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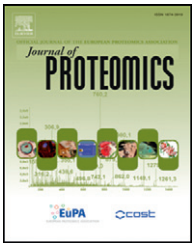

Review

Glycomics of pediatric and adulthood diseases of the central nervous system

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ABSTRACT

Glycosylation consists in the covalent linkage of a carbohydrate structure to membrane bound and secreted glycoconjugates. It is a common post-translational modification that serves multiple functions in cell differentiation, signaling and intercellular communication. Unlike DNA/RNA/protein, the addition of complex carbohydrates is not-template driven and it is conceivable that both genetics and environmental factors might interact to influence glycosylation machinery in several pathological processes.

Over the last few decades, the recognition of Congenital Disorders of Glycosylation (CDG) as an increasing number of genetic diseases of glycosylation with almost constant nervous system involvement, dramatically illustrated the consequences of abnormal glycosylation as improper CNS development and function. In addition, CDG recognition contributed to postulate that aberrant glycosylation processes might play a role in multifactorial, complex CNS diseases. On this context, CNS glycomics explores the effects of possible aberrant glycosylation to identify potential glyco-biomarkers useful for the diagnosis and ultimately for potential intervention strategies in neurological diseases.

Up to date, CNS glycomics is an emerging, still uncharted area because of the specificity of CNS glycosylation, the complexity of the neurological disorders and for the inaccessibility and invasiveness of disease relevant samples. Here we review current knowledge on clinical glycomics of nervous system diseases, starting with CDG to include those pediatric and adulthood neuropsychiatric diseases where some evidences suggest that multifactor determinants converge to dysregulate glycosylation. Conventional and mass spectrometry-based high throughput technology for glyco-biomarker detection in CNS diseases is reported.

Contents

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1. Introduction

Glycosylation is a post-translational modification that serves many processes through the covalent linkage of oligosaccharide chains (glycans) to membrane bound and secreted glycoconjugates [1]. Glycan structures play roles in intercellular communication processes including differentiation, development, inflammation and metastasis [2,3]. Major glycosylation routes are N- and O-glycosylation that can be classified according to the glycan-peptide linkage region [4]. The N-glycosylation is a highly ordered process encompassing different cellular compartments (cytosol, endoplasmic reticulum and Golgi) in sequence and leading to the attachment of carbohydrate structures (N-glycans) to the asparagine (Asn) residue of the polypeptide chain [5]. All N-glycan types share a common core $Man_3GlcNAc_2$. Based on the additional sugars attached, N-glycan structures are distinguished in complex, hybrid and high mannose types.

In O-glycosylation, N-acetylgalactosamine (GalNAc) is commonly attached to the hydroxyl groups of serine (Ser) and threonine (Thr) residues by the action of tissue specific polypeptide GalNAc-transferases. Extension of the GalNAc residue leads to the synthesis of mucin-type O-glycans. Eight different core structures of mucin-type O-glycans are known (Fig. 1A); cores 1–4 are the most common in humans. Possible elongation and termination of the mucin-type cores may generate a variety of structures, including a number of siaylated O-glycans. In addition, unique types of protein O-glycosylation have recently been reported, such as O-linked fucose, O-linked glucose, O-linked GlcNAc and O-linked mannose [6,7].

As the glycan synthesis is not template driven, it is expected that a variety of cellular stressors might affect glycosylation, so that this latter might mirror the status of the cell itself. Gene-knockout studies have shown the importance of protein N-glycosylation and O-mannosylation for neural tissue development in early embryogenesis [8,9].

Proteomics of cerebrospinal fluid (CSF) was validated for biomarker detection in various adulthood neurological diseases including Motor Neuron Disease (MND) [10], multiple sclerosis [11,12], Creutzfeldt Jakob Disease (CJD) [13] and Alzheimer's Disease (AD) [14], while it had only limited application to pediatric CNS disorders.

The impact of improper glycosylation on human health is illustrated in the Congenital Disorders of Glycosylation (CDG) [15]. CDG a an increasing group of monogenic, mainly autosomal recessive disorders, caused by mutations in different genes involved in the N- and/or O-glycosylation and in lipid glycosylation. More than 40 CDG types have been identified [15] and almost all these genetic glycosylation defects are associated to neurological disability and mental retardation of variable degree [15,16]. Thus, it has been recommended to screen for CDG in any unexplained neurological syndrome, particularly when associated with other organ disease [17].

More recently, the importance of glycosylation in the development and functional maintenance of the CNS has been indicated not only for monogenic disorders as CDG but also for heritable neurological and psychiatric diseases with multifactor determinants. On this regard, the recent identification of Copy-Number Variations (CNVs) in the human genome pointed to recognition of positional candidate genes underlying complex genetic disorders, including Autism Spectrum Disorders (ASD) [18,19]. ASD are childhood-onset disorders of neurodevelopment characterized by significant impairment of communicative abilities and social interaction as well as repetitive and restricted behavior and interests. Gene-network analysis of susceptibility gene in autism patients revealed an overrepresentation of genes related to glycobiology, indicating that dosage alterations in these genes could contribute to the autism phenotype [20]. On this scenario the identification at the proteomic levels of the effects of this dysfunction could be important to identify potential glyco-biomarkers and ultimately to discover pharmacological intervention strategies for autism and other pediatric neuropsychiatric disorders.

Further lines of evidence support a critical role for glycosylation changes in aging processes as well as in AD and related disorders through dysregulation of brain tau protein phosphorylation [21,22], amyloid production and clearance [23–25], and differential glycoform expression of targeted glycoproteins implicated in AD [26,27].

The glycoproteome represents one of the most important subproteomes in tissues and bodily fluids. However, macro- and microheterogeneity of glycoprotein structures hamper glycoproteome studies that are still lagging behind the knowledge about other macromolecules. In addition to glycoproteomic analyses, methods for structural characterization of N-linked and O-linked glycans represent a research area of increasing interest [28].

Structural elucidation of secretory glycoproteins isolated from human CSF indicated a possible specificity of CNS protein glycosylation as already proven in brain tissues of different mammalian species [29]. The hypothesis of "brain type" glycosylation for CNS native glycoproteins was verified by glycoform analyses of beta-trace/prostaglandin D2 synthase (β-TP), a secretory glycoprotein predominantly intrathechally synthesized and secreted by the glia-rich fractions of the brain. Indeed, β-TP carbohydrate structural features include "brain type" glycosylation characterized by large amounts of biantennary N-glycans with bisecting GlcNAc, and proximal fucosylation (α1,6 fucosylation at the chitobiosyl core). Additional structural characteristics of the "brain type" glycans comprise a significant amount of peripheral fucose (α1,3 linked to GlcNAc on glycan antennae) resulting in a Lewis^x (Le^x) or sialyl Lewis^x (sLe^x) epitope, as well as the presence of N-acetylneuraminic acid (NeuAc; sialic acid) in α2,3 or α2,6 linkage [30]. These traits are distinct from that observed in "serum-type" glycosylated proteins bearing complex glycans with almost absent bisecting GlcNAc and peripheral fucosylation. NeuAc, when present, is predominantly α2,6-linked. Typical features of "brain type" and "serum type" N-glycans are illustrated in Fig. 1B. Further insights on brain-specific glycosylation were obtained by structural elucidation of the CSF asialo-transferrin fraction. This represents almost 30% of CSF transferrin (Tf) and it turned out to contain "brain-type" complex agalacto-biantennary glycans [31]. CSF glycome analyses might serve as a tool to unravel defect restricted to the CNS. A valuable example is the glycoproteomic approach that led to identify a novel form of cerebellar ataxia named CAFSA (Cerebellar Ataxia with elevated cerebrospinal Free Sialic Acid). This is characterized by progressive cerebellar ataxia, cognitive and/or behavioral deterioration and peripheral neuropathy, increased Free Sialic Acid (FSA) in CSF and normal FSA levels in urine, plasma and fibroblasts [32].

Owing to its own specificities, CSF glycoproteomic analyses are still emerging as potential tool for biomarker discovery in neurological diseases. Here we review clinical and experimental data indicating the impact of glycosylation in CNS diseases both in adulthood and pediatric age as summarized in Table 1, with particular emphasis on the most innovative strategies applied for glycosylation analyses in these disorders. We first describe those monogenic disorders affecting directly the glycosylation patterns (CDG) as these represent an established paradigm connecting altered glycosylation and CNS impairment with a wide range of clinical manifestations. Then we will discuss an increasing number of complex trait diseases of the CNS where current knowledge suggests both genetic and environmental factors converging to dysregulate glycosylation.

