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ORIGINAL ARTICLE

# IGF system in children with congenital disorders of glycosylation

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## Summary

**Objective** The function of IGF system components is affected by their glycosylation status *in vitro*. However, little is known about the role of glycosylation status of these components *in vivo*. In this study we determined the impact of glycosylation on the endocrine IGF system in children with the rare syndrome of congenital disorders of glycosylation (CDG).

**Design** Analyses of serum samples from children with CDG and healthy controls.

**Patients** Children with CDG ($N = 12$) were recruited as part of a separate clinical study of mannose therapy at the Mayo Clinic. Serum from control children ($N = 11$) were obtained as routine samples before discard.

**Measurements** Levels and glycosylation state of components of the IGF system and ability to form physiologically relevant ternary complexes composed of IGF, IGFBP-3, and an acid-labile subunit (ALS).

**Results** Serum levels of IGF-1, IGF-2, ALS, and IGFBP-3 were reduced ($P < 0{\cdot}05$) in children with CDG when compared to controls. Immunoblot analysis showed incomplete glycosylation of ALS and IGFBP-3 and impaired ternary complex formation in CDG. Partial normalization of ALS and IGFBP-3 glycosylation was associated with improvement in linear growth in a child with CDG-Ib during initiation of oral mannose therapy.

**Conclusions** Inadequate glycosylation of IGFBP-3 and ALS has a negative effect on the function of these proteins *in vivo*. This study provides the first evidence in humans for the importance of glycosylation on components of the IGF system.

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## Introduction

Protein glycosylation involves the addition of oligosaccharides to proteins during translation. These sugars help to stabilize proteins and are involved in multiple protein–protein interactions. Glycoproteins have been shown to be important components of the hormone cascades regulating growth, metabolism and sexual development.[1,2]

The IGFs are important for growth in children. The IGFs are not glycosylated. Their transport and actions, however, are modulated by IGFBPs, several of which are glycosylated. Of the six known IGFBPs, IGFBP-3 and IGFBP-4 are *N*-glycosylated and IGFBP-5 and IGFBP-6 are *O*-glycosylated. IGFBP-1 and IGFBP-2 are nonglycosylated.[3] In the serum, > 90% of IGF is found in a protein complex of approximately 150 kDa. This complex is comprised primarily of IGF, an acid-labile subunit (ALS) and IGFBP-3, although IGFBP-5 also has been detected in ternary complex.[4] ALS is an 85 kDa glycoprotein with approximately 20 kDa made up of N-linked oligosaccharides. The formation of the ternary complex has been suggested to be important in determining the stability of IGFs in serum[5,6] and their effects on growth.[6,7]

Previous *in vitro* studies have indicated the importance of glycosylation on the stability and function of the components of the IGF ternary complex. The conservation of the seven *N*-glycosylation sites in the ALS gene from mouse to human suggests an important role for *N*-glycosylation in ALS function.[8] Enzymatic deglycosylation of ALS reduces the affinity of ALS for IGFBP-3–IGF complexes by 50–100% depending on the extent of deglycosylation.[9] IGFBP-3 has three sites of *N*-glycosylation. Mutation of each site separately and all three together had no significant effect on ternary complex formation *in vitro*.[10] Recombinant IGFBP-3 expressed in *Escherichia coli* (nonglycosylated) and CHO cells (glycosylated) have identical IGF binding activity.[11,12] However, insufficient protein glycosylation has been hypothesized to increase the protease-susceptibility of ALS and IGFBP-3·[10] Indeed, nonglycosylated IGFBP-3 has a shorter half-life than glycosylated IGFBP-3 when administered to rats.[13]

Congenital disorders of glycosylation (CDG, formerly Carbohydrate Deficient Glycoprotein Syndrome, CDGS) are a group of rare clinical disorders due to defects in the enzymes and other proteins responsible for providing the substrates necessary for protein glycosylation and for processing of protein-linked oligosaccharides.[14,15] CDG-Ia is caused by deficiency of phosphomannomutase 2 (PMM2 deficiency) required for conversion of mannose-1-phosphate to mannose-6-phosphate.[16,17] CDG-Ib is caused by deficiency of the phosphomannose isomerase (MPI deficiency) required for conversion of fructose-6-phosphate to mannose-6-phosphate.[16,18] The partial deficiency of these enzymes leads to incomplete N-linked glycosylation of numerous proteins in this syndrome. Analysis of transferrin glycosylation by immunoisoelectric focusing and more recently by tandem mass spectroscopy has been used as a diagnostic test for CDG.[19,20] Postnatal growth failure is common in children with CDG despite normal or elevated levels of GH.[1,21] In a prospective study of growth in children with CDG-Ia, Kjaergaard *et al.*[22] found a progressive decline in length/height from a mean of 0 SDS at birth to a mean of –1·8 SDS at 2 years of age. Height continued to show a downward trend after the age of 2 years. The abnormal glycosylation and clinical symptoms in CDG-Ib can be treated with oral mannose.[23–25]

