consortium for Functional Glycomics glycan structures central database and Glycoworkbench v2.1 (Ceroni et al., 2008). Selected ions were fragmented by MALDI TOF/TOF MS/MS to further elucidate the corresponding glycan structures by comparison with model compounds (Dextra Laboratories Ltd., U.K.). Statistical analyses were accomplished using a commercially available software program (SPSS for Windows, version 18.0.1; SPSS Inc/IBM)

Results

CSF N-glycan profiling

MALDI MS N-glycan profiles of control and patient CSF are compared in Figure 1. MALDI mass spectrum in Figure 1A is representative of CSF N-glycan profiles in healthy individuals. It consists, as a whole, of about ninety different N-glycan species (individually reported in Table S1) with the base peak at *m/z* 2792.2 corresponding to the complex biantennary disialylated glycan. Reference CSF N-glycan pattern was in line with that reported for healthy individuals aged between 2 and 50 (Fogli et al., 2012).

Patient's CSF showed definite different glycomic pattern (Figure 1B) with the most intense molecular ions, at *m/z* 2081.0, 2285.1 and 2459.2, consistent with asialo-, fucosylated complex N-glycans bearing bisecting GlcNAc. MS/MS fragmentation of the main species at *m/z* 2081.0 $(GlcNAc)_3$ $(Fuc)_1$ $(Man)_3$ $(GlcNAc)_2$ confirmed the corresponding molecular structure is the asialo-, agalacto-, core-fucosylated bisected glycan (data not shown). Likewise, peaks at *m/z* 2285.1 and 2459.2 were assigned to bisected asialo- monogalacto-structures with one and two fucose residues, respectively (Figure 1B).

Relative intensities of 43 major N-glycans, falling into five separate families, were evaluated in patient and in control samples, showing a decrease of biantennary sialylated structures and an increase of asialo- bisected and fucosylated structures (Figure 2).

MALDI-TOF mass spectra of permethylated plasma N-glycans from the patient was unremarkable with respect to healthy individuals (Figure S1), suggesting that observed changes of N-glycosylation are not systemic and may reflect CNS disease in the patient.

Discussion

We studied CSF protein N-glycosylation of a LOTSD patient presenting with CDD. We found a decrease of sialylated species in spite of an increase of asialo-, core-fucosylated bisected N-glycans. The findings suggest a general undersialylation of brain N-linked glycoproteins in the patient. Glycosylation impacts on many important cellular processes including cellular recognition, protein function and immunogenicity thus implicating several possible pathomechanisms to the ganglioside storage disorder (Barone et al., 2012). The causative link between GM2 gangliosidosis and impaired N-glycosylation, as deduced by herein reported N-glycomic CSF profile, may include several causative factors as: 1) misregulation of proteins and intracellular trafficking 2) depletion of precursor pools 3) altered regulation of proteins and enzymes by an increased load of lipids. Pathomechanism of GM2 gangliosidosis is related to a progressive impairment of the physiological turnover of membrane glycolipids (gangliosides) within the endocytotic-lysosomal pathways (Sandhoff and Harzer, 2013) Alterations of composition and function of membranes may influence the glycosylation process, as the membrane bound glycosyltransferase enzymes, mostly the one in the late Golgi compartments, seem to be affected. Moreover, the ganglioside content of membrane was proven to affect glycosylation of membrane glycoproteins by regulating glycosyltransferase activities, as indicated by *in vitro* studies based on incorporation of sugars into endogenous protein acceptors (Merritt and Morrè, 1980). Despite being a well-known cause of developmental delay in childhood, very early symptoms of TSD might be overlooked. The present patient had a psychiatric