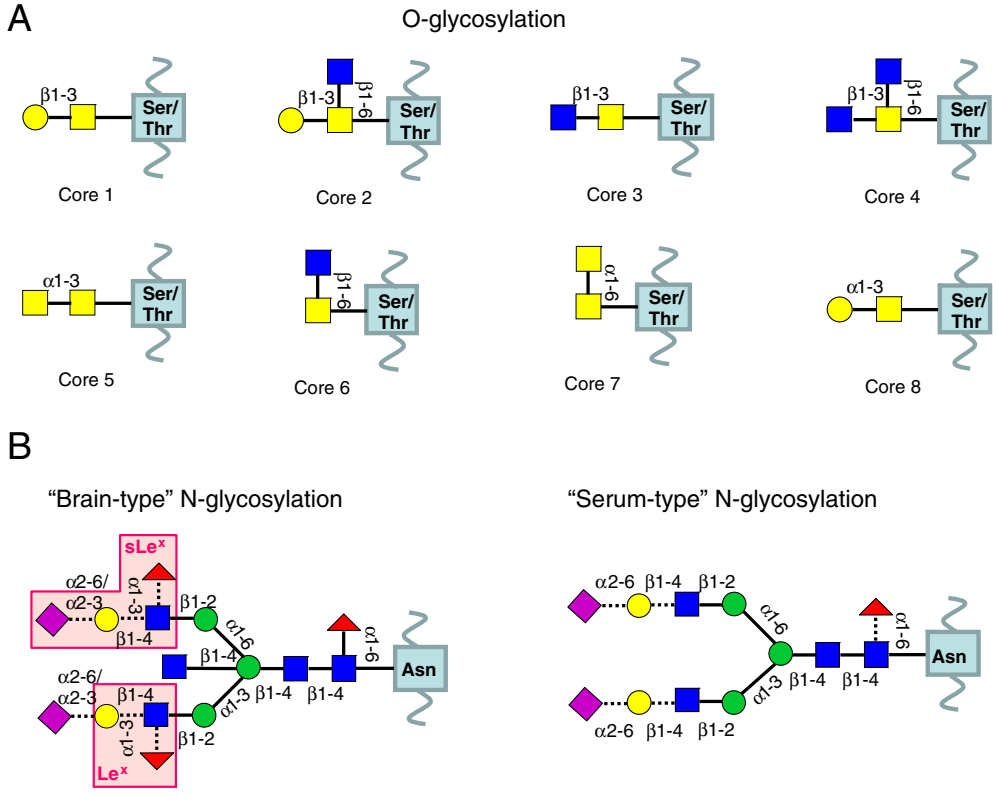


Fig. 1 – O-linked and N-linked glycan structures: A) core structures of O-linked glycans. B) Typical features of "brain type" and "serum type" complex biantennary N-glycans. Dotted linkages are related to additional "brain type" and "serum type" structural characteristics.

2. Glycosylation in pediatric CNS disorders

2.1. *Congenital Disorders of Glycosylation*

CDG is a human genetic disorder caused by defects in protein N-glycosylation and/or O-glycosylation and in lipid glycosylation. These diseases have a prominent nervous system involvement including CNS malformative conditions, disorders of ocular movements, eye structural anomalies and visual defect of variable degree, hearing loss, seizures and stroke-like episodes and peripheral neuropathy. Mental retardation is almost constant from borderline developmental delay to the absence of any psychomotor development. An increasing number of CDG types are characterized by epileptic encephalopathy, microcephaly and severe visual impairment. In addition, a subset of congenital muscle dystrophies that exhibit severe CNS malformation, result from O-mannosylation

Table 1 – Glycomic findings in neuropsychiatric disorders. Samples (serum, CSF, or tissue) and glycosylation altered subjects (increase or decrease of particular glycans, glycoproteins, proteins, glycan-related enzymes/genes) in pediatric and adulthood diseases of the central nervous system.

Serum/CSF	Cell/tissue
Congenital Disorders of Glycosylation (CDG) (N-glycosylation defects)	
Serum: defective glycosylated glycoforms of Tf in CDG-I and CDG-II [17,34]. Quantitative and qualitative changes of Tf-glycans and total serum N-glycans in CDG-II [40–42]. CSF: defective glycosylation of β-TP protein in PMM2-CDG [43] and ALG6-CDG [44]. Truncated β-TP glycan structures in MGAT2-CDG [43].	Enzyme and gene defects localized along the N-glycosylation pathway and detectable in leukocytes and/or fibroblasts [15,16,34].
CDG (N- and O-glycosylation defects)	
Serum: defective glycosylated glycoforms of Tf and apoC-III glycoprotein.	Defects in proteins with functions in N-glycosylation and O-glycosylation (core-1 mucin-type O-glycans), i.e. COG7 deficiency, detected in fibroblasts and with genetic testing [39]
CDG (O-mannosylation defects) muscular dystrophy-dystroglycanopathies	
–	Decrease of glycosylated α-dsytroglycan detectable in muscle tissue. Deficiencies of know (POMT1, POMT2, POMGNT1, LARGE) or putative (FKTN and FKRP) glycosyltransferases, detected with genetic testing [16]
Vanishing White Matter disease	
CSF: decrease of asialo-Tf glycoforms bearing brain-type biantennary glycan structure composed of 2 GlcNAc, 3 Man, 3 GlcNAc and 1 Fuc. Detection of asialoglycan $Man_5GlcNac_2$ attached to Asn^{432} residue of asialo-Tf [48].	Brain tissue. increase of foamy Periodic Acid Schiff (PAS)-positive glial cells [46].
Autism	
–	Dosage changes in genes involved in N-glycosylation (*B3GALT6*, *B4GALT1*, and *GCNT2*), O-glycosylation (*LARGE*, *GALNT9* and *GALNTL5*) and lipid glycosylation (*ARSA*) [20].
ADHD	
Serum: increased antennary fucosylation of biantennary glycans and decreased levels of some complex glycans with three or four antennas [54].	–
Alzheimer's Disease	
Serum: decrease of digalactosylated core-α-1,6-fucosylated biantennary glycan. Increase of α1,3-fucosylated trigalactosylated glycans. [patent application publication, May 5, 2011]. CSF: altered levels of full-length Reelin and Reelin 180 kDa fragment. Abnormal glycosylation pattern of 180-kDa reelin [26]. Increase of sialylated O-glycans in Tyr10 of APP/Aβ glycopeptides [23]. Decrease of TTR brain-specific isoform [76]. Quantitative changes of apolipoprotein E, clusterin, α-1-β-glycoprotein and α-1-AAT. Decreased glycosylation of one specific α-1-antitrypsin isoform [80,82]. Decrease of WGA-reactive Tf glycoforms [88,89].	Brain tissue: glycosylation changes in CRMP-2 [72], increase of glycosylated isoforms of AChE and BuChE [27]. Abnormal ConA- and the WGA-affinity of several glycoproteins in hippocampus and inferior parietal lobe [93,94]. Upregulation of MGAT3 enzymes [25].
Idiopathic normal pressure hydrocephalus	
CSF: increase of Tf-2 (serum type)/Tf-1 (brain-type) glycoforms ratios [90].	–
Multiple sclerosis	
Serum: modification of plasma acute-phase proteins glyco-isoforms [102].	Human T cell blasts: abnormal MGAT enzymes expression. Decrease of glycan branching and surface expression of the glycoprotein autoimmune inhibitor CTLA-4 [100].
Schizophrenia	
Serum: increase in male patients of tetraantennary tetrasialylated glycans bearing polylactosamine with A4G4LacS4 extension and triantennary trisialylated containing the SLe^x epitope [105]. CSF: decrease of bisecting and sialylated glycans levels.	Prefrontal cortex: down-regulation of beta-N-acetylglucosaminyltransferase III and beta-galactoside alpha-2,3/6-sialyltransferases [106].

defects of alpha-dystroglycan that impairs its ligand-binding activity and results in muscle degeneration and failure of neuronal migration [33]. As glycoconjugates are ubiquitous, multiorgan failure during infancy and a variable combination of extra neurological symptoms may also occur in CDG patients.