In this study, we used CDG as an *in vivo* model of defective protein glycosylation to investigate the impact of glycosylation on stability and function of the components of the IGF system. We analysed the levels of the serum components of the IGF ternary complex, and determined the degree of glycosylation of these components and their ability to form ternary complexes. Finally, we investigated the impact of mannose therapy on the glycosylation of ALS and IGFBP-3 in a child with CDG-Ib during a period of remarkable catch-up growth. These data provide evidence for the importance of glycosylation on the stability and function of the components of pathways, such as the IGF system, involved in growth.

## Subjects and methods

Children diagnosed with CDG by transferrin isoelectric focusing and subsequent enzymatic analyses were recruited as part of an Institutional Review Board (IRB)-approved study of mannose therapy. Separate IRB approval was granted to use stored serum samples, which were consented by the patients for future studies, for biochemical analyses. Normal control sera in the age-range of the CDG children were obtained at the central processing laboratory just prior to discard. Health status was confirmed by chart review. Mean ± SD age for controls was 8·3 ± 4·8 years (range 22 months–18 years). Mean age ± SD for CDG was 8·1 ± 6·6 years (range 21 months–26 years).

### *Determination of serum levels*

Serum levels of total IGF-1, total IGF-2, free IGF-1 and IGFBP-3 were determined using immunoradiometric assay (IRMA) kits as directed by the manufacturer [Diagnostic Systems Laboratories, Inc (DSL), Webster, TX]. Serum levels of total ALS were determined using an enzyme-linked immunosorbent assay (ELISA) kit as directed by the manufacturer (DSL).

### *Immunoblotting*

Serum (2 μl) was diluted in four times Sample Buffer and resolved on 5–15% gradient gels via sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions. Proteins were transferred to nitrocellulose or polyvinyldifluoride (PVDF) membranes by semidry technique and blocked in Tris Buffered Saline with 0·2% Tween (TBST) and cow's milk (5%). The membranes were incubated with polyclonal rabbit antihuman IGFBP-3 (kindly provided by Dr M. Binoux, Paris, France) 1 : 1000 or goat antihuman ALS (DSL) 1 : 1000 overnight at 4 °C followed by goat antirabbit or rabbit antigoat Immunoglobulin G horseradish peroxidase (HRP) conjugate for 60 min at room temperature. Subsequent TBS washes were followed by development with Enhanced Chemiluminescence products (Amersham, Arlington Heights, IL).

### *2-D electrophoresis*

The isoelectric points of glycosylated ALS (pI = 4·5) and enzymatically desialylated ALS (pI = 7·0) have been previously described.[9] Serum (1 μl) was equilibrated overnight with pH 4–7 focusing strips (Bio-Rad, Hercules, CA) under reducing conditions. 2-D electrophoresis was performed using a ramping current over 16 h. Strips were then applied to a 7·5–15% SDS-PAGE gradient gel for molecular weight resolution. Gels were then transferred and immunoblotted as described above.

### *Western ligand blotting*

Serum (2 μl) was diluted in four times Sample Buffer and resolved by SDS-PAGE under nonreducing conditions. Proteins were transferred to PVDF membranes by semidry technique. The membrane was washed in 3% NP-40/TBS and blocked in TBS/0·5% BSA. The membranes were incubated with $^{125}$I-IGF-1 ($1 \times 10^6$ cpm) overnight at 4 °C. Subsequent TBST/TBS washes were followed by air drying of the membrane. The membranes were then exposed to film using a Dupont enhancing screen at –70 °C.

### *IGFBP-3 proteolysis*

Proteolytic fragments of IGFBP-3 were quantified in serum using previously published ELISAs.[26] The assay utilizes a common monoclonal capture antibody combined with a polyclonal or two different monoclonal detection antibodies for detecting total, fragment and intact IGFBP-3 immunoreactivity. The reaction is quantified using a colourimetric assay involving HRP antibody conjugates.

### *Size exclusion chromatography*

Serum (200 μl) was resolved by fast performance liquid chromatography (FPLC) using a Superose 12 column with 50 mM Tris–HCl (pH 7·4) plus 150 mM NaCl at a flow rate of 0·5 ml/min The column was calibrated using molecular weight standards: blue dextran, transferrin, bovine serum albumin, carbonic anhydrase, and cytochrome c (Sigma, St. Louis, MO).

**Table 1.** Components of the IGF System

| | Control ($n = 11$) | CDG-Ia ($n = 12$) |
|---|---|---|
| ALS total (mg/l) | 19·63 ± 1·65 | 9·74 ± 1·09* |
| IGFBP-3 (μg/l) | 3563 ± 306 | 2069 ± 192* |
| IGF-2 total (μg/l) | 677 ± 63 | 404 ± 35* |
| IGF-1 total | | |
| Median (range) (μg/l) | 195 (25–586) | 70 (17–311)* |
| IGF-1 Free | | |
| Median (range) (μg/l) | 2·34 (0·75–6·65) | 1·03 (0·51–3·97) |

Measurement of the serum components of the IGF system in CDG-Ia patients compared to healthy controls. Total and Free IGF-*I*-values were expressed as median and range due to lack of a normal distribution.
*$P < 0·05$.

### *Statistical analysis*

Results are presented as mean ± SEM unless otherwise stated. Analyses using *t*-test and ANOVA were performed using JMP® software (SAS Institute, Cary, NC).

## Results

Analysis of the serum from CDG-Ia patients and controls showed a reduction in each of the major components of the IGF system (Table 1). Total ALS was 50% lower in children with CDG than controls ($P < 0·05$). The serum levels of IGFBP-3 were similarly reduced ($P < 0·05$). The amount of total IGF-1 and IGF-2 was 40–60% lower in children with CDG than the controls ($P < 0·05$). Therefore, the components of the ternary complex were significantly reduced in children with CDG. The free IGF-1 was reduced by 66%, but this was not statistically significant ($P = 0·09$).

In order to investigate the mechanism by which the components of the ternary complex were reduced in children with CDG, we determined the glycosylation status of these proteins. ALS glycosylation was evaluated by immunoblot analysis. In children with CDG-Ia, incomplete ALS glycosylation was demonstrated by the presence of multiple lower molecular weight bands in children with CDG compared to approximately 85 kDa ALS seen in normal controls (Fig. 1a). These are reminescent of partial enzymatic deglycosylation of ALS *in vitro*.[9] Differential glycosylation of ALS was also demonstrated using 2-D gel electrophoresis (Fig. 1b). This method emphasizes the reduction in charge and molecular weight of ALS in the setting of incomplete glycosylation in sera from patients with CDG-Ia.

IGFBP-3 glycosylation was evaluated by immunoblot and ligand blot analyses. In normal controls, glycosylated IGFBP-3 migrates as a doublet at 38 and 46 kDa. In Fig. 2, there is a relative reduction in the intensity of the 38 and 46 kDa bands in patients with CDG-Ia by both immunoblot (Fig. 2a) and ligand blot (Fig. 2b). In both analyses, there is a 30 kDa band which may represent unglycosylated IGFBP-3, a proteolytic fragment of IGFBP-3 or a combination of the two. This band is increased in CDG-Ia in the ligand blot. The 30 kDa proteolytic fragment of IGFBP-3 has been shown previously to bind IGF, but with low affinity; it is not normally detected by ligand blot with radiolabelled IGF-1. Based upon previous *in vitro* data,[11,12] the unglycosylated form of IGFBP-3 would be expected to bind IGF normally.

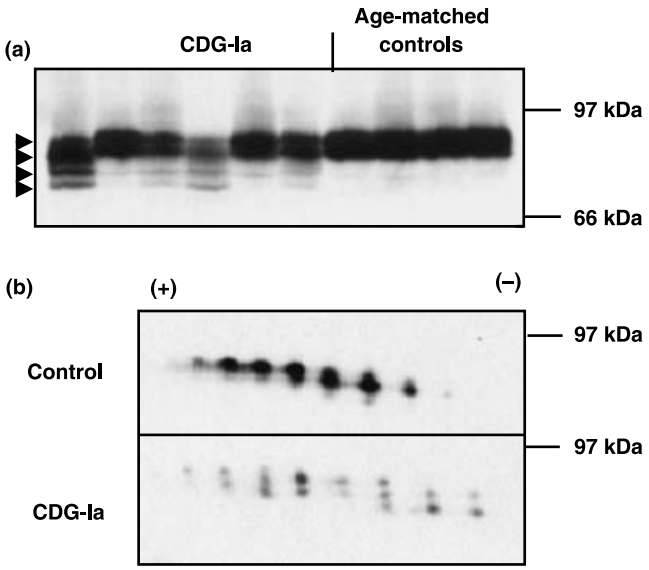



**Fig. 1** ALS immunoblot. (a) ALS detected in immunoblot of 2 μl of serum from CDG-Ia patients and controls. Incomplete ALS glycosylation is indicated by arrowheads in CDG-Ia. (b): ALS detected in immunoblot of 1 μl of serum from CDG-Ia patient and age-matched control separated by 2-D gel electrophoresis. Positions of molecular weight markers are indicated at the right.

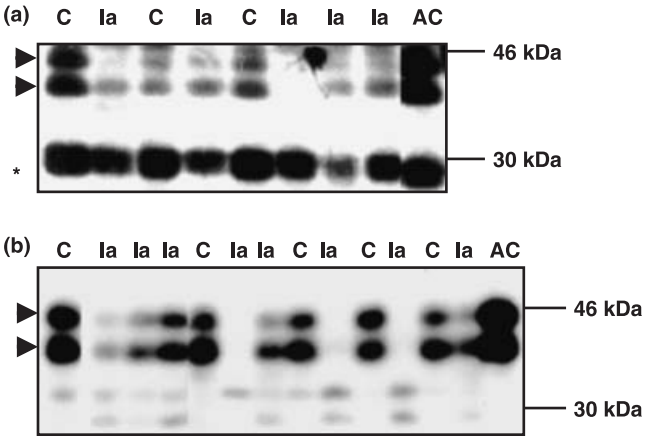



**Fig. 2** IGFBP-3 immunoblot and ligand blot. IGFPB-3 detected by (a) immunoblot and (b) ligand blot of 2 μl of serum from CDG-Ia patients and controls. AC, adult control; C, age-matched control; Ia, CDG-Ia. Positions of molecular weight markers are indicated at the right.

In order to assess the role of proteolysis on serum IGFBP-3 levels, we used previously reported ELISAs that can discriminate between intact and proteolysed IGFBP-3 (Table 2). Total IGFBP-3 was reduced by 43% and intact IGFBP-3 was reduced by 53% ($P < 0·05$). The ratios of fragmented to intact IGFBP-3 (50%) and of fragmented to total IGFBP-3 (39%) were both significantly increased. These data

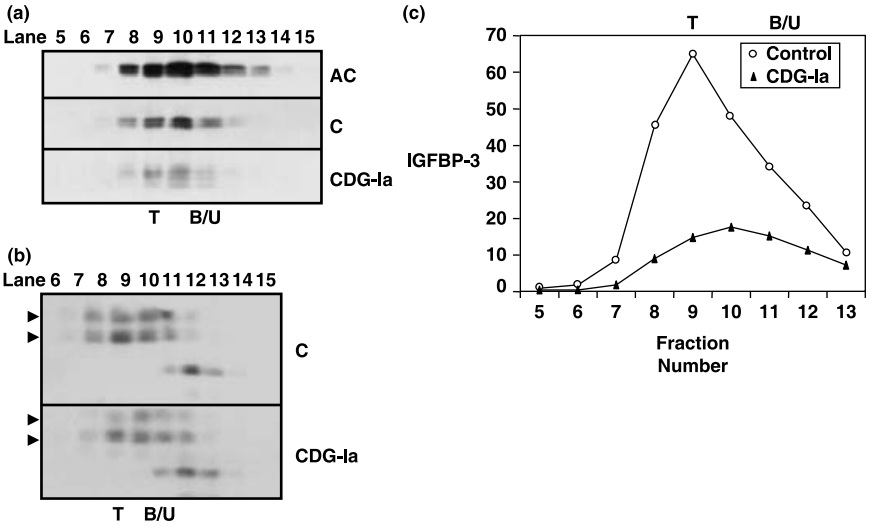



**Fig. 3** Ternary complex formation. Ternary complex formation detected in fractions of serum (250 µl) separated by size exclusion chromatography by (a) ALS immunoblot (b) ligand blot of IGFBPs and (c) immunoradiometric assay for IGFBP-3. Expected elution peaks of ternary complex (T) and binary complex/unbound IGFBP-3 (B/U) based upon molecular weight standards are indicated. AC: Adult Control, C: Age-matched control.

**Table 2.** IGFBP-3 proteolysis

| IGFBP-3 | Control ($n = 8$) | CDG-Ia ($n = 10$) |
|---|---|---|
| Total (mg/l) | 2·15 ± 0·18 | 1·22 ± 0·13* |
| Intact (mg/l) | 1·27 ± 0·17 | 0·60 ± 0·09* |
| Fragment (mg/l) | 1·59 ± 0·11 | 1·28 ± 0·16 |
| Fragment/intact | 1·65 ± 0·34 | 2·48 ± 0·30* |
| Fragment/total | 0·79 ± 0·07 | 1·10 ± 0·12* |

Measurement of total and intact IGFBP-3 in sera from CDG-Ia patients compared to healthy controls. *$P < 0·05$.

suggest that proteolysis may contribute to the reduction of serum IGFBP-3 levels in children with CDG-Ia.

Previous *in vitro* data show that enzymatic deglycosylation of ALS can reduce its affinity for the IGF-IGFBP-3 binary complex leading to reduced ternary complex formation.[9] To investigate ternary complex formation in CDG, size exclusion chromatography was performed. The elution fractions were examined for the presence of ALS by immunoblot (Fig. 3a) and of IGFBP-3 by ligand blot (Fig. 3b). The column elution fractions containing both ALS and IGFBP-3 represent formation of the ternary complex. Although ALS shows a similar elution profile in each of the samples (Fig. 3a), elution of IGFBP-3 is delayed in serum from CDG-Ia children. This delay in elution of IGFBP-3 likely represents a dissociation of ALS and IGFBP-3 with the total amounts of the ternary complex formed in CDG-Ia reduced compared to controls. This could also represent the presence of ternary complexes composed of incompletely glycosylated ALS and IGFBP-3. Similar results were obtained from immunoradiometric assay of elution fractions for IGFBP-3 (Fig. 3c).

A child with CDG-Ib with adequate nutrition showed a remarkable period of catch-up growth following initiation of oral mannose therapy (Fig. 4a,b). Prior to mannose therapy, the serum values of ALS (8·21 mg/l), IGFBP-3 (1443 µg/l) and total IGF (73 µg/l) were reduced similar to those seen in children with CDG-Ia (Table 1). In this child with CDG-Ib, ALS glycosylation detected by immunoblot shows relative improvement after 1 month of mannose therapy (Fig. 4c, Lanes 1 and 2). IGFBP-3 glycosylation detected by immunoblot showed similar improvement following 1 month of mannose therapy (Fig. 4d, Lanes 1 and 2). Subsequent analyses of the impact of mannose therapy on protein glycosylation and ternary complex formation were not possible due to lack of available samples.

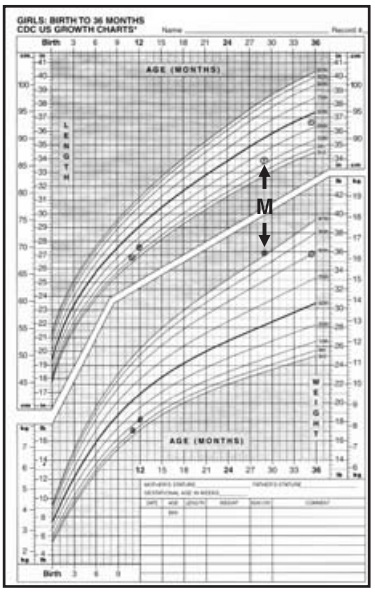
## Discussion

Protein glycosylation is important for protein stability at the intracellular and extracellular levels. N-linked oligosaccharides are important in protein–protein interactions involved in receptor–ligand and cell to cell signalling. As a result, deficiencies in protein glycosylation can impact multiple organ systems. Previous *in vitro* studies have shown the importance of glycosylation on the stability and function of the components of the IGF ternary complex.[8–10,13] However, *in vivo* studies were not available.

In this study, we analysed the components of the IGF system in CDG, a human condition associated with abnormal protein glycosylation and progressive growth failure. We show that levels of the glycosylated components of the serum IGF ternary complex, ALS and IGFBP-3, as well as the nonglycosylated ligand IGF-1, are all reduced in children with CDG-Ia. Each of these components is GH-dependent, and children with CDG-Ia have previously been shown to have normal or elevated GH secretion.[1] Thus, GH deficiency is not the primary driver of the reduced IGF-1, IGFBP-3, and ALS levels in these children with CDG.

The reduction of ALS and IGFBP-3 in the serum of children with CDG-Ia is accompanied by a reduction in the degree of glycosylation of these proteins (Figs 1, 2 and 4). Proper glycosylation is important in protein–protein interactions. Using immunoblot analysis after 2-D electrophoresis, we have shown that a reduction in ALS glycosylation is accompanied by a neutral shift in the isoelectric point of ALS (Fig. 1b). This change in ALS charge has been previously described following enzymatic desialylation of human ALS and has been correlated with a reduction in the affinity of ALS for the IGFBP-3-IGF binary complex.[9] Thus, glycosylation may regulate both the binary complex binding function and availability

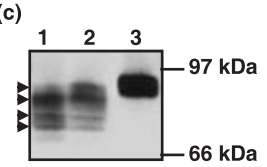
(a)




(b)

| Age | Height | % | Weight | % | GV | % |
|---|---|---|---|---|---|---|
| 2y5m | 86·6 | 22 | 16·3 | 22 | 7·6 | 22 |
| 2y11m | 93·0 | 100 | 16·2 | 100 | 12·6 | 100 |

(c)




(d)

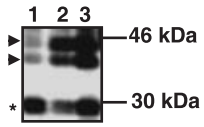



**Fig. 4** Patient with CDG-Ib before and after mannose treatment. (a) Growth chart, M: mannose treatment (b) Height (in cm), weight (in kg) and growth velocity (GV) (c) ALS immunoblot (d) IGFBP-3 immunoblot. For (c) and (d), 2 μl serum from a child with CDG-Ib before and after 1 month of mannose therapy resolved by nonreducing SDS-PAGE. lane 1: before mannose, lane 2: after 1 month of mannose therapy, lane 3: adult control.

of ALS in the serum. One might speculate that a change in the charge state of ALS might also affect its interaction with proteases making ALS more susceptible to degradation, although proteolytic fragments were not detected by immunoblot. Incomplete glycosylation of IGFBP-3 has been shown to increase the risk of proteolysis *in vitro*[10] and to lead to increased clearance *in vivo*.[13] Using assays specific for intact and fragmented IGFBP-3, our data suggest that IGFBP-3 proteolysis plays a role in the reduction of the levels of this protein in CDG-Ia.

The dissociation of IGFBP-3 from ALS in size-exclusion chromatography fractions from CDG-Ia (Fig. 3a,b) suggests that incomplete glycosylation leads to a reduction in ternary complex formation. Previous data suggested that *in vitro* ALS affinity for the binary complex of IGFBP-3 and IGF-1 is reduced by enzymatic deglycosylation.[9] This study provides human *in vivo* data to support this finding.

In CDG-Ib, a condition treatable with oral mannose, we have shown that short-term mannose therapy led to partial correction of the glycosylation defect in ALS and IGFBP-3 (Fig. 4). Long-term studies of the impact of mannose therapy on the glycosylation status and function of the components of the ternary complex were not possible in this retrospective study due to sample availability. The increase in glycosylation correlated with the beginning of a period of accelerated linear growth in this child. We hypothesize that the increase in glycosylation increases the levels of the components of the ternary complex and their ability to bind one another. Thus, the ternary complex would be stabilized, the half-life of IGF prolonged, and perhaps delivery of IGF to the target tissues would be facilitated. The fact that the nutritionally sufficient child with CDG-Ib had improved growth with improved protein glycosylation suggests that poor nutrition may play a lesser role than incomplete glycosylation in the growth failure in children with CDG.

This study in a rare syndrome of CDG represents the first *in vivo* human study of the impact of protein glycosylation on IGF ternary complex formation. Although the sample size is small and quantities limiting, the data comparing CDG children with healthy controls in the same age range support the hypothesis that impaired glycosylation leads to reduction in the levels of the components of the IGF ternary complex. We have provided data showing that incomplete glycosylation of IGFBP-3 is associated with an increase in proteolysis. In addition, incomplete ALS glycosylation appears to impair ternary complex formation *in vitro* and *in vivo*. The combination of these mechanisms would lead to a reduction in the half-life of the circulating IGFs.

## Acknowledgement

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