Congenital disorders of glycosylation type Ia and Ib

Genetic, biochemical and clinical studies

Susanne Kjærgaard

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H-S Rigshospitalet, Department of Clinical Genetics, Copenhagen.

Correspondence: Susanne Kjærgaard, John F. Kennedy Institutet, Gl. Landevej 7, 2600 Glostrup, Denmark.

Official opponents: Professor, PhD Niels A. Jensen and Associate Professor, Doctor of Dental Surgery Henrik Clausen.


1. INTRODUCTION

Congenital disorders of glycosylation (CDG), formerly known as carbohydrate-deficient glycoprotein syndromes, are a rapidly expanding group of inherited multisystem disorders resulting from defects in N-linked carbohydrate side-chains (glycans) of proteins. The molecular basis of several types of CDG has been elucidated during the past few years, and 12 types have been recognized by February 2003. CDG type Ia (CDG-Ia, OMIM #212065), by far the most common, is the focus of this thesis, and less emphasis is placed on CDG type Ib (CDG-Ib, OMIM #602579). CDG-Ia is caused by mutations in PM M2 and the resulting deficiency of the enzyme phosphomannomutase (PM M). Clinical manifestations include psychomotor retardation, hypotonia, failure to thrive, ataxia, liver dysfunction and dysmorphic features. CDG-Ib is due to mutations in MPI, which lead to deficiency of the enzyme phosphomannomutase isomerase (PM I). Manifestations of CDG-Ib include hepatic fibrosis, protein-losing enteropathy, thrombosis and hypoglycaemia, but no primary neurological impairment.

PM M and PM I both play a role in maintaining the intracellular supply of mannose derivatives required for glycosylation of proteins, and deficiency of one of the enzymes leads to hypoglycosylation.

The aims of the studies were:

1. to characterise Danish CDG-Ia patients biochemically and molecularly
2. to study the effect of PM M2 mutants on phosphomannomutase function in vitro
3. to investigate the origin of the predominant PM M2 mutations R141H and F119L by haplotypic analysis
4. to characterise the clinical phenotype of CDG-Ia due to the predominant R141H/F119L PM M2 genotype
5. to study the effect of mannose therapy in patients with CDG-Ia and CDG-Ib.

2. BACKGROUND

2.1 BIOSYNTHESIS AND FUNCTIONS OF N-GLYCANS

The widespread post-translational glycosylation of proteins affect their properties and functions. Most secreted proteins and membrane proteins (receptors and transporters) as well as some intracellular proteins (e.g., lysosomal enzymes) have glycan side chains, which are added to asparagine (N-glycosylation) or serine/threonine polypeptide residues (O-glycosylation). Involving 30–40 enzymatic and transport steps, N-glycosylation runs through three cellular compartments: the cytosol, the rough endoplasmic reticulum (rER) and the Golgi apparatus (Figure 1). In the first part of this pathway PM M and PM M1 participate in the synthesis of guanosine diphosphomannose (GDP-mannose) from mannose-6-P. The nucleotide sugars donate sugars attached to the dolichyl-phosphophate to form the lipid-linked oligosaccharide (LLO) precursor. Two N-acetylglucosamines and five mannoses are added to the LLO precursor, which is anchored to the cytosolic membrane of the rER. Subsequently, the LLO precursor is flipped to the luminal side of rER, where more sugars are added sequentially using dolichol-P-mannose/glucose as donors. When the branched oligosaccharide moiety contains two N-acetylglucosamines, nine mannoses and three glucoses, it is linked to selected asparagine residues of the nascently translated polypeptide. Next, three glucoses and one mannose are removed sequentially, and the glycoprotein is transferred to the Golgi apparatus for final modification. Six additional mannoses are removed and replaced by two or more each of N-acetylglucosamine and galactose, and the biosynthesis of the oligosaccharide branches are terminated by the addition of sialic acid residues.

Certain glycoproteins are further modified by fucosylation or phosphorylation. Finally, the glycoproteins are secreted into the body fluids, membrane glycoproteins are incorporated in cell membranes, and lysosomal enzymes are taken up by lysosomes.

Glycans participate in the folding of glycoproteins in the rER and modify their stability, action and clearance. Glycans are essential for the function of cell surface receptors, as signals for protein targeting, mediators of cell-to-cell interaction, mediators of protein turnover and as protection of polypeptides from proteases.

2.2. CDG TYPES AND NOMENCLATURE

Several human disorders due to defects in the N-glycosylation pathway have recently been characterised. The disorders fall into two groups based on the localisation of the enzymatic defect (Participants, First International Workshop on CDG 1999).

Group I CDG (CDG-I) refers to defects in the initial steps of N-linked protein glycosylation. These defects affect the assembly of LLO and/or its transfer to asparagine residues on nascent polypeptides. Group II CDG (CDG-II) refers to defects in the processing of protein-bound glycans or the addition of other glycans to the polypeptides. Untyped patients are assigned to CDG-x until their genetic defect is established. Currently known CDG types, the corresponding enzyme deficiencies, and the genes are listed in Table 1.

2.3. ISOELECTRIC FOCUSING OF TRANSFERRIN

A SCREENING TEST FOR CDG

An individual glycoprotein has a heterogeneous but consistent population of N-linked glycans, which can be separated and visualised by isoelectric focusing (IEF) and Western blotting/immunoprecipitation (Stibler et al, 1998, Niehues et al, 1998). Various glycoforms of the protein are each represented by a band in the IEF pattern. Transferrin is only one of many hypoglycosylated proteins in CDG, but its microheterogeneity on IEF has turned out to be a sensitive biochemical marker of most N-glycosylation defects (Stibler et al, 1998).

Normally, the predominant glycoform of transferrin contains four oligosaccharide branches (tetrasialotransferrin) on two N-linked glycans. Hypoglycosylation of proteins results in partial deficiency of the terminal, negatively charged sialic acid, and hence in a cathodal shift of the IEF pattern. In patients with CDG-I the proportions of glycoforms with zero (asialo-) and two (disialotransferrin) oligosaccharide branches are increased with a corresponding decrease in tetrasialotransferrin – a “type I” pattern (Figure 2) (Stibler et al, 1991b).

Dried blood spots and serum can be used for IEF (Stibler and Cederberg 1993). A false-positive IEF pattern may be seen when...
using EDTA-anticoagulated plasma, and abnormal IEF pattern not related to CDG may be seen in serum from patients with untreated galactosemia, untreated hereditary fructose intolerance or alcohol abuse (Jaeken et al, 1996, Stibler 1991a, Stibler et al, 1997). Amino acid polymorphisms of transferrin can alter its IEF pattern (Stibler et al, 1988). IEF of serum transferrin from parents of a CDG-suspected individual will differentiate between a transferrin protein variant and abnormal glycosylation.

Normal IEF pattern has been reported in a few cases with enzymatically and genetically proven CDG-Ia (Fletcher et al, 2000, Dupre et al, 2001). Abnormal glycosylation of transferrin (and other glycoproteins) tends to improve slightly with increasing age in CDG-Ia, which might obscure the diagnosis in a minority of older patients (Dupre et al, 2001, Stibler et al, 1998). It is remarkable, and still unexplained, that abnormal glycosylation of serum transferrin (and other glycoproteins) is not detectable by IEF in CDG-Ia fetuses and preterm newborns until the 36th gestational week (Clayton et al, 1993, Stibler and Skovby 1994a). IEF analysis cannot differentiate types of CDG, and additional biochemical and genetic analyses are needed to reach a specific diagnosis. Quantitative determination of Carbohydrate-Deficient Transferrin (CDT) is an alternative screening method, which measures the amount of di-, mono- and asialo-transferrin (Stibler et al, 1991a). However, elevated values need to be confirmed by IEF.

2.4. PATIENTS
By February 2003, 32 patients from 26 unrelated Danish families had been diagnosed with CDG-Ia, 17 of whom were born in 1990-1998. This corresponds to an incidence of 1:41 000 newborns. We know of two additional, deceased patients with clinical manifestations highly suggestive of CDG-Ia, but biological materials were not available for confirmation. All these patients had a “type I” pattern IEF of serum transferrin (Figure 2) (Kjaergaard et al, 1998a, Westphal et al, 2001b, Grubenmann et al, 2002).

3. CONGENITAL DISORDER OF GLYCOSYLATION TYPE IA
3.1. PHOSPHOMANNOMUTASE DEFICIENCY
Phosphomannomutase deficiency was established as the cause of CDG-Ia in 1995 by the detection of PMM activities ≤ 10% in fibroblasts/leukocytes/liver tissue from patients and of intermediate activities in their parents (Van Schaftingen and Jaeken 1995). PMM is a cytosolic enzyme that catalyses the conversion of mannose-6-P to mannose-1-P (Figure 1). PMM requires mannose-1,6-biphosphate or glucose-1,6-biphosphate as a cofactor, which serves to phosphorylate the catalytic site of PMM. Mannose-1-P is required for the synthesis of GDP-mannose, the donor of mannose to the growing LLO precursor. PMM deficiency results in depletion of the mannose-1-P and GDP-mannose pools in fibroblasts (Körner et al, 1998a, Rush et al, 2000). Hence, the incorporation of mannose into LLO is reduced, and glycoproteins lacking complete N-linked glycans are synthesized (Wada et al, 1992, Yamashita et al, 1993, Powell et al, 1994, Krasnewich et al, 1995). Fibroblasts with PMM deficiency do not accumulate mannose-6-P, which suggests that...
PMI maintains an equilibrium between mannose-6-P and fructose-6-P (Figure 1) (Körner et al, 1998a).

PMM activities in extracts of cultured fibroblasts from Danish CDG patient have been measured by the assay described by Van Schaftingen and Jaeken (1995): the reduction of NADP to NADPH was measured spectrophotometrically following coupled enzymatic reactions catalysed by PMI, phosphoglucoisomerase and glucose-6-P dehydrogenase, and mannose-1-P was used as substrate (Figure 3). PMM activities in cultured fibroblasts ranged from undetectable to 15% of controls, thereby documenting PMM deficiency in all patients (Kjaergaard et al, 1998a).

Grünewald et al, (2001) pointed out a possible pitfall in the diagnosis of PMM deficiency after observing CDG-Ia patients with high residual PMM activity overlapping control values in cultured fibroblasts. This problem was not encountered in assays of fresh leukocytes and liver tissue. The variability of PMM activity among different cell types points to a cell-specific effect, as yet unexplained. One possibility is that mutant PMM or the isozyme (see below) become overexpressed in rapidly dividing fibroblasts with high protein synthesis as opposed to fully differentiated leukocytes with little protein synthesis (Grünewald et al, 2001).

PMM is a homodimer of 30 kDa subunits, whose tertiary structure is not yet known (Kepes and Schekman 1988, Pirard et al, 1997). Two isozymes of PMM with 66% sequence homology have been identified: PMM1 and PMM2. They catalyse the same reaction at approximately equal rates, but PMM2 is more substrate-specific (Pirard et al, 1999a). PMM2 is the defective enzyme in CDG-Ia. The role of PMM1 is still a puzzle, and no disorder has so far been associated with defects of PMM1. Studies in normal rat on the tissue distribution/expression of PMM1 and PMM2 by Western/Northern blot analyses showed that PMM2 is the only isozyme detectable in most tissues except brain and lung, where PMM1 accounts for approximately 66% and 13% of the total activities, respectively (Pirard et al, 1997).

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### Table 1. Currently known CDG types, corresponding enzyme deficiencies, and genes.

<table>
<thead>
<tr>
<th>Type</th>
<th>Deficiency</th>
<th>Gene</th>
<th>Reference</th>
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<tbody>
<tr>
<td>CDG-Ia</td>
<td>Phosphomannomutase</td>
<td>PMM2</td>
<td>Van Schaftingen 1995</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Matthijs 1997</td>
</tr>
<tr>
<td>CDG-Ib</td>
<td>Phosphomannosiosemerase</td>
<td>MPI</td>
<td>Niehues 1998</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Jaeken 1998</td>
</tr>
<tr>
<td>CDG-Ic</td>
<td>Dolichyl-P-Glc:Man, GlcNAc2-PP-dolichyl α-1,3-glucosyltransferase</td>
<td>ALG6</td>
<td>Körner 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Imbach 1999</td>
</tr>
<tr>
<td>CDG-Ig</td>
<td>Dolichyl-P-Man, GlcNAc2-PP-dolichyl α-1,3-mannosyltransferase</td>
<td>ALG3</td>
<td>Körner 1999</td>
</tr>
<tr>
<td>CDG-Ie</td>
<td>Dolichol-P-Man synthase 1</td>
<td>DPM1</td>
<td>Imbach 2000</td>
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<td></td>
<td></td>
<td></td>
<td>Kim 2000</td>
</tr>
<tr>
<td>CDG-If</td>
<td>Dolichol-P-Man utilisation defect</td>
<td>MPDU1</td>
<td>Schenk 2001</td>
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<td></td>
<td></td>
<td></td>
<td>Kranz 2001</td>
</tr>
<tr>
<td>CDG-Ig</td>
<td>Dolichyl-P-Man:Man, GlcNAc2-PP-dolichyl α-1,6-mannosyltransferase</td>
<td>ALG12</td>
<td>Chantret 2002a</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Grubenmann 2002</td>
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<tr>
<td>CDG-Ih</td>
<td>Dolichyl-P-Glc:Man, GlcNAc2-PP-dolichyl α-3-glucosyltransferase</td>
<td>ALG8</td>
<td>Chantret 2003</td>
</tr>
<tr>
<td>CDG-IIa</td>
<td>UDP-GlcNAc:α-6-D-mannoside β-1,2-N-acetylgulcosaminyltransferase II (GlcNAcT II)</td>
<td>MGAT2</td>
<td>Jaeken 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tan 1996</td>
</tr>
<tr>
<td>CDG-IIb</td>
<td>α-1,2-glucosidase I</td>
<td>GCS1</td>
<td>De Praeter 2000</td>
</tr>
<tr>
<td>CDG-IIc</td>
<td>GDP-fucose transporter</td>
<td>FUCT1</td>
<td>Lübbe 2001</td>
</tr>
<tr>
<td>CDG-IIe</td>
<td>N-acetylgalcosamine β-1,4-galactosyltransferase I</td>
<td>B4GALT1</td>
<td>Hansske 2002</td>
</tr>
<tr>
<td>CDG-x</td>
<td>Genetic basis unknown</td>
<td></td>
<td>电影节</td>
</tr>
</tbody>
</table>

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**Figure 2.** IEF pattern of serum transferrin from a normal control, CDG-Ia, CDG-Ib- and CDG-Ig patient. The number of sialic acid residues (negative charges) is given at the right. The anode and cathode are given at the left. The duplicate bands in the CDG-Ig lane are probably due to a genetic variant of transferrin at the polypeptide level.
et al, 1999a). In contrast, Northern blot analysis of normal human tissues showed widespread expression of both PMM1 and PMM2 (Matthijs et al, 1997a and b). The highest expression of PMM2 was seen in pancreas and liver, organs with a predominant role in glyco-protein synthesis. PMM2 was weakly expressed in brain, unlike PMM1. How these observations correlate with the severe CNS involvement of CDG-Ia is not known. However, low PMM activity measured in liver, fibroblasts and leukocytes of CDG-Ia patients indicates that PMM1 contributes little to the total PMM activity in these cell types, and that it does not rescue PMM2 deficiency.

3.2. THE PMM2 GENE

CDG-Ia is inherited as an autosomal recessive trait, and a locus has been assigned to chromosome 16p13 by linkage analysis (Jaeken et al, 1994). Two genes both encoding a PMM (PMM1 and PMM2) indicates a strong conservation from yeast to man, reflecting a fundamental role of PMM (Matthijs et al, 1997b).

A processed pseudogene, PMM2ψ, located on chromosome 18p, has also been identified. Caution is therefore required when designing primers for mutation analysis, as identical mutations have been reported in PMM2 and PMM2ψ (Schollen et al, 1998).

3.3. PMM2 MUTATIONS

Molecular analysis has revealed a large number of mutations in CDG-Ia patients worldwide. Seventy-one PMM2 mutations have been reported in patients from 24 countries, including Denmark (Figure 4) (Kjaergaard et al, 1998a and 1999, Kondo et al, 1999, Matthijs et al, 2000, Tróndrud et al, 2000, Imtiaz et al, 2000, De Lonlay et al, 2001). These observations correlate with the severe CNS involvement of CDG-Ia patients harboured the F119L mutation, and 83% of the patients were compound heterozygous for these mutations (Kjaergaard et al, 1998a). Surprisingly, we did not observe homozygosity for either of the two predominant mutations. The absence of homozygotes expected on the basis of Hardy-Weinberg equilibrium was statistically highly significant. Subsequently, we and others have detected homozygosity for F119L, but homozygosity for R141H has been conspicuously absent in results of genotype analysis (Matthijs et al, 1998a and 2000, Kjaergaard et al, 1998a and 1999, Bjursell et al, 2000, Imtiaz et al, 2000, De Lonlay et al, 2001). These observations...

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**Figure 3.** Enzymatic reactions involved in the assays for determination of PMM activity in fibroblasts and of serum mannanose.

**Figure 4.** Position of the reported PMM2 mutations. Mutations underlined have been identified in Danish patients with CDG-Ia.
suggest that homozygosity for R141H is not compatible with life or, less likely, leads to another clinical phenotype.

3.4. RELATIONSHIP BETWEEN PMM2 GENOTYPE AND RESIDUAL PMM ACTIVITY

The extensive allelic heterogeneity worldwide and the somewhat variable residual PMM activity in different cell types from the same patient complicate the study of the correlation between genotype and residual PMM activity. Furthermore, PMM activity is low in extracts of control fibroblasts, and differentiation of PMM activities below 0.5 nmol/mg/min is not reliable, which make it difficult to compare residual activities of various mutant fibroblast cell lines. We and others observed profoundly decreased PMM activity in fibroblasts from all patients (Kjaergaard et al., 1998a, Imtiaz et al., 2000). In general, there was no clear correlation between genotype and residual PMM activity except for the R141H/F119L genotype, which invariably was associated with very low PMM activity (Kjaergaard et al., 1998a, Grünewald et al., 2001). Certain genotypes (R141H/C241S, F157S/C241S, R141H/T237M) were associated with a relatively high residual PMM activity in fibroblasts (30-70% of controls) (Grünewald et al., 2001).

3.5. IN VITRO EXPRESSION STUDIES

To investigate the impact of PMM2 mutations on the catalytic activity of the enzyme, wild type and mutant PMM2 were expressed in E. coli (Kjaergaard et al., 1999, Pirard et al., 1999b). We cloned PMM2 cDNA into a PET3a expression vector, and the six different mutations identified at the time (R141H, F119L, C192G, G117R, T237R and D223E) were each introduced into PMM2 cDNA by site-directed mutagenesis. Specific activities of recombinant PMM2 suggested the presence of two types of mutations. One type resulted in virtually null activity, and the other type resulted in PMM2 with decreased activity and/or altered properties such as affinity for the substrate and stability. The R141H protein had no residual PMM2 activity, and the F119L protein had approximately 25% of normal activity, higher K_m for mannose-1-P and less thermostability (Kjaergaard et al., 1999). The lack of correlation may also be explained by difficulties in comparing activities due to the relatively low sensitivity of the PMM assay and the low PMM activity in control fibroblasts.

So far, no CDG-Ia patient has been found to have two PMM2 mutations conveying virtually no PMM2 activity when expressed in vitro (Kjaergaard et al., 1999, Pirard et al., 1999b, Vuillaume-Parrot et al., 1999 and 2000, Westphal et al., 2001a). Even though one should be cautious applying the results of in vitro studies in E. coli to the in vivo situation in man, the expression studies support the hypothesis that homozygosity for R141H (or another "severe" mutation) impairs PMM2 activity to a degree incompatible with life, whilst F119L (or another "mild" mutation) conveys a phenotype compatible with survival due to residual PMM2 activity. The fate of zygotes homozygous for R141H is unknown. The absence of patients harbouring two null alleles points to an essential role of PMM2 in normal cellular functions, and suggests that such zygotes are lost in early pregnancy. An absolute requirement for residual activity is compatible with the glycosylation, however deficient, of circulating proteins in CDG-Ia patients and with the apparent lack of other biopathways to synthesize mannose-1-P and GDP-mannose.

Within one of the three extremely conserved protein sequences (motifs) delineated in PMM2, the phosphorylation site (see p. 7) has been assigned to an aspartate residue at position 12 (Aravind et al., 1998, Collet et al., 1998). The motifs may be involved in the catalytic site of PMM2, but none of the mutations identified in Danish patients affects the amino acids within these motifs. Indeed, mutations distant to the presumed catalytic site may influence conformation and thereby activity of the enzyme. More knowledge of the structure of PMM2 is needed to predict the impact of a mutation on the enzymatic function of PMM2.

3.6. GENETIC EPIDEMIOLOGY

CDG-Ia occurs worldwide. The reported incidence is highest in Denmark (1:41 000 newborns) followed by Sweden (1:80 000), the Netherlands and Belgium (Mathijs et al., 2000, Kjaergaard et al., 2001). R141H, by far the most frequent PMM2 mutation, is present in 73% of Caucasian patients (Mathijs et al., 2000). R141H and F119L make up the vast majority of mutations of Scandinavian patients (Kjaergaard et al., 1998a, Bjursell et al., 2000). Genotypes are much more heterogeneous in other Western European countries (Mathijs et al., 2000). The predominance of two mutations among Danish patients reflects the genetic homogeneity of the Danish population also seen in other diseases, e.g., cystic fibrosis (Schwartz et al., 1993) and galactosmia (M. Schwartz, unpublished data).

Prior to cloning of the PMM2 gene one specific haplotype was shown to be markedly overrepresented among patients from southwestern Sweden, Southern Norway and Eastern Denmark, indicating one shared ancestral mutation (Bjursell et al., 1997). In order to investigate the origin of the two predominant mutations, haplotype analysis of the Danish CDG-Ia families was performed using markers defining the Scandinavian founder haplotype and one additional closely linked marker (D16S 3020, located within 20kb of PMM2) (Kjaergaