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# Global serum glycoform profiling for the investigation of dystroglycanopathies & Congenital Disorders of Glycosylation

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ABSTRACT

The Congenital Disorders of Glycosylation (CDG) are an expanding group of genetic disorders which encompass a spectrum of glycosylation defects of protein and lipids, including *N*- & *O*-linked defects and among the latter are the muscular dystroglycanopathies (MD). Initial screening of CDG is usually based on the investigation of the glycoproteins transferrin, and/or apolipoprotein CIII. These biomarkers do not always detect complex or subtle defects present in older patients, therefore there is a need to investigate additional glycoproteins in some cases. We describe a sensitive 2D-Differential Gel Electrophoresis (DIGE) method that provides a global analysis of the serum glycoproteome. Patient samples from PMM2-CDG ($n = 5$), CDG-II ($n = 7$), MD and known complex *N*- & *O*-linked glycosylation defects ($n = 3$) were analysed by 2D DIGE. Using this technique we demonstrated characteristic changes in mass and charge in PMM2-CDG and in charge in CDG-II for α1-antitrypsin, α1-antichymotrypsin, α2-HS-glycoprotein, ceruloplasmin, and α1-acid glycoproteins 1&2. Analysis of the samples with known *N*- & *O*-linked defects identified a lower molecular weight glycoform of C1-esterase inhibitor that was not observed in the *N*-linked glycosylation disorders indicating the change is likely due to affected *O*-glycosylation. In addition, we could identify abnormal serum glycoproteins in LARGE and B3GALNT2-deficient muscular dystrophies. The results demonstrate that the glycoform pattern is varied for some CDG patients not all glycoproteins are consistently affected and analysis of more than one protein in complex cases is warranted. 2D DIGE is an ideal method to investigate the global glycoproteome and is a potentially powerful tool and secondary test for aiding the complex diagnosis and sub classification of CDG. The technique has further potential in monitoring patients for future treatment strategies. In an era of shifting emphasis from gel- to mass-spectral based proteomics techniques, we demonstrate that 2D-DIGE remains a powerful method for studying global changes in post-translational modifications of proteins.



## 1. Introduction

Congenital Disorders of Glycosylation (CDG) are a group of rare inborn errors of metabolism and include some forms of dystroglycanopathies. They are an expanding family of predominantly autosomal recessive multi-systemic disorders [1]. Since glycosylation is a complex biological process involving many pathways, any defect may result in defective glycosylation. Hence there are >60 distinct disorders identified to date [2,3]. These are sub-grouped into defects of protein *N*-glycosylation, protein *O*-glycosylation, lipid glycosylation, GDP-anchor glycosylation and multiple glycosylation defects [4]. CDG have a very broad range of clinical symptoms that can involve many organs with a wide spectrum of clinical severity [5] making initial clinical assessment challenging. The most common form of CDG results primarily from defects in the phosphomannomutase 2 gene (termed PMM2-CDG) this disrupts early steps in the glycan assembly and attachment of glycans to proteins resulting in the complete absence of *N*-linked glycans on some

*Abbreviations:* 2D DIGE, 2-dimensional differential gel expression; CDG, Congenital Disorders of Glycosylation; TFN, transferrin; MD, muscular dystrophy; IEF, isoelectric focusing; MW, molecular weight; COG, conserved oligomeric golgi.

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glycosylation sites (macroheterogeneity). CDG type II disorders are a result of defective remodeling of glycans resulting in truncated and abnormal glycan structures (microheterogeneity). Several CDG-II causing mutations are in genes involved in the Conserved Oligomeric Golgi (COG) complex [6].

*O*-glycosylation is an even more complex process [7]. The clinical spectrum within a single disorder and among the different inborn errors of *O*-glycan metabolism is vast and the disorders described to date may represent only a subset [8]. One such class of *O*-linked CDG are the dystroglycanopathies [9]. Alpha dystroglycanopathy is a large glycoprotein of approximately 156 kDa in skeletal muscle and is heavily N and *O*-glycosylated as well as *O*-mannosylated [10]. Many of the described mutations for muscular dystrophy affect the *O*-glycosylation pathways for the biosynthesis of α-dystroglycan and new types of CDG have been described that result in defects in the *O*-mannosyl glycosylation pathway [11–14].

Traditionally the isoelectric focusing (IEF) pattern of serum transferrin is used to detect disorders of protein *N*-glycosylation and is usually the first step in screening for CDG. Transferrin is a 79 kDa serum glycoprotein with two *N*-linked glycans and is highly abundant in serum. A positive transferrin result needs to be followed up by further enzyme and/or genetic testing to identify the specific type of CDG. A similar IEF test to help diagnose *O*-linked disorders is performed on apolipoprotein-CIII which has a single *O*-linked glycan which only captures abnormalities of core-1mucin type *O*-glycosylation [15]. Both of these tests require specialist laboratories to run and interpret the results for a conclusive diagnosis.