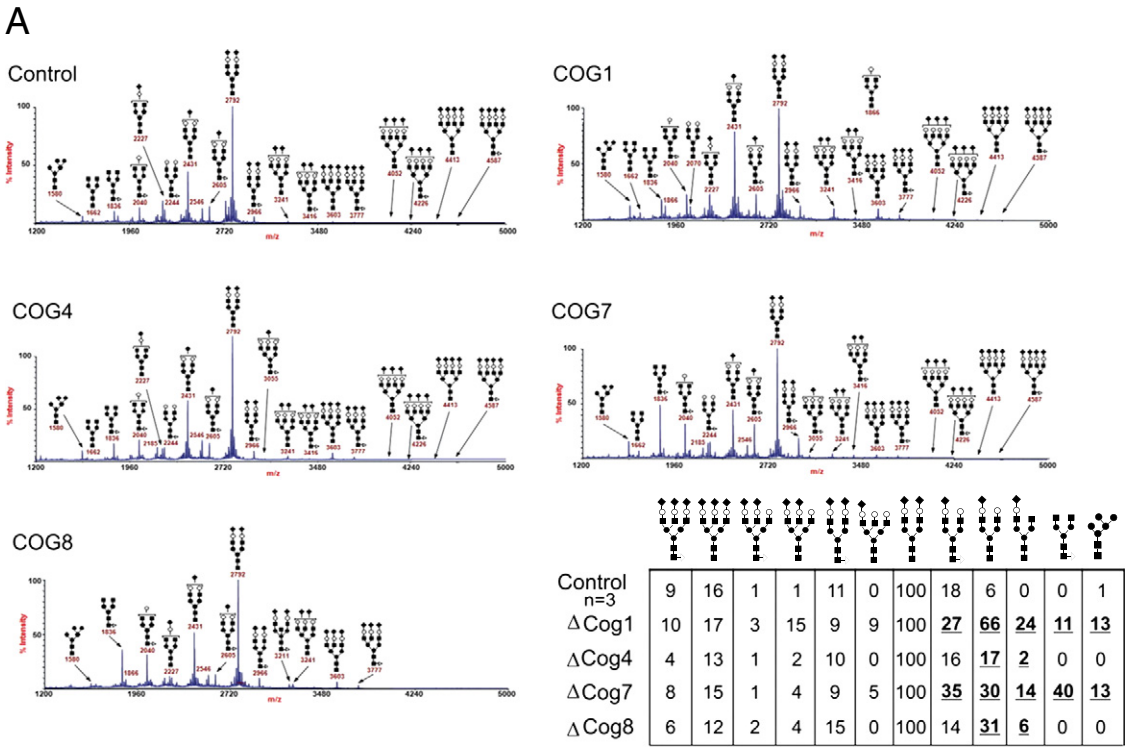
To date, some 45 CDG types have been described based on the specific glycosylation defect. As this number is expected to rise in the short-term, a practical classification encompassing all the known and the upcoming CDG has been recently proposed. It includes four categories, namely (i) defects of protein N-glycosylation, (ii) defects of protein O-glycosylation, (iii) defects of lipid glycosylation and of GPI anchor glycosylation, (iv) and defects in multiple glycosylation pathways and in other pathways [15,34]. PMM2-CDG (formerly CDG-Ia) is the most common N-glycosylation disease and it is due to deficiency of phosphomannomutase 2 (PMM2) enzyme, required for the synthesis of GDP-mannose in the very early steps of N-glycan synthesis. First line laboratory diagnosis of CDG is based on demonstration of abnormally glycosylated glycoforms of serum Tf by IEF, which allows charge separation of Tf isoforms differing for the numbers of terminal sialic acid residues. Tetrasialo-Tf is the main species in healthy individuals: in CDG-I, a decrease of tetrasialo- and an increase of disialo- and asialo-Tf are observed (type I pattern), whereas in CDG-II also monosialo- and trisialo-Tf are increased (type II pattern) [35]. Recognition of the causative defects is obtained by combination of lipid-linked oligosaccharide profile in fibroblasts (CDG-I defects), MALDI-MS analyses of plasma N-glycans (CDG-II defects) and putative gene identification by targeted molecular analyses. Identification of glycosylation defects in CDG was elucidated by glycoform characterization of serum derived glycoproteins such as Tf, α-1 antitrypsin and IgG. In such a context, ESI-MS and MALDI-MS have become valuable tools for CDG diagnosis and characterization [36]. CDG-I defects result in the defective assembly of the full-length oligosaccharide $Glc_3Man_9GlcNAc_2$-PP-dolichol that finally will be transferred onto nascent glycoproteins by the oligosaccharyltransferase complex. As a consequence, underoccupancy of N-glycosylation sites onto glycoproteins is the hallmark feature of CDG-I. Such defects result in measurable molecular mass shifts with respect to the fully glycosylated glycoprotein. In PMM2-CDG, the MS profile of serum Tf allows the identification of a recognizable pattern with two abnormal Tf glycoforms differing from the fully glycosylated tetrasialo-species, for the molecular mass of one or both the entire N-glycan moiety (~2.2 kDa), giving rise respectively to monoglycosylated, disialo-Tf and aglycosylated, asialo-Tf [37,38]. CDG-II is characterized by impaired N-glycan processing in the Golgi resulting in abnormal truncated species observable by MALDI-MS analyses of total plasma N-glycans or serum Tf N-glycans. Mutations in the Conserved Oligomeric Golgi (COG) complex are the most common among known causative defects of CDG-II [39]. COG defects lead to incorrect localization of Golgi enzymes required for proper glycan stepwise maturation, thus impairing either N- and/or O-glycosylation. The COG complex is a hetero-octameric oligomer. Currently, mutations in six out eight subunits have been found and identified as COG1-CDG, COG4-CDG, COG5-CDG, COG6-CDG, COG7-CDG and COG8-CDG [15]. MALDI-MS N-glycan analyses in serum of COG-deficient patients showed defective N-glycan sialylation including, in some specific defects, reduced galactosylation and increase of abnormal mannosylated structures [40], as shown in Fig. 2.

Recently, we had reported on the impact of MS in human genetic glycosylation defects, focusing on development and application of a method for CDG diagnosis based on MALDI-MS of immunopurified intact serum glycoproteins and N-glycan analyses in different CDG-I and II diseases [36,41,42].

N-glycosylation study in the CNS of CDG patients was approached by CSF glycoform analyses of brain-derived β-TP [43,44]. Due to its high CSF specificity, β-TP has been long considered a biomarker for the diagnosis of CNS disorders and for CSF leakage determination. In healthy individuals, SDS-PAGE/western-blot analysis of CSF β-TP showed a single band of about 24 kDa, accounting for a polypeptide chain with biantennary N-linked oligosaccharides at Asn^{29} and Asn^{56} glycosylation sites, whereas in patients with PMM2-CDG three different bands were identified corresponding to di- (24 kDA), mono- (22 kDA), and unglycosylated (20 kDa) forms of this protein. MALDI-TOF MS analysis of immunopurified CSF β-TP from PMM2-CDG patients showed a dominant ion peak at 23.3 kDa as well as two signals at 21.1 and 18.8 kDa, indicating the absence of carbohydrate chains from one or both the glycosylation sites. Structural features of β-TP glycans on MALDI-TOF analyses were almost identical in PMM2-CDG and control subjects and indicated the occurrence of "brain-type" carbohydrate structures. By contrast, distinct MALDI spectra of β-TP protein were obtained from MGAT2-CDG (N-acetylglucosaminyltransferase II deficiency, CDG-IIa) patients, showing a single peak at a molecular mass slightly lower than the normal control (22.45 kDa), thus suggesting that both glycosylation sites were occupied by truncated defective glycan moieties [43]. Such defect was compatible to that reported in serum glycoproteins from patients with MGAT2-CDG [45]. In the search of a diagnostic marker for genetic N-glycosylation defects in the brain, Grünewald and colleagues [44] confirmed these hypotheses and extended CSF β-TP glycosylation studies to patients with different CDG-I subtypes. In particular, analyses on patients with ALG6-CDG (CDG-Ic) and CDG-Ix (unknown defect) demonstrated that β-TP hypoglycosylation was essentially attributable to the mono-glycosylated isoform, as, in contrast to PMM2 deficiency (CDG-Ia), the a-glycosylated isoform was nearly absent. Based on the above mentioned findings, β-TP was found as a suitable CSF glycoprotein with "brain-type" structural features to analyze N-glycosylation defects in the CNS of patients with CDG.

2.2. *Childhood-onset Ataxia and Central Hypomyelination or Vanishing White Matter disease*

Glycoproteomic analyses was fundamental for the discovery of disease biomarkers in patients with a recently discovered inherited white matter disorder, named Childhood-onset Ataxia and Central Hypomyelination (CACH) or Vanishing White Matter (VWM) disease. This condition is due to mutations in the five genes encoding subunits of translation initiation factor 2B (eIF2B) leading to an abnormal control of protein translation [46]. VWM disease usually starts in the



Control n=3	9	16	1	1	11	0	100	18	6	0	0	1
ΔCog1	10	17	3	15	9	9	100	**27**	**66**	**24**	**11**	**13**
ΔCog4	4	13	1	2	10	0	100	16	**17**	**2**	0	0
ΔCog7	8	15	1	4	9	5	100	**35**	**30**	**14**	**40**	**13**
ΔCog8	6	12	2	4	15	0	100	14	**31**	**6**	0	0

Fig. 2 – MALDI mass spectra of serum N-glycans of COG deficient patients. Adapted from [40] with permission from Oxford University Press.

first years of life with a gait disorder (ataxia) in children with normal or delayed development. The course is marked by regression of motor abilities and cerebellar symptoms as dysmetria, tremor and dysarthria. In children older than 5 years, disease onset is characterized by progressive spastic diplegia and a longer survival. In addition, prenatal onset with additional systemic features and adult form were reported. Hallmark feature in CACH/VWM disease is a diffuse decrease signal of white matter on T1-weighted MR study with no gadolinium enhancement and cystic breakdown of the myelin or cavitation seen on MR Fluid Attenuated Inversion Recovery (FLAIR) sequences. Neuropathological studies indicated the disease process affects the glial cells, with oligodendrocyte reduction and formation of foamy Periodic Acid Schiff (PAS)-positive cells, suggesting abnormal glycosylation. However, abnormal glycoforms were not found in knockout models [47].

An interesting glycoproteomic work, allowing identification of specific Tf glycoforms in CSF of VWM patients, was conducted via 2-DE followed by MS analyses of the obtained gel-separated spots [48]. Notably, Tf 2-DE profile was consistent with a reduction of basic Tf spots (pI 6–6.5) in all studied VWM (from 0.5% to 5% of total CSF Tf) with respect to control samples (ranging from 8% to 35%). An accurate peptide/glycopeptide mapping, afforded by nanospray FT-MS of the in-gel tryptic digest from either the basic or the acidic 2-DE spots, and subsequent data processing by GlycoMod tool (an on-line software for assignments of sugar moieties composition by their observed mass: http://us.espasy.org/tools/glycomod/), allowed prediction of the putative structure at each glycosylation site. Based on this study, the acidic Tf (sialo-Tf) was found likely to bear at both Asn^{432} and Asn^{630} the same biantennary "serum type" glycan structure composed of 2 GlcNAc, 3 Man, 2 GlcNac, 2 Gal, and 2 NeuNAc. Structural elucidation of tryptic glycopeptides obtained from asialo-Tf spots indicated the occurrence of biantennary glycan structure composed of 2 GlcNAc, 3 Man, 3 GlcNAc and 1 Fuc attached to Asn^{630} glycosylation site. This particular structure concurs with the so called "brain type" N-glycan moiety, previously identified in human CSF [30]. In addition, a distinct carbohydrate structure linked to Asn^{432} residue, corresponding to the asialoglycan $Man_5GlcNac_2$, was identified. Interestingly, this glycan structure was also found in serum Tf of patients with CDG type II [49] (Sturiale L and Garozzo D, unpublished data), pointing to a demannosylation defect of the glycosylation pathway in CDG patients.

While it is presumed that sialo-TF in CSF might reflect serum-derived Tf that can cross the blood–brain barrier, the origin of CSF asialo-Tf is unclear. This glycoform could arise from partial metabolic degradation of serum sialo-Tf [50] or the asialo Tf glycoform is actually synthesize de novo in the brain compartment. The decrease of asialo-fraction of the total Tf is considered a valuable marker for diagnosis of CACH/VWM disease for preliminary screening before gene sequencing, although the role of Tf glycosylation changes in the pathophysiology of this disorder still remains unknown.

2.3. *Autism Spectrum Disorders and Attention-Deficit Hyperactivity Disorder*

Recently, altered glycosylation and ensuing differences in receptor function have been considered among predisposing

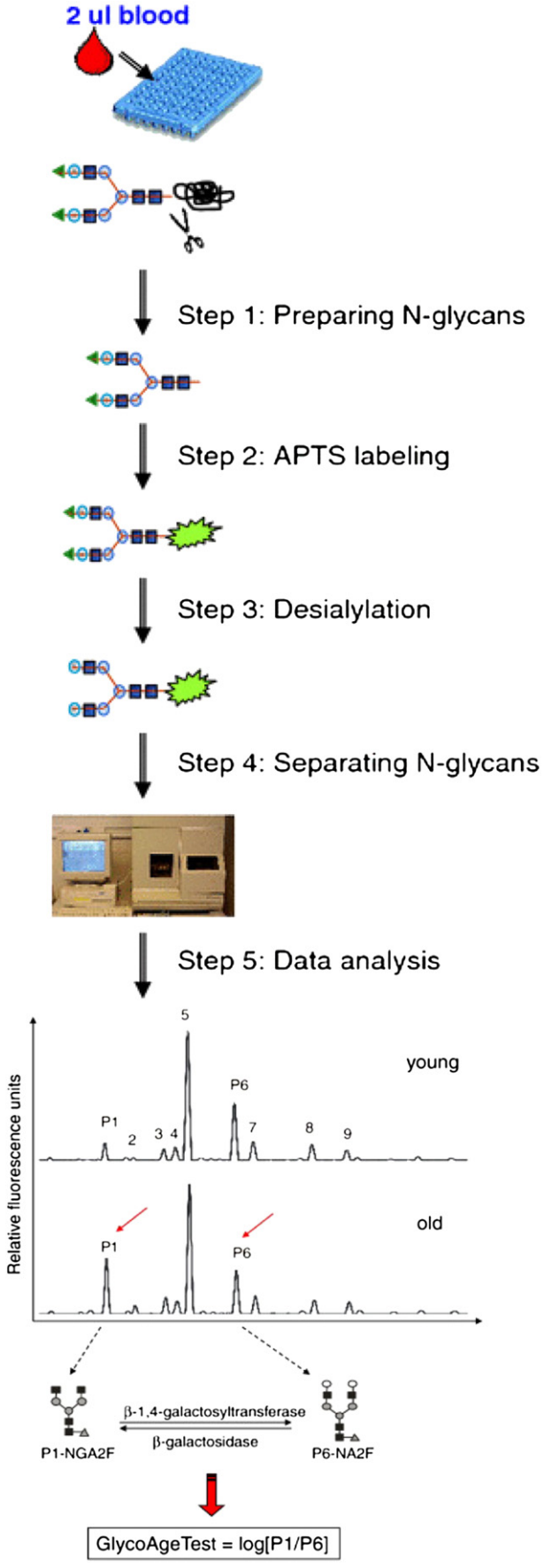
factor in different psychiatric disorders including schizophrenia, bipolar affective disorder and autism [51,52,20].

ASD include autism, Asperger syndrome, and pervasive developmental disorder-not otherwise specified. They all are characterized by impairment of communicative abilities, social interactions and restricted patterns of behavior and interests (according to the Diagnostic and Statistical Manual of Mental Disorders-fourth edition-text revision, DSM-IV-TR). In order to investigate molecular basis of infantile autism, it is important to delineate almost homogeneous clinical phenotypes to limit variability in clinical features. On this regard, two autism patient groups might be distinguished: non-complex autism and complex-autism. This latter is characterized by the occurrence of additional features including dysmorphic features, growth disorders and congenital anomalies while the non-complex autism patients have little or none of the mentioned anomalies. The use of powerful tools for ranking candidate genes within disease susceptibility loci for ASD allowed to delineate affected gene networks and associated biological pathways [19]. In this context, gene-network analyses identified a cluster of seven glycobiology-related genes in CNVs detected in a sample of patients with non-complex infantile autism, including genes operating in N-glycosylation (*B3GALT6*, *B4GALT1*, and *GCNT2*), O-glycosylation (*LARGE*, *GALNT9* and *GALNTL5*) and lipid glycosylation (*ARSA*) [20]. The identified glycobiology genes were not affected by copy number changes in the control cohort and in patients with complex autism; also, these glycosylation related genes are expressed in developing murine brain regions known to be altered in the human autistic brain. Thus, it is suggested that genomic gains or losses in glycosylation pathways might contribute to autism indicating that the ratios of the enzymes encoded by these genes are regulated in the brain and dysregulation could result in aberrant sugar chains on their protein substrates [20].

Attention-Deficit Hyperactivity Disorder (ADHD) is a highly heritable, multifactorial neurodevelopmental disease with childhood onset. It is characterized by deficits in attention, hyperactivity, increased impulsivity and emotional dysregulation. Alterations in structural and functional neuroanatomy in ADHD patients have been suggested by clinical, neuropsychological and brain imaging studies. Genetic basis is supported from linkage and association studies indicating the dopamine transporter gene (*DAT1*) and *DRD4* dopamine receptor gene as susceptibility loci. However, interactions between molecular and risk factors in developing ADHD remain elusive so far [53]. A recent study investigated the plasma N-glycome in a large sample of patients with ADHD and ASD [54]. The employed strategy, based on a method for high throughput quantitative analysis of total plasma glycans, involved sugars release in a 96-well microtiter plate by peptide-N-glycosidase F (PNGase F) followed by fluorescent labeling of the released glycans with 2-aminobenzamide (2-AB) and their subsequent separation by hydrophilic interaction chromatography (HILIC). In addition, the glycan structures were separated according to the number of sialic acids by weak anion exchange HPLC and further characterized after exoglycosidase digestion. This approach allowed establishing significant correlations between plasma glycan changes and ADHD: in particular, a relative increase of peripheral fucosylation of biantennary glycans and a decrease of branching including tri- and tetraantennary glycans were observed. No significant alteration was found in plasma N-glycome of ASD patients. On the other hand, the observed glycosylation changes in children with ADHD might be of relevant interest, because glycan branching plays a role in the adaptive processes of cell membrane and membrane-receptor clearance and properties [55,56].

3. Glycosylation and aging

The analyses of biological processes that act physiologically during aging might unravel important clues to understand the pathomechanisms of neurodegenerative disorders with onset in elder population. The aging process is known to reflect modification in different cellular signaling pathways and transcription factors, including genomic instability, DNA repair, and oxidative stress. These same processes play a role not only in aging but also in age-related diseases, such as AD. Efforts have been devoted to detect biomarkers of the biological age as better reliable predictors of life expectancy than individual chronological age. For this reason, age-related changes of the glycoproteins during aging currently represent an active research area [57]. First evidences in this field came from the observation that Gal content of serum IgG decreases during aging [58]. As the non-galactosyl IgG binds less effectively to the Fc-receptors, this alteration could be meaningful for the immunodeficiency observed in aged subjects. Recently, age-related changes of plasma N-glycans were searched by high-throughput serum N-glycosylation analyses performed in healthy volunteers at different ages, in age-related diseases (dementia) and in a genetic condition of DNA repair deficiency with premature aging (Cockaine syndrome). This large-scale analysis was made possible with the use of an ultra-sensitive technique for standard DNA sequence adapted for the study of serum N-glycan profile, named “DNA Sequencer Adapted-Fluorophore Assisted Carbohydrate Electrophoresis” (DSA-FACE) [59–61]. The procedure, that employs just two blood microliters, includes three main steps: N-glycan labeling, N-glycan profiling through the identification of the nine most prominent glycan structures, and association analyses between specific glycans and aging process. The analyses of hundreds of healthy samples from different age groups (from 20 to 60 years) showed several glycan structures of serum to be closely related to aging. In particular, the biantennary agalactosylated structure with core α1,6-fucosylation (NGA2F) increased with age, while the biantennary digalactosylated, core α-1,6-fucosylated structure (NA2F) decreased [59]. Therefore, the log ratio of NGA2F and NA2F species was considered as a potential aging biomarker named GlycoAgeTest. It was found that the serum GlycoAgeTest in healthy control samples remained stable up to the age of 40 years, increased by the age 50 and even more so after the age of 60 years, reaching the highest level in nonagenarian group (90–99 years) [61]. Fig. 3 schematizes the whole DSA-FACE procedure leading to distinct N-glycan profiles. Those reported in the figure are representative of differences between young and old healthy subjects.



The increase of agalactosylated N-glycan in serum during aging might represent a decrease of β-1,4-galactosyltransferase or a greater abundance of β-1,4-galactosidase activity, as age-related phenomena [61]. Thus, it is conceivable that the same mechanism reflecting aging in serum is acting in CNS independently of age.

Application of the GlycoAgeTest to dementia patients showed a higher value with respect to age-matched controls, therefore suggesting that their biological age corresponded to a higher chronological age. The validity of GlycoAgeTest was confirmed also with the study of patients with a premature aging syndrome named Cockaine syndrome, due to a genetic defect of the DNA repair system. In six studied patients with Cockaine syndrome with age ranging from 3 to 20 years, the GlycoAgeTest was in the same range as in the nonagenarian people, thus indicating the occurrence of premature aging in Cockaine syndrome [61]. Thus, evaluation of N-glycan shift in serum could be used to have a measure of aging process as well as an indicator of age-related diseases such as dementia.

Two further independent studies [62,63] confirmed plasma glycan changes related with increasing age and showed more profound associations between glycan pattern and age in females than in males. Such associations might reflect specific hormonal influences related to aging and menopause in female subjects. In particular, Knezevic et al. [63], by performing high-throughput HILIC–HPLC analysis of the 2-AB-labeled glycans released from plasma proteins by PNGase F, were able to identify a higher number of individual glycans thanks to the robustness of the applied method. Interestingly, they found also some statistically significant correlations including those between specific glycosylation patterns with cholesterol and lipoprotein levels, thus suggesting that plasma protein glycosylation might be influenced by the lipid status. A method based on dual HILIC–HPLC separation was also adopted in an even more recent study to profile N-glycans from the total protein pool in plasma after their labeling with 2-aminobenzoic acid (2-AA) [64]. N-glycosylation changes with aging were evaluated to mark familial longevity in a large cohort of nonagenarian siblings (n: 1671) and controls (n: 744). A typical chromatogram of plasma derived N-glycans labeled with 2-AA is shown in Fig. 4 that reports also the 26 more abundant structures identified according to their relative chromatographic retention times. Results revealed that nongalactosylated glycans increased with age, while core fucoylsated asialo- and monosialo-digalactosylated glycans decreased. In particular, two N-glycan features, namely digalactosylated asialo-, and monogalactosylated monosialo-glycoform (indicated in Fig. 4 with red arrows) were found to mark familial longevity as they were more abundant in plasma of the offspring of long-lived individuals, as compared to controls. Although in the aforementioned approach, total plasma glycans may reflect altered glycosylation of more than one glycoprotein in plasma related to aging process, it could not be excluded that these changes may also be related to variations of glycoprotein expression itself. For this reason, it

Fig. 3 – Schematic overview of the N-glycan analysis via DSA-FACE technique.
Adapted from [61] with permission from Elsevier.

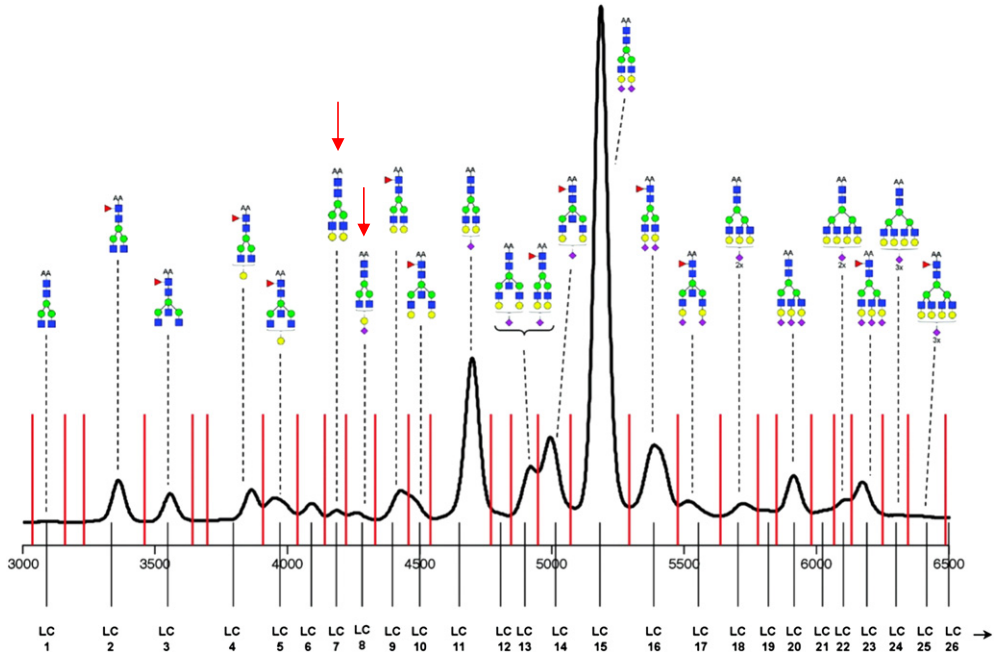


Fig. 4 – Typical HILIC–HPLC chromatogram, with fluorescence detection, of plasma derived N-glycans labeled with 2-AA. Red arrows indicate species associated with familial longevity.
Adapted with permission from [64]. Copyright (2011) American Chemical Society.

might be relevant to perform also parallel analyses of quantitative protein profiles and/or to monitor age-related glycosylation changes in individual target glycoproteins. On the whole, high-throughput methods applied for the N-glycan profile in serum represent a robust and sensitive tool for the analyses of large sized samples in the development of serum glycome markers.