onset of TSD with initially normal social-communicative development, and the onset of premonitory signs with agitation and anxiety preceding regression after the age 36 months. At age 54 months she came to our observation because of overt regression in multiple domains including progressive loss of verbal language and severe impairment of social and adaptive functioning and enuresis. Regression was accompanied by a repetitive pattern of atypical behaviours and motor stereotypes. Capturing the natural history through a "trajectory" dimension, the child was considered affected by CDD . Analyses of symptoms in a large cohort of patients with LOTSD indicated that behavioral and psychiatric disturbances occurred at onset of disease in almost 12% of subjects at a mean age of 5,3 ± 4,1. Along with disease progression, psychiatric disturbances were observed in almost 36-46% of patients of variable ages (13,2 ± 8,2) (Maegawa et al., 2006). Psychotic symptoms were mostly reported and included auditory and visual hallucinations and paranoia and/or catatonia usually accompanied with impaired consciousness, memory disturbance and inability to perform self-care. Mood disturbances may be a prominent and early manifestation of the illness with reported abnormalities of mood, mania or euphoria, unipolar depression and atypical manic-depressive psychosis (MacQueen et al., 1998). The disease course, highlighted by the present patient, is characterized by progressive gait and speech disturbances, cognitive impairment, extrapyramidal features and muscle wasting. Herein findings highlight possible relationships between the early onset of psychiatric disturbance featuring CDD in the patient and defective protein glycosylation in the CNS. Several studies have shown the importance of glycosylation for synaptic function and the co-occurrence of ASD in the presence of sialylation deficiency in the CNS. The expression of sialyltransferase (ST) genes involved in sialylation of brain glycoproteins is strongly regulated by higher expression during the neonatal and infantile ages with a gradual decrease from toddler to adult thus implicating a fine-tuning control of sialylation on brain connectivity (Varki, 2008; Schnaar et al., 2014). Single nucleotide polymorphisms (SNPs) in polysialyltransferase gene ST8SIA2 have previously shown genome-wide significant association with ASD (verbal subtype) (Anney et al., 2010). Haploinsufficiency of ST8SIA2 was associated

with ASD (Kamien et al., 2014), as well as schizophrenia and bipolar disorders (McAuley et al., 2012). Glycomic analyses in patients with schizophrenia indicate a general decrease of sialylated glycans in the CSF (Stanta et al., 2010). This is consistent with the decreased expression of ST6GAL1 and ST3GAL2 in the prefrontal cortex of schizophrenia patients, resulting in deficiency of beta-galactoside alpha-2,3/6-sialyltransferases in the Golgi responsible for the attachment of sialic acids to the biantennary and triantennary complex N-glycan structures (Narayan et al., 2009). The defective CNS sialylation in patients with schizophrenia concurs with the decrease of sialylated glycans in this patient with LOTSD, as psychosis is a common neuropsychiatric disorder in patients with LOTSD. The present study strengthens the existing link between brain glycosylation disorders and neuropsychiatric phenotypes. Analyses of molecular pathomechanisms in monogenic diseases might enhance current knowledge and become functional for molecular therapeutic approaches in related complex genetic neuropsychiatric disorders. We highlight the occurrence of CDD as an early sign of TSD because of possible therapeutic developments and family counselling.