While serum transferrin IEF has proven to be a useful test for defective *N*-glycosylation [5], it only identifies differences in charge and lacks information on changes in molecular weight (MW). In addition, secondary glycosylation disorders including galactosaemia, hereditary fructose intolerance, bacterial infections [16] and transferrin polymorphisms must be excluded before considering a diagnosis of CDG. Other methods of CDG investigation include isoelectric focusing of other glycoproteins or investigation of the total glycan profile from glycans removed from the glycoproteins using PNGase F digestion and MALDI analysis [17]. There are a number of suspected CDG cases which do not have altered transferrin IEF profiles or for whom traditional tests provide inconclusive results. Therefore a secondary test to confirm the frontline IEF tests currently used for complex cases is needed. Therefore we have optimised the 2-Dimensional –Difference Gel Electrophoresis (2D-DIGE) method [18] to examine the global serum glycoproteome. 2D-DIGE is an ideal method to investigate changes in post-translational modifications of proteins since multiple samples can be run simultaneously, eliminating gel-to-gel variations and allowing for direct overlaid comparison. This is particularly useful in interpreting subtle or complex CDG type II cases in which the changes in IEF may be subtle. The ability to include confirmed PMM2-CDG and CDG-II samples as internal standards makes interpretation more reliable. Subtle changes in charge and molecular weight can also be observed allowing the detection of a range of defects in post-translational modifications including those of glycosylation that traditional 2D PAGE methods would not detect. To our knowledge this is the first time this technique has been applied to such a large cohort of diverse CDG patient samples. We observed glycoform profile changes for *N*-linked and *O*-linked disorders in looking at various *N*-glycosylated glycoproteins such as α1-antitrypsin, α-1-antichymotrypsin, and α1-acid glycoproteins 1 & 2 and *N*- and *O*-glycosylated α2-HS-glycoprotein and C1-esterase inhibitor.

## 2. Materials and methods

### 2.1. Patient samples

The study was approved by the local NHS Research Ethics committee (London-Bloomsbury) ref: 12/LO/0905. Samples used in this study were anonymized surplus serum remaining from routine diagnostic analysis and were stored and used for the purpose of improving methods for CDG diagnosis. Serum samples were obtained after separation from the cells by centrifugation (3000 *g*/10 min). All samples were stored frozen at −80 °C prior to analysis.

Patient age-range at sampling was between four months and five years. CDG patients included were diagnosed and treated at Great Ormond Street Hospital Metabolic Unit. Muscular dystrophy patients were diagnosed by the Dubowitz Neuromuscular Centre team at Great Ormond Street Hospital and previously, at the Hammersmith Hospital, London.

Table 1 summarises the disease samples used in this study and details the defect(s) where known. Briefly, five genetically confirmed PMM2-CDG and seven CDG patients with a type II transferrin IEF pattern were included, as was serum from three muscular dystrophy patients; one with LARGE mutation; one with a B3GALNT2 mutation and one an unexplained dystroglycanopathy. Three patients with *N*- & *O*-linked glycosylation defects and two patients with inconclusive transferrin or apolipoprotein CIII IEF profiles and with suspected CDG disorders were analysed to test the method developed. Ten age matched anonymized control samples were selected from surplus serum of patients visiting GOSH for other purposes.

### 2.2. 2-Dimensional difference gel electrophoresis (2D DIGE)

For each sample 40 μl of serum was depleted for albumin and IgG using a ProteoPrep Albumin/IgG Depletion Kit (Sigma-Aldrich, Dorset, U.K.). Depleted protein was eluted in 50 mM ammonium bicarbonate buffer pH 7.8 and assayed for protein concentration using the bicinchoninic acid assay kit (Sigma-Aldrich, Dorset, U.K.). Protein was aliquoted into required amounts and freeze dried.

Freeze-dried serum protein (50 μg) was labeled with Cy-Dye DIGE Fluor minimal dye (GE Healthcare) at a concentration of 200 pmol of dye/50 μg of protein. Samples were labeled with Cy3, Cy5, or Cy2. Control protein consisted of pooled sera from five normal subjects, this was used as the internal standard run on all gels and represent the 'normal' glycoform profile to compare patient samples with. All three labeled samples, (pooled control and two patients), were combined and resolved on one gel. Combined samples were added to IPG strip rehydration buffer (7 M Urea, 2 M Thiourea, 2% CHAPS, 50 mM DTE and 1% 4–7 IPG buffer) with pH 4–7 or 3–6.5 immobiline drystrips. IEF strips were left at room temperature for 12 h to rehydrate prior to analysis and run the following day on an IPG multiphor (GE Healthcare). Each IEF strip was focused for between 90 and 100,000 V h. After IEF all strips were flash frozen using liquid nitrogen and stored at −80 °C. IEF strips were re-equilibrated prior to separation by PAGE using a method previously described [19]. PAGE was carried out on 10% and 15% acrylamide gels using piperazine diacrylamide as the cross-linker to improve the resolution of proteins. Gels were scanned in a Typhoon Variable Mode Imager (GE Healthcare) using appropriate lasers and filters with photomultiplier (PMT) voltage between 550 and 600 V. 2D DIGE gel images were analysed using Progenesis Same Spots software version 4.2 (Non-Linear Dynamics, Ltd). All protein identities were confirmed by in-gel digestion of proteins.

### 2.3. In-gel proteolytic digestion and identification of proteins

In-gel digestion of proteins was performed according to published methods [19,20]. All analyses were performed as described previously [21] using a nano-Acquity UPLC and QTOF Premier mass spectrometer (Waters Corporation, Manchester, UK).

Data were analysed using ProteinLynx Global Server (PLGS) version 2.4 (Waters Corporation, Manchester, UK) with a downloaded Uniprot Human Proteome database. Search settings allowed a minimum three ion matches per peptide, seven ion matches and three peptides matches per protein. Modifications taken into account included up to three missed cleavage sites for trypsin with carboxymethylated cysteine as a