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ORIGINAL ARTICLE

Proceedings of workshop: "Neuroglycoproteins in health and disease", INNOGLY cost action

Esther Llop[1] · Ana Ardá[2,3] · Elsa Zacco[4] · Roisin O'Flaherty[5] · María-Salud García-Ayllón[6,7,8] · Massimo Aureli[9] · Moran Frenkel-Pinter[10] · Celso A. Reis[11,12,13] · Ole K. Greiner-Tollersrud[14] · Inmaculada Cuchillo-Ibáñez[7,8,15]

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Abstract
The Cost Action "Innovation with glycans: new frontiers from synthesis to new biological targets" (INNOGLY) hosted the Workshop "Neuroglycoproteins in health and disease", in Alicante, Spain, on March 2022. This event brought together an european group of scientists that presented novel insights into changes in glycosylation in diseases of the central nervous system and cancer, as well as new techniques to study protein glycosylation. Herein we provide the abstracts of all the presentations.

Keywords Glycosylation · Cancer · Neurodegenerative diseases · Lectins · Glyco-enzimes · Mass-spectrometry · NMR

Introduction

The Workshop "Neuroglycoproteins in health and disease" took place at Universidad Miguel Hernández de Elche, in Alicante, Spain, in March 2022 for 2 days. This workshop was part of the activities of the Cost Action "Innovation with glycans: new frontiers from synthesis to new biological targets" (INNOGLY).

Initiatives such as this Workshop inside INNOGLY emerge to provide a successful space to enhance a better understanding of the mechanisms behind the diseases involving glycosylation. The workshop brought together a total of

✉ Inmaculada Cuchillo-Ibáñez
icuchillo@umh.es

1 Department of Biology, Biochemistry and Molecular Biology Unit, University of Girona, 17003 Girona, Spain

2 Ikerbasque, Basque Foundation for Science, Maria Diaz de Haro 3, 48013 Bilbao Bizkaia, Spain

3 CIC bioGUNE, Basque Research & Technology Alliance, Bizkaia Technology Park, Bld. 800, 48160 Derio, Bizkaia, Spain

4 Italian Institute of Technology, Genova, Italy

5 Department of Chemistry, Maynooth University, Maynooth, Co. Kildare, Ireland

6 Laboratorio de Investigación, Hospital General Universitario de Elche, Fundació para el Fomento de la Investigación Sanitaria y Biomédica de la Comunitat Valenciana, Alicante, Spain

7 Instituto de Neurociencias de Alicante, Universidad Miguel Hernández de Elche-CSIC, Sant Joan d'Alacant, Spain

8 Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Madrid, Spain

9 Department of Medical Biotechnology and Translational Medicine, University of Milano, via Fratelli Cervi 93, 20090 Segrate, MI, Italy

10 The Center for Nanoscience and Nanotechnology, Institute of Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel

11 Instituto de Investigação E Inovação Em Saúde, Universidade Do Porto, Porto, Portugal

12 Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Porto, Portugal

13 Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade Do Porto, Porto, Portugal

14 Institute of Medical Biology, UiT The Arctic University of Norway, Tromsø, Norway

15 Instituto de Investigación Sanitaria y Biomédica de Alicante (ISABIAL), Alicante, Spain

11 internationally renowned scientists from Italy, Norway, Portugal, Ireland, Israel, The Netherlands and Spain, with active research and expertise in this area. This "in person" meeting also placed value on the importance of personal encounters to enhance brain storming and allow formation of future collaborations.

The study of glycosylation is a challenging field where many questions remain to be answered; however, it is already well established glycoconjugates play a dominant role in physiological and pathological processes. Aberrant glycosylation has been recognized as a feature of many mammalian diseases, including hereditary disorders, immune deficiencies, neurodegenerative diseases and cancer. Scientists participating in the workshop shared their latest investigations about alterations in glycosylation in cancer (Celso A Reis and Esther Llop), in the central nervous system (Massimo Aureli, Roisin O'Flaherty, Moran Frenkel-Pinter, Mª Salud García-Ayllón and Inmaculada Cuchillo-Ibáñez), and in hereditary syndroms (Ole K Greiner-Tollersrud). Classical and new methodological approaches to characterize glycoproteins were also discussed, and the scientists participating in the workshop showed powerfull techniques based on lectins (Ana Ardá, Esther Llop and Lorenzo Albertazzi) and spectrometry (Ole K Greiner-Tollersrud, Roisin O'Flaherty) but also presented the concept of new small glycoconjugates as tools (Elsa Zacco).