4. Glycosylation and age-related diseases: Alzheimer's Disease

4.1. *Overall framework and glycoproteomic approaches, applications and advances*

AD represents about 65–70% of all dementia types and it typically affects elders over 70 years of age. The disease is characterized by progressive loss of cognitive functions, representing a disabling disorder and a major health concern. The cause of AD is unknown and neither preventive nor therapeutic tools are available so far. In clinical practice, diagnosis of "possible" or "probable" AD is currently obtained by standardized clinical criteria, including NINCDS-ADRDA [65] and DSM-IV. The use of structural biomarkers based on neuroimaging is impaired because of the difficulty to discern by visual interpretation between normal aging process and dementia. Hence, definitive diagnosis still relies on post-mortem brain tissue analyses. AD pathological hallmarks are β-amyloid protein (Aβ) deposits derived from the abnormal cleavage of the ubiquitous amyloid precursor protein (APP) in the brain parenchyma and cerebral blood vessels and the presence of neurofibrillary tangles. Tau proteins are associated microtubule proteins found in neurofibrillary tangles and related to degenerating and dying neurons.

Currently known CSF protein biomarkers of AD comprise β-amyloid 1–42 (Aβ42) peptide, total tau (t-tau) and phosphorylated tau protein (p-tau). High levels of t-tau proteins and even more of p-tau in the CSF have been associated with AD diagnosis and progression [66]. Combined clinical examination and elevation of CSF t-tau or p-tau protein with diminished CSF Aβ42 levels in comparison to controls, have become valuable diagnostic tools during the recent years predicting more than 80% of the AD cases [67,68]. Deviation of such biomarkers improves prediction of progression from mild cognitive impairment (MCI) to dementia. However, progression from very mild to more pronounced cognitive impairment is not reflected by biomarker variations. Based on this observation it is suggested that, at present, the measured biomarkers are not early disease markers [69]. Additional diagnostic tools would be important to improve an early and correct diagnosis of AD, as early and definitive diagnoses are crucial for therapeutic intervention when possible disease-modifying drugs would be available.

Several evidences support a role for glycosylation abnormality in AD and other neurodegenerative disorders. On this regard, it was demonstrated that human brain tau protein is modified by O-GlcNAcylation, a type of protein O-glycosylation by which the monosaccharide β-N-acetylglucosamine attaches to Ser/Thr residues by an O-linked glycosidic bond. O-GlcNAcylation acts on nucleoplasmic and cytoplasmic proteins and it is reciprocally related to protein phosphorylation. Reduced glucose uptake/metabolism, as seen in AD brain, results in decreased O-GlcNAcylation and consequently hyperphosphorylation of tau itself in an animal model [21,22].

Abnormal CSF levels and improper glycosylation of the structural brain glycoprotein reelin have been implicated in pervasive developmental disorders as autism and in neurodegenerative diseases. Reelin is a 420 kDa signaling glycoprotein expressed postnatally in the GABAergic cortical interneurons, with main functions in cytoarchitectonic pattern formation of different brain areas during development, and in maintaining synaptic plasticity. Interestingly, the glycosylation pattern of reelin is different in plasma and CSF thus supporting intrathecal synthesis of CSF reelin [26]. Reduced reelin levels have been demonstrated in serum and brain areas of autistic patients in line with some of the brain structural and cognitive deficits observed in the disorder [70]. Altered CSF levels of full-length reelin and reelin 180 kDa fragment have been recognized in AD, as well as in other neurodegenerative disorders such as frontotemporal dementia, progressive supranuclear palsy and Parkinson's disease [26,71]. Moreover, the glycosylation pattern of CSF 180-kDa reelin was different in AD versus non-demented controls thus suggesting both quantitative and qualitative changes of CSF reelin in AD [26].

Glycosylation influences also the biological activity of asp-2 that is crucial for the formation of amyloid 39–42 β-peptides, and glycosylation changes have been observed in Collapsin Response Mediator Protein 2 (CRMP-2) that regulates the assembly and polymerization of microtubules associated with neurofibrillary tangles in AD [72].

Furthermore, it was demonstrated that acetylcholinesterase (AChE), a critical enzyme in AD pathogenesis and targeted in current clinical management of AD, shows an increase of glycosylated isoforms in post-mortem brain and in CSF of AD patients; glycosylated AChE and butyrylcholinesterase levels increase as a matter of disease duration in AD patients [27].

Additional studies relied on glycosylation analyses of APP, pivotal molecule which proteolytic processing is considered decisive for aggregating amyloid peptides/glycopeptides in AD. APP is a membrane glycoprotein with several N- and O-glycosylation sites. As protein O-glycosylation can modulate the action of proteases by influencing the accessibility of the cleavage sites [73], particular attention has been devoted to investigate site-specific O-glycosylation, of APP/Aβ-peptides. In 2009 Perdivara and co-workers reported on the identification of three APP O-glycosylation sites (Thr-291, Thr-292 and Thr-576) elucidating types, composition and structure of the O-linked glycans at each site by nanoHPLC-tandem MS, using Electron Transfer Dissociation (ETD) and CID as fragmentation techniques [74]. A more recent work, also conducted via LC–MS/MS, allowed recognition of a series of APP/Aβ glycopeptides in CSF, revealing a varied O-glycosylation pattern hitherto unknown, with a specific highly sialylated O-glycan structure attached to tyrosine 10 (Tyr10) most abundant in AD patients [23]. The impact of Tyr10 glycosylation onto APP proteolytic processing warrants further investigations.

Based on the indication that glycoprotein glycans affect protein stability, conformation and trafficking, Akasaka-Manya and colleagues conducted a straightforward analysis of N-glycan structures in normal and mutant human APP [24]. Mutant APP has an increased rate of Aβ42 and Aβ40 secretion and was found to have a higher content of N-glycans with bisecting GlcNAc residues and core–fucose residues compared to wild-type APP. Noteworthy, increased levels of N-acetylglucosaminyltransferase III (MGAT3), responsible for synthesizing a bisecting GlcNAc residue, were found in Neuro2a cells after Aβ treatment. Also, MGAT3 mRNA expression levels are increased in the brains of AD patients [25]. Considering that upregulation of MGAT3 protein leads to enhanced clearance of Aβ in mononuclear cells [75] and decreases Aβ production in Neuro2a cells [25], it was suggested that upregulation of MGAT3 in AD brains and increased N-glycan bisecting GlcNAc residues might represent a protective factor by reducing Aβ production in AD [25], therefore offering a novel therapeutic target to prevent or ameliorate AD. At the glycoproteomic level, it could be postulated that the increase of bisecting N-glycans in CSF is a phenomenon related to AD. For this reason it would be of interest to map bisecting protein glycoforms in CSF of controls and in subjects with different ranges of severity, from MCI to overt AD cases.

4.1.1. *Glycosylation analysis of cerebrospinal fluid in Alzheimer's Disease and in other elderly dementias*

CSF is a continuous with the extracellular fluid of the brain, however almost 80% of CSF proteins are plasma proteins, emanating from serum by passage over the blood–brain barrier. Most of the CNS specific glycoproteins are low in abundance and most proteomic techniques are biased toward abundant proteins, including albumin, haptoglobin, and IgG. Elimination of plasma proteins by affinity chromatography may greatly enhance detection sensitivity for CNS specific glycoproteins. In a pioneering work, Davidsson et al. [76] established a procedure for detecting brain-specific proteins in CSF, including three affinity chromatography steps aimed to reduce the most abundant serum proteins from CSF. Thereafter, four major peaks were detected by micro-reversed HPLC and characterized as beta2-microglobulin, cystatin C, transthyretin C and asialo-Tf by western blotting and ESI-MS. Also, the procedure identified a brain-specific isoform of transthyretin (TTR) with pI 5.7 that turned out to be less present in AD patients CSF. TTR is a tetrameric glycoprotein with molecular mass of about 55 kDa. It is able to bind to beta-A4 protein, a major component of the senile plaques [77]. Thus, the reduction of brain-specific TTR isoform in CSF of AD patients is thought to be related to the absorption of TTR to the amyloid deposits in the senile plaques, suggesting that disequilibrium of proteins normally binding to beta-A4 protein might enhance amyloid formation in the brain [78].

2-DE coupled with MS analysis of the in-gel digested protein glycoforms is widely utilized for structural determination and comparative analyses of glycoproteins in the study of neurodegenerative disorders. Sample handlings as prefractionation (mostly albumin depletion) prior to 2-DE separation with micro-narrow pH range IEF, enable to improve detection of different glycoprotein isomers [79–81]. Fig. 5 reports the 2-DE fractionated glycoproteome from one AD patient wherein circles mark spots corresponding to individual glycoforms that differ in intensity from controls [82]. As a further step, Sihlbom et al. analyzed tryptic digest from the excised spots by FT-ICR MS and additional MS/MS fragmentation with CID. This technique, besides the identification of the altered protein glycoforms in AD, thanks to the

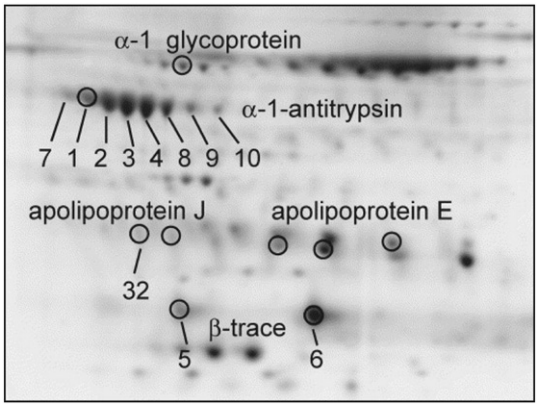


Fig. 5 – 2-DE (pH 4.7–5.9) of CSF (300 μl) from one AD patient. Circles mark spots corresponding to individual glycoforms that differ in intensity from controls.
Reproduced from [82] with permission from Elsevier.

excellent mass accuracy, enabled structural characterization of site-specific N-glycosylation and reduced the number of possible different isoforms of glycopeptides resulting from oligosaccharide combination. Although no AD specific glycoform was attributable to the analyzed proteins, altered levels of several CSF glycoproteins, such as apolipoprotein E, apolipoprotein J (clusterin), α-1-β-glycoprotein and α-1-antitrypsin, were found in AD patients, showing also decreased glycosylation of one specific α-1-antitrypsin isoform [80,82].

Structural glycomics of CSF in neurodegenerative processes has been approached also by alternative procedures that exploit glycoprotein interaction with lectins. Lectin-affinity chromatography is based on the properties of these proteins to specifically recognize distinct oligosaccharide epitopes. Thus, the method allows the isolation and discrimination of glycan structures among different glycoproteins or glycopeptides. ConA binds mannosyl and glycosyl residues containing unmodified hydroxyl groups at C3, C4, and C6, therefore having prominent affinity for high-mannose, complex and terminal glucose N-glycan structures; wheat germ agglutinin (WGA) recognizes glycostructures comprising terminal GlcNAc and sialic acids, characteristic of both N- and O-glycosylation; *Arachis hypogaea* (PNA) and *Datura stramonium* agglutinin (DSA) specifically bind to glycans containing β-Gal and GlcNAc respectively. Lectin-affinity protocols take advantage of a number of solid supports for lectins and of different chromatographic tools such as tubes, columns and microfluidic channels [83]. Double- and multi-lectin chromatography are used for a complete enrichment of glycoproteins from biological fluids prior to MS analyses [84,85]. Currently, the most advanced upshot of lectin-based strategies consists on microarray that makes use of a chip (LeeChip™) containing 45 different lectins, for a direct and sensitive profiling of glycoproteins. Further applications of lectin microarray include cell profiling (for direct analyses of cell glycome) and indirect glycoprotein profiling (through antibody overlay) [86].

Finally, lectin affinity purification and hydrazide chemistry used in combination and followed by 2D LC–MS/MS, allowed the identification, with high confidence, of 216 glycoproteins in human CSF [87].

Glycosylation changes in AD were investigated by lectin-affinity chromatography that revealed reduced levels of some WGA-reactive glycoproteins in CSF from these patients with respect to controls. In particular, a less staining of CSF Tf was observed, thus suggesting that glycosylation of Tf might be a biological marker for the diagnosis of AD [88]. On this regard, Taniguchi et al. [89] concurred to demonstrate a lower WGA-binding activity of CSF Tf in a group of AD patients. The same study takes account of further analysis by IEF that supported the hypothesis of possible glycosylation changes of CSF Tf in AD.

A strategy based on SDS gel electrophoresis of CSF Tf was used to identify potential biomarkers for idiopathic normal pressure hydrocephalus (iNPH), a senile dementia associated with ventriculomegaly [90]. By this technique it was possible to distinguish two isoforms, indicated as Tf-1 and Tf-2, that differ for their glycan composition. Glycan analyses revealed that Tf-1 had "brain type" biantennary asialo- and agalacto-complex type N-glycans (GlcNAc-terminated glycans), whereas Tf-2 had "serum-type" sialyl-terminated biantennary N-glycans. It was demonstrated that the Tf-2/Tf-1 ratios in CSF from iNPH patients were significantly higher than those of controls ($p=0.0019$) and Alzheimer's patients ($p=0.0010$), thus suggesting such parameter as diagnostic index to discriminate iNPH from AD and possibly other dementias. More recently, the same research group developed a high-throughput ELISA method with combination of lectins and an anti-Tf antibody (TfAb), to measure different Tf glycoforms in CSF thus enabling identification of Tf-1 and Tf-2 species [91].

4.1.2. *Brain glycosylation analysis in Alzheimer's Disease*

Basing on methods that employ classical "bottom-up" proteomic protocols (as schematized in Fig. 6), Butterfield and Owen emphasized the efficacy of lectin-affinity chromatography to pick out glycosylation changes of brain proteins in AD and MCI [92]. Two separate studies regarding respectively the ConA- and the WGA-fractionated proteome revealed altered levels for a variety of glycoproteins in hippocampus and inferior parietal lobule including chaperones, synaptic, cytoskeletal, cell signaling and protease inhibitor proteins, all consistent with the pathology and progression of AD [93,94].

Results on individual glycoconjugates were deeply discussed, with particular focus onto the normal biological role of the identified glycoproteins and how their variations in glycosylation could influence and/or dysregulate cellular functionality at different clinical stages of AD and MCI.

4.1.3. *Serum glycosylation analysis in Alzheimer's Disease*

High-throughput methods for N-glycome analyses in serum might become advantageous in AD biomarker research, by avoiding invasive CSF sample collection. On this regard, the ultra-sensitive strategy for large scale study of blood N-glycome by DSA-FACE was applied for biomarker discovery in AD [patent application publication, May 5, 2011]. Total blood N-glycans were released, labeled and analyzed by using DSA-FACE as described for the GlycoAgeTest [59,60]. Desialylated blood N-glycome was analyzed in AD patients,

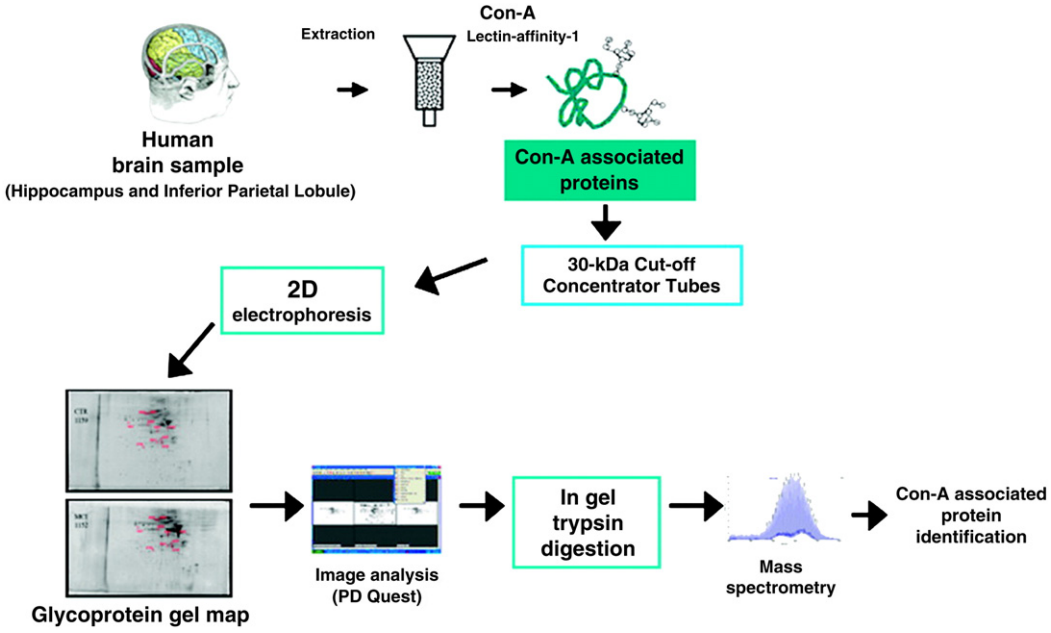


Fig. 6 – Flowchart of the proteomic strategy adopted in the study of brain glycosylation: Co-A lectin affinity chromatography coupled to 2DE afforded identification altered levels of brain glycoproteins in AD and MCI patients. Reprinted with permission from [93]. Copyright (2009) American Chemical Society.

non-AD dementia patients and healthy age-matched subjects. The N-glycans detected in serum were quantified by normalizing the height of each peak to the sum of the heights of all peaks in the profile. A quantitative variation of two of the glycan structures (more pronounced in females) was found related to dementia. The level of peak corresponding to NA2F (digalactosylated core-α-1,6-fucosylated biantennary oligosaccharide) was decreased only in AD patients, with 90% of specificity and 70% sensitivity. This finding served for identifying AD from the non-AD and control groups. Moreover, signal corresponding to a branching α1,3-fucosylated trigalactosylated triantennary glycan (NA3Fb), was significantly enhanced ($p<0.006$) in the female dementia patients (AD and non-AD) with 90% of specificity and 60% of sensitivity [patent application publication, May 5, 2011]. Significant relationships between serum N-glycan variations, CSF biomarker levels and data emerging from standard cognitive evaluation procedures as mini-mental state examination (MMSE), were recognized in female dementia patient. Serum amount of NA2F was positively correlated to the CSF levels of Aβ-42 and negatively correlated to the levels of p-tau protein, whereas in male patient's serum NA2F levels were substantially related only with CSF total t-tau level [95].

5. Glycosylation in other CNS diseases

In addition to aging and age-related neurodegenerative disorders, evidences of glycosylation changes have been emerging for other diseases of the CNS. In particular some distinct studies explored the relationships between altered glycosylation pathways and pathomechanisms in multiple sclerosis and in severe mental disorders such as schizophrenia.

5.1. *Multiple sclerosis*

Multiple sclerosis is a chronic inflammatory demyelinating disease of the CNS that is assumed to be an autoimmune disorder in which both genetic and environmental factors play a role [96]. Genetic studies suggest an epistatic model of interaction between independent alleles and the environment which might affect a common biochemical pathway with a combined deleterious effect leading to immune dysregulation and disease onset.

First evidence that deficiency of the Golgi enzyme N-acetylglucosaminyltransferase V (MGAT5, responsible of N-glycan branching), dysregulates T-cell response in mice, was reported by Demetriou and colleagues in 2001 [97]. Subsequent works sustained the hypothesis of a causative role for defective N-glycosylation, due to N-acetylglucosaminyltransferase I, II and V (MGAT1, -2 and -5) impairment, in the mouse model of immune-mediated neurodegenerative diseases, including multiple sclerosis [98].

MGAT enzymes ensure N-glycan branching by sequential reactions that rely on the supply of UDP-GlcNAc by the hexosamine pathway [99]. Disturbed N-glycosylation via MGAT impairment might promote loss of self-tolerance by reducing glycan branching and surface glycoprotein expression of the autoimmune inhibitor Cytotoxic T-Lymphocyte-associated Antigen-4 (CTLA-4) in human T cell blasts [100]. Also, MGAT1 dysfunction might enhance the exposition of cryptic mannose linkage on the cell surface of oligodendrocytes

that mimic microbial and pathogen glycans, then activating chronic innate immune responses [101]. Multiple sclerosis risk allele interleukin-2 receptor-α (IL2RA) and interleukin-7 receptor-α (IL7RA) lead to down-regulation of MGAT1, decrease branching and CTLA-4 surface expression, ultimately increasing multiple sclerosis risk. As a counterpart, environmental factors such as vitamin D3/sunshine and metabolism, might enhance MGAT1 leading to increased branching, CTLA-4 surface expression, and decrease multiple sclerosis risk [100]. Such compelling evidences suggest that multifactor determinants might converge to affect the efficiency of the N-glycan processing in the Golgi and ultimately influencing the risk to develop multiple sclerosis, as visualized in Fig. 7.

As a further approach, glycomics study relied on the investigation of acute-phase proteins in order to identify potential serum/plasma associated glyco-biomarkers of inflammatory processes related to multiple sclerosis [102].

5.2. *Schizophrenia*

Schizophrenia is a rather common psychiatric disorder characterized by disorganized speech and thinking including hallucinations and delusions. It is a multifactorial disease with complex inheritance pattern, several candidate genes for susceptibility and an unclear etiology including possible different biological causes [103,104]. The lack of empirical diagnostic tests, by delaying identification of the affected patients, encouraged the research of molecular biomarkers as a possible target for both environmental and genetic determinants to enable timely diagnosis.

Investigations of glycoproteome in serum and in CSF of patients at disease onset revealed specific alteration when compared to healthy individuals [105]. A detailed and accurate study based on quantitative separation of total glycan pool by NP-HPLC and subsequent 2-AB labeling, pointed to a general decrease of bisecting and sialylated glycans in CSF of schizophrenia patients. Interestingly, individual glycan structures such as the tetraantennary tetrasialylated glycan with a polylactosamine extension and the triantennary trisialylated glycan containing the SLe^x epitope, exhibit appreciable gender specificity. In fact a sizeable increase of these species was found on both high abundant and low abundant serum protein fractions in male schizophrenia subjects. These results were converging with changes in the gene expression levels of Golgi glycosyltransferases found in prefrontal cortical samples from schizophrenic subjects [106] and moreover permitted to formulate new hypothesis on the pathophysiological mechanism underlying schizophrenia.

6. Concluding remarks

Clinical and experimental data converge to indicate the impact of glycosylation in CNS diseases both in adulthood and pediatric age. CDG, as human genetic diseases of glycosylation, illustrates the effects of improper glycosylation on CNS development and function. In CDG patients glycosylation of studied brain-derived intrathecal glycoproteins such as β-TP, is also defective. In several CNS disorders

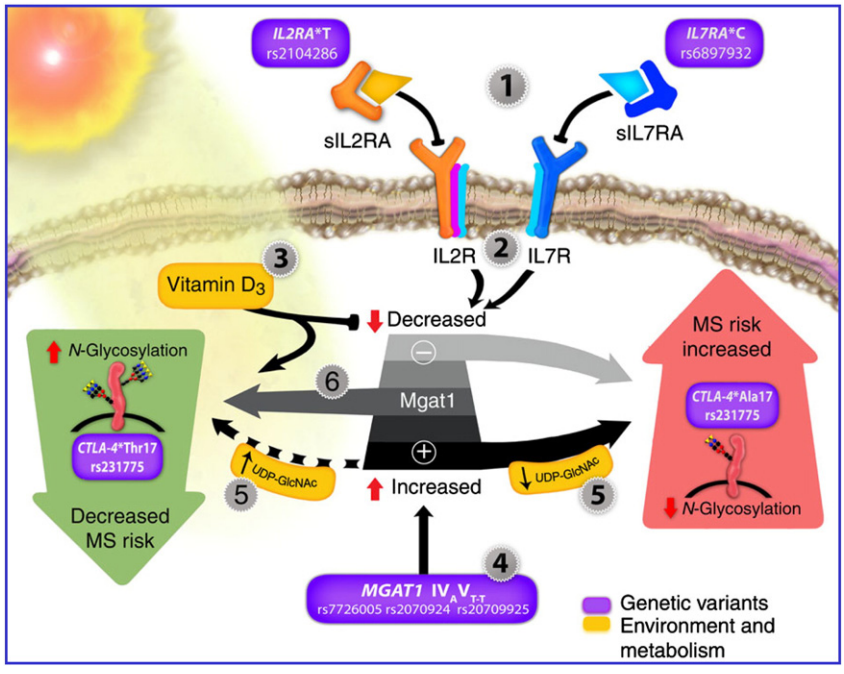


Fig. 7 – Multiple sclerosis genetic background variants (IL-7RA, IL-2RA, MGAT1 and CTLA-4) might interact with multiple environmental factors (sunlight/vitamin D_3 and metabolism) having as a final target a common pathway that is Golgi N-glycosylation.
Reproduced from [100] with permission from Nature Publishing Group.

with multifactor determinants, genetic and environmental factors combine to dysregulate glycosylation.

2-DE-MS including FT-MS allowed prediction of putative structure at each protein glycosylation site, indicating modification of CSF glycoforms in neurodegenerative disorders including AD. As a different approach, the investigation of the whole CSF glycan pool by NP-HPLC and 2-AB labeling was remarkable in that it showed a decrease of some individual structures in severe mental disorders as schizophrenia.

High-throughput methods for serum N-glycome profile in neuropsychiatic diseases were put forward for large sample analyses. Employed strategies include HILIC separation of 2-AB labeled N-glycans that resulted in the identification of disease-associated plasma N-glycan profile in children with ADHD, an almost frequent childhood psychopathological disorder. The same technique and additional blood DSA-FACE investigation were tools to detect age-related changes of N-glycan profile in the development of serum glycome markers of aging and age-related CNS disorders. It is conceivable that future application of current strategies for CNS glycomics might include genome-wide copy number variation analyses to highlight functional glycosylation gene network in multifactorial neuropsychiatric diseases, as demonstrated for autism.

Comprehensive population studies might reveal further glyco-phenotype associated to specific diseases or disease endophenotypes.

Acknowledgments

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