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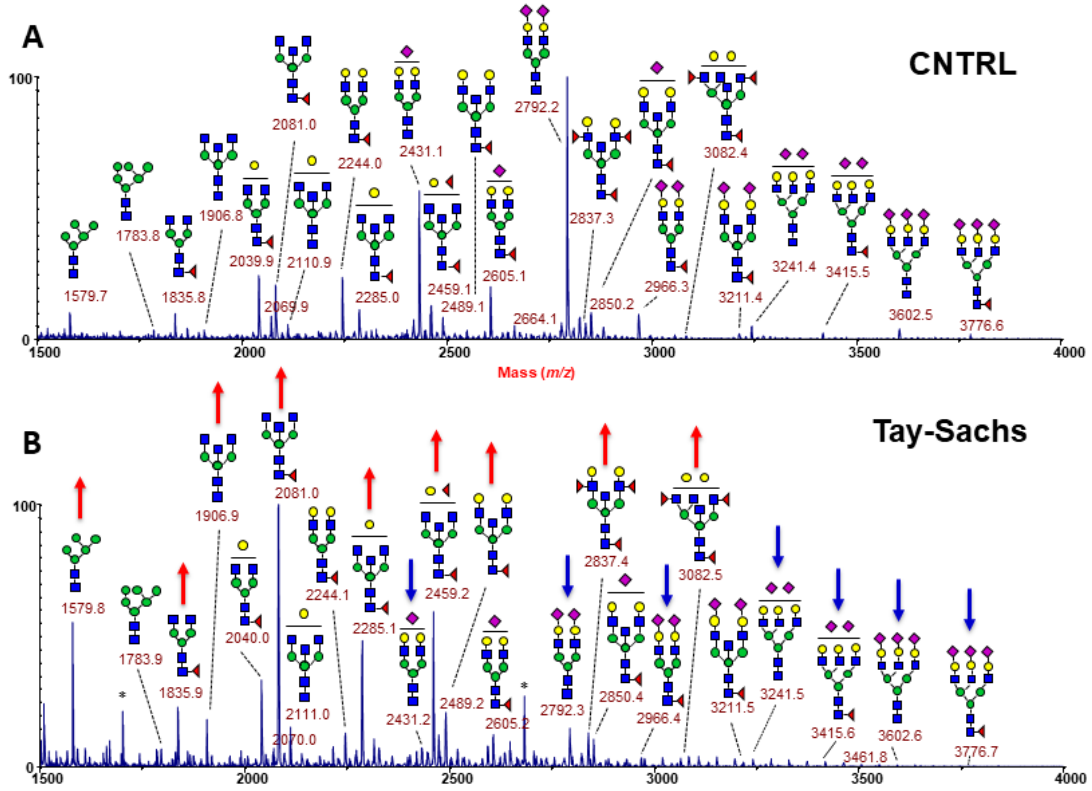
Legend to Figures

Figure 1. MALDI spectra of permethylated CSF N-glycans in healthy subject (A) and in the patient (B). Complete peak annotation, including additional minor structures of control samples, are reported in Table S1. Fucose (Fuc): triangle, Mannose (Man): green circle, N-acetyl glucosamine (GlcNAc): square, Galactose (Gal): yellow circle; Sialic acid (NeuAc): diamonds. Asterisks refer to impurities.

Figure 2. Relative intensities (%, +/-SEM) of N-glycan types in CSF from TSD patient lying outside the 99% confidence interval calculated for relative intensities of control samples.


A
CNTRL
100
0
1579.7
1783.8
1835.8
1906.8
2039.9
2069.9
2081.0
2110.9
2244.0
2285.0
2431.1
2459.1
2489.1
2605.1
2664.1
2792.2
2837.3
2850.2
2966.3
3082.4
3211.4
3241.4
3415.5
3602.5
3776.6
1500
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2500
3000
3500
4000
Mass (m/z)
B
Tay-Sachs
100
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1579.8
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2459.2
2489.2
2605.2
2792.3
2837.4
2850.4
2966.4
3082.5
3211.5
3241.5
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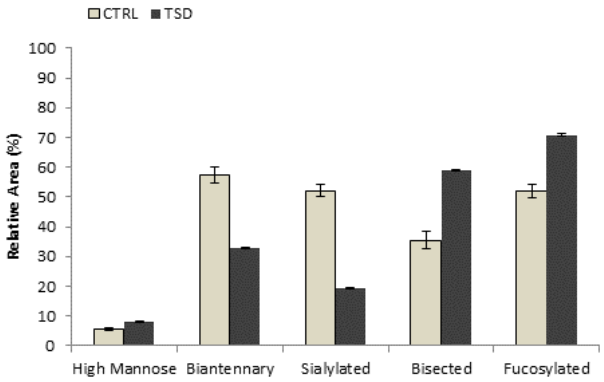
Figure 1.

Figure 2



N-glycan families	Relative Intensity (%) ±SD	99% Confidence Interval
High Mannose	8.11±0.22	3.94 – 7.02
Biantennary	32.77±0.24	47.38 – 67.34
Sialylated	19.36±0.17	44.74 – 59.63
Bisected	58.98±0.05	25.41 – 45.48
Fucosylated	70.86±0.36	44.49 – 59.56