Overall, the workshop provided evidences that the Glycosciences has a promising role in improving human health and consequently, advances in research have to foster applications into clinical practice.

The topics covered key themes that could be grouped into 2 categories: "Glycosylation in diseases", and "Methods in Glycoscience". The proceedings of every talk are included below.

Glycosylation in diseases

"*Plasma membrane-associated glycohydrolases: glyco-enzymes involved in neuronal differentiation and senescence*", Massimo Aureli

The classical concept that the biosynthesis and catabolism of the glycosphingolipids are associated with the Golgi apparatus and with the lysosomes respectively, has been partially overcome by the presence of enzymes involved in glycosphingolipids metabolism that are able to exert their activity directly at the plasma membrane level. These enzymes include the sialidases and sialyltransferases, β-hexosaminidases and β-N-acetyl-galactosyltransferases, β-galactosidases, and the β-glucocerebrosidase GCase and the non-lysosomal β-glucosylceramidase (NL-Gase). The association of sialidases and sialyltransferases with the synaptosomal membrane was firstly described in the 70 s [1–5], suggesting that a sialylation–desialylation cycle exists for gangliosides at plasma membrane level and might be involved in defining the functional role of glycosphingolipids in neurons. Interestingly, this aspect was later strengthened by the discovery of "sphingomyelin cycle" and of the "N-acetylgalactosamine cycle" [6]. Despite the origin of the glycosyltransferases is still not defined, most of the glycohydrolases, except for the sialidase Neu3 and of the NL-Gase, are the same N-glycoproteins present in the lysosome that can reach the plasma membrane via the lysosomal exocytosis [7]. Interestingly these enzymes can exert their activity also at the external side of the plasma membrane thanks to the presence of specific proton exchangers that locally create the proper acidic microenvironment [8]. The local changes of the sphingolipid composition could exert an important effect on the dynamics of the plasma membrane and consequently on the cell homeostasis. For instance, the head group of an amphiphilic compound, together with the volume of the hydrophobic backbone, determines the packing parameter of the monomer inserted into the membrane. In glycosphingolipids, this parameter is determined by the ratio between the volume occupied by the oligosaccharide chain and by the ceramide. The enzymatic removal of sugars from the glycosphingolipids leads to a higher packing parameter resulting in a less curved surface. On the other hand, the enzymatic addition of sugars reduces this packaging parameter and promotes an enhanced curvature [9, 10].

All these aspects allow the formation of "lipid rafts" with specific size and dynamics that are important players especially in the neuronal social life including the differentiation and the senescence.

"*N-Glycosylation in central nervous system disorders*", Roisin O'Flaherty

The central nervous system is highly controlled and fine-tuned by glycosylation; glycans play significant roles in neurodevelopment, neural transmission, excitability, and plasticity [11–14]. Emerging evidence highlights the impact of protein N-glycosylation in central nervous system disorders: glycans and glycan genes including those that encode transcription factors, cytokines, glycosyl transferases and glycosidases play critical roles in Parkinson's disease (PD), Alzheimer's disease (AD), neuroinflammatory models, depressive disorders and addiction studies [15–21]. Two distinct technologies were presented. The first describes the characterisation of the rodent N-glycome in the striatal and substantia nigra regions of rodent brains as an *in vivo* model for PD, healthy rodent tissue N-glycomes described in detail in [18]. In our study, we demonstrated distinct glycan motifs characteristic of PD in an animal model. In a separate study, a glycoanalytical technology was presented, described in detail in [15] to assess the relative changes in

N-glycosylation in a large population (n = 1516) for participants with/without major depression. The participants were defined as depressed according to the Goldberg Depression Scale (D ≥ 2) [22]. In the study, an artificial intelligence (AI) Shapley Additive exPlanations (SHAP) plot model was employed combining over 300 variables (age, gender, health indicators, cytokines, questionnaires etc.) to predicts self-reported depression with 84% accuracy. Most strikingly, 11 of the top 20 contributors to the model directly link glycosylation related features to depression. Taking this into account, considering multivariate analysis of their glycan peaks indicates glycosylation is significantly altered (p = 2.53e-11) in those participants with self-reported depression, and the fact that congenital disorders of glycosylation (CDGs), which involve mutations in glycosylation enzymes and associated genes, often present with neurological symptoms in 80% of cases [21], despite their clinical heterogeneity strongly suggests that glycosylation abnormalities are central to central nervous system disorders such as major depression. Collectively these data present strong evidence for future study of protein N-glycan pathways in central nervous system disorders.

"*Establishing the role of global protein glycosylation alterations in Alzheimer´s disease*", Moran Frenkel-Pinter

Deviations from the normal nucleoplasmic protein O-GlcNAcylation, as well as from normal protein sialylation and N-glycosylation in the secretory pathway, have been reported in Alzheimer's disease (AD) [23–28]. However, the interplay between the cytoplasmic protein O-GlcNAcylation and the secretory N-/O-glycosylation in AD has not been described. We have presented a comprehensive analysis of the N-, O-, and O-GlcNAc–glycomes in AD-affected brain regions as well as in AD patient serum [27]. We detected marked differences in levels of glycan involved in both protein O-GlcNAcylation and N-/O-glycosylation between patients and healthy individuals and revealed brain region–specific glycosylation-related pathology in patients. These alterations are not general for other neurodegenerative conditions, such as frontotemporal dementia and corticobasal degeneration. The alterations in the AD glycome in the serum could potentially lead to novel glyco-based biomarkers for AD progression [29, 30]. Strikingly, negative interrelationship was found between the pathways of protein O-GlcNAcylation and N-/O-glycosylation, suggesting a novel intracellular cross-talk.

The mechanism leading to this negative correlation between the pathways of protein O-GlcNAcylation and N-/O-glycosylation is unknown. Ngoh *et al*. reported that ER stress up-regulates O-GlcNAc signaling, which in turn results in cardio-protection [31]. They therefore suggested that up-regulation of O-GlcNAcylation might be a protective cellular response aimed at reducing ER stress-induced cell death. Along these lines, we propose that following ER stress and ERAD, reported to occur in AD patients, the UPR machinery up-regulates the O-GlcNAcylation pathway. Another mechanism that could account for the negative correlation between O-GlcNAcylation and N-/O-glycosylation pathways could be the GlcNAc moiety itself, which is common to both pathways. The brain of AD patients exhibits lower uptake of glucose into the cells due to insulin resistance and decreased levels of various glucose transporters. Therefore, the intracellular availability GlcNAc, which is derived from glucose through the hexosamine biosynthesis pathway, is limited in AD patients. The limited availability of GlcNAc which is shared as a substrate by different glycosylation pathways and may result in their competition for this resource.

"*Alterations in acetylcholinesterase glycosylation in Alzheimer's disease*", María-Salud García-Ayllón

Acetylcholinesterase (AChE) is a serin protease that hydrolyses the neurotransmitter acetylcholine at cholinergic synapsis. However, other functions not related with its cholinergic activity as neurogenesis, oxidative stress, and inflammation has been attributed to AChE [32]. This variety of functions could be related with the rich polymorphism of the protein that displays several molecular forms with specific tissue and cellular locations [33]. Moreover, this polymorphism is enriched with different glycosylation patterns. AChE is a glycoprotein with 3 N-glycosylation sites that are conserved in mammals [34]. Glycosylation of AChE is not required to form oligomers, but to result in a correct folding of the enzyme. Abnormal glycosylation leads AChE to remain in the endoplasmic reticulum and hence, reduce its enzymatic activity [35]. Several studies have demonstrated that cholinergic system is the most affected in Alzheimer disease (AD) with a loose of cholinergic innervation and a reduction of the neurotransmitter acetylcholine and the activity of the cholinergic elements AChE and the enzyme choline acetyltransferase, in charge of the acetylcholine synthesis [36]. However, in spite of the decrease on AChE activity, our group demonstrated that AChE protein levels were preserved in AD brain probably as result of an increment of an inactive AChE pool, which could induce vulnerability to the pathological brain [37]. The increase on AChE inactive variants could be related with alterations in its glycosylation. Indeed, it has been described alterations in the glycosylation of several proteins like tau, reelin [38]. Thus, we decided to study the glycosylation of AChE in AD brain by lectin-binding analyses. Previous studies of Prof Sáez-Valero demonstrated that the glycosylation of enzymatically active AChE was altered in the brain and cerebrospinal fluid of AD patients [39, 40]. The fraction not recognized by the lectin Con A (a terminal mannoses specific lectin) was higher in brain and CSF samples of AD patients,

indicating a reduction of the active AChE with mannose terminal residues. The alteration was attributed to an increase in the expression of a small subset of AChE in AD probably as result of the variation in the levels of beta-amyloid [41]. We further study in AD brain samples the glycosylation of enzymatic active AChE and AChE protein measuring enzymatic activity and protein by western blotting respectively after the lectin binding assays. We analyzed the interaction with two lectins that bind to mannose residues, Con A and LCA, being the difference between both lectins in the specify of LCA to mannoses in oligosaccharides with a fucose core. According with Dr. Saez-Valero's results, the amount of AChE activity not recognized by the lectins was higher in AD samples. About AChE protein, most of the protein remains in the unbound fraction for both lectins and in accordance with enzymatic activity interaction, the amount of AChE protein not recognized by the lectins was higher in AD brain samples. In conclusion, in AD brain the amount of AChE with terminal mannoses oligosaccharides is lower than in non-demented brain samples.

"*Glycosylation-dependent mechanisms in Alzheimer´s disease*", Inmaculada Cuchillo Ibáñez

Many proteins related to Alzheimer´s disease (AD) are glycosylated and this includes the major pathological hallmarks of AD: tau and the amyloid beta peptide (Aβ). Hyperphosphorylated tau is the main component of the neurofibrillary tangles, and Aβ forms the senile plaques, being both types of aggregates typically found in the brain of individuals with AD. We present a study of glycosylation of three proteins, i) reelin, a glycoprotein that regulates tau phosphorylation, ii) APP, the protein precursor of Aβ and iii) apoE.

i) Glycosylation of reelin is important for its function and capacity to bind to its receptor ApoER2 and consequently to control tau phosphorylation. We have characterized the glycosylation pattern of reelin in plasma, cerebrospinal fluid (CSF) and frontal cortex using specific lectins. We have found differences between plasma and CSF reelin that indicate a different cellular origin of the reelin protein in these two fluids, but also differences in reelin glycosylation in frontal cortex of Alzheimer respect to that in control individuals. In cultured cortical cells, treatment with an mannosidase inhibitor or with Aβ changed the glycosylation pattern of reelin, and this different glyforms of reelin had less capacity to bind to ApoER2 and to activate its signalling cascade, resulting in an increase of tau phosphorylation [38, 42–44].

ii) Two N-glycosylation sites have been identified in APP, together with eight sites for O-glycosylation and sites for O-GlcNAcylation [45]. We present our studies on the glycosylation of two specific soluble fragments of APP, sAPPα and sAPPβ, in the frontal cortex. These fragments originate from APP cleavage by two different enzymes (α-secretase and β-secretase). We used lectins but also pan-specific antibodies to discriminate fragments from two sources, neurons and glia (KPI variants) [46]. Lectin binding assays identified differences in the glycosylation of sAPPβ species, not sAPPα, derived from neurons and glia. When the lectin-binding pattern was compared between the frontal cortex of individuals with Alzheimer´s disease and controls, significant differences were evident in sAPPα glycosylation. Our analysis of the lectin binding to sAPPα and sAPPβ suggests that glycosylation dictates the proteolytic pathway for APP processing, and this may influence on the generation of Aβ and, consequently, on the pathological progression of AD.

iii) ApoE is the primary cholesterol carrier in the brain. There are three isoforms of apoE in humans, apoE2, apoE3 and apoE4. The allelic variant APOEε4 is the major genetic risk factor for late-onset AD. Glycosylation of apoE affects its protein function, receptor binding and hydrophobicity. We studied the fully-glycosylated apoE (mature form) and the inmature forms of apoE in the CSF of individuals with Alzheimer. We have observed that in individuals with AD and genotype APOEε3/3 and APOEε3/4 the native forms of apoE is more predominant that in control individuals with the same genotype. However, in individuals with Alzheimer's disease and genotype APOEε4/4 the mature form of apoE was predominant respect to the native forms presents in the CSF. Taking in account that APOEε4 is a risk factor for AD, the fact that apoE4 is fully glycosylated and therefore, mature, is an element that could contribute to the pathological progression of the AD.

Overall, the study of the glycosylation of these three crucial proteins in Alzheimer´s disease demonstrates the high impact that alterations in this post-translational modification can exert on the base of the disease, and deserve further studies.

"*Altered glycosylation in cancer: dissecting the molecular mechanism and understanding the clinical implications*", Celso A. Reis

Alterations of glycosylation are common molecular alterations with major biological implications for disease progression [47]. Recent studies applying glycomic and glycoproteomic strategies in cancer have provided novel information in the understanding key molecular roles that glycans play in cancer biology. These studies revelled how glycosylation impacts the activation of oncogenic tyrosine kinase receptors in gastrointestinal cancer cells, such as the expression of terminal sialylated O- and N-glycans on receptors RON, MET, EGFR and HER2 (ErbB2) [48–51] and the aberrant expression of truncated O-glycans (STn antigen) affecting key players in the biology of the tumour such as CD4 [48]. These glycosylation changes are capable of inducing major phenotypic aggressive features of the cancer cell [52]. Finally, we reported the glycoproteomic

map of the HER2 in gastric cancer cells which disclosed a site-specific glycosylation profile of this oncogenic receptor in gastric cancer cells [50]. We further disclosed how this HER2 glycosylation affects the biology of the receptor and the sensitivity of HER2-dependent gastric cancer cells to the therapeutic humanized monoclonal antibody trastuzumab, currently used in the clinics [50]. These results highlight the functional aspects of glycosylation modifications occurring in cancer and supports their potential application as biomarkers for patient stratification, personalize medicine and for novel and improved therapeutic applications [49, 53].

"*Determination of altered Prostate Specific Antigen glycoforms in prostate cancer: clinical implications*", Esther Llop

Prostate Cancer (PCa) is the most common type of cancer and the second leading cause of cancer death in men in western countries [54]. Nowadays, PCa diagnosis is mainly based on digital rectal examination and prostate specific antigen (PSA) test, which consists of measuring the concentration of this glycoprotein in serum. Multi-parametric magnetic resonance imaging is also becoming a widely used technique for the diagnosis of PCa. In PCa, but also in prostate benign diseases such as benign prostatic hyperplasia (BPH), there is a disruption of the prostatic epithelium that leads to an increase in serum PSA levels. Therefore, PSA is a biomarker that lacks cancer specificity and the search for new diagnostic, predictive and prognostic biomarkers for PCa is needed. Novel non-invasive biomarkers with better capacity to distinguish PCa from BPH and for PCa risk stratification are required to reduce PCa overdiagnosis and overtreatment. It is well- known that aberrant protein glycosylation is one hallmark of cancer and the search of altered PSA glycoforms in serum constitutes a promising approach for improving diagnosis of clinically significant (aggressive) PCa [55]. We have developed a Sambucus nigra agglutinin (SNA) lectin affinity chromatography to separate the PSA glycoforms containing α2,6-linked sialic acid (α2,6-SA PSA) from PSA glycoforms containing α2,3-linked sialic acid (α2,3-SA PSA) [56]. PSA glycosylation from a 79 serum samples (29 BPH and 50 PCa with different risk) were analysed using this methodology, which requires 0.75 mL of serum and PSA levels ≥ 2 ng/mL. The results showed a significant increase in the percentage of α2,3-sialylated PSA glycoforms from high risk PCa subjects. Receiver- operating characteristic analysis of the glycobiomarker (α2,3-SA PSA) yielded higher potential to differentiate high-risk PCa from BPH and indolent PCa than PSA and Prostate health index (PHI) in the cohort of this study [AUC 0.97 vs 0.84 with 82% sensitivity] [57]. A high-throughput methodology should therefore be developed in order to confirm these promising results in a larger cohort of patients and then be translated into clinics to detect aggressive PCa and guide urologists in their clinical decision-making.

Methods in Glycoscience

"*Characterization of N-glycan structures of brain glycoproteins by Q Exactive mass spectrometry: A comparison between secreted and lysosomal proteins*", Ole K. Greiner-Tollersrud

All secretory and lysosomal proteins are synthesized and folded in the endoplasmic reticulum and usually modified co-translationally with a common $Man_9GlcNAc_2$-glycan structure that are N-linked to Asn-X-Thr/Ser sequons. Processing in the Golgi apparatus leads to an large array of different N-glycans in mature glycoproteins, that are both developmentally and cell specific. Stress conditions, as cancer, infections etc. can affect the glycan processing, resulting in N-glycans being a valuable biomarker for a number physiological and pathological conditions. The use of glycoanalytical methods with increased specificity and sensitivity has revealed novel N-glycan structures, which deviate from known intermediates of the classical glycan processing pathway *en route* through Golgi. We have developed a method to enrich lysosomal glycoproteins in porcine brain extracts, and compared the N-glycan structures of intact tryptic glycopeptides from known lysosomal and secretory glycoproteins, using Q Exactive mass spectrometry after in gel trypsination of protein bands from SDS/PAGE. Comparing the N-glycan structures from 15 lysosomal and 15 secretory glycoproteins from porcine brain, the lysosomal glycoproteins consistently exhibited a group of apparently truncated N-glycans structures that were absent in secretory glycoproteins. A common feature was that they could be explained by N-glycan trimming in the lysosomes, by known acid exoglycosidases. As they cannot be classified into the common groups of structures, high mannose, complex or hybrid types they constitute a new N-glycan group. This group can be used as a biomarker for the lysosomal localization of a protein, as we have shown for adenosine deaminase 2. They are also a potential biomarker of physiological and pathological conditions.

"*Tryptophan-galactosylamine conjugates inhibit and disaggregate amyloid fibrils of Aβ42 and hIAPP peptides while reducing their toxicity*", Elsa Zacco

Aberrant self-assembly of proteins into amyloid fibrils is hallmark of many diseases, including Alzheimer's disease (AD) and Type-2 diabetes Mellitus (T2DM). Aggregation of specific peptides, such as Aβ42 in AD and hIAPP in T2DM, leads to the formation of insoluble deposits that cause cellular dysfunction and is associated to the pathogenesis of the disease. While amyloidogenic proteins lack a consensus sequence which directly correlates with their tendency to aggregate, such polypeptides always contain aromatic residues in their hydrophobic core. Aromaticity

and hydrophobicity are essential in potentially directing proteins towards their aberrant self-assembly. The use of small molecules inhibitors of protein aggregation directed towards these key aromatic amino acids may represent a promising tool for slowing down or reverting the formation of toxic amyloid species. In this work, we show, both *in vitro* and *in cell*, that small hybrid molecules derived from the residue tryptophan conjugated to different derivatives of galactosylamine can significantly inhibit the formation of Aβ42 and hIAPP amyloid fibrils [58]. The same conjugates also display the ability to disassemble their pre-formed fibrils in a dose-dependent manner. Tested in multiple mammalian cell lines, the tryptophan-galactosylamine hybrids can reduce the cytotoxicity induced by Aβ42 and hIAPP aggregates. We believe that these amino acid-glycan conjugates may serve as a scaffold for the development of therapeutics towards AD and T2DM, diseases showing a significant co-morbidity during the latest stages of life.

"*NMR applied to glycoproteins*", Ana Ardá

Glycosylation is one of the most common protein post-translational modifications in higher animals, entailing significant effects on protein stability and function. Studies on glycoprotein glycan profile analysis and its relationship with biological function are however hampered by the lack of dedicated tools for detailed glycan analysis on intact glycoproteins and studies on the involvement of these glycan moieties in glycoprotein interactions with glycan binding proteins (lectins).

Over the last few decades different efforts have been invested towards the development of new strategies for the use of Nuclear Magnetic Resonance (NMR) as a tool to report on key structural aspects on glycoprotein glycans. Most of these strategies are based on the incorporation of 13C-labels on the glycan moieties, for which different experimental protocols and schemes may be applied [59–62].

In our group we have recently produced different glycoproteins with homogeneous 13C-glycan labelling, allowing an unprecedent detailed glycan profile characterization. We have exploited the obtained NMR glycan signatures to monitor glycoprotein binding to human lectins. The Receptor Binding Domain (RBD) of the viral protein Spike from SARS-CoV-2 [63] and the extracellular domain Spike trimer (unpublished) have been studied following this protocol, as well as the soluble subunit of the human Fce Receptor [64]. Likewise, the glycosylation of the human glycoprotein CD14, which has been recently reported to be a Galectin-4 binding partner [65], has been characterised revealing interesting structural–functional relationships (unpublished). These studies reveal that NMR represents a powerful source of information for studying the role of glycans on glycoproteins, and that in combination with other tools (for instance gene-editing methods) and techniques (MS) might represent a great opportunity to better understand Glycosylation and its impact on specific biological events.

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Declarations

Ethical statement Not applicable.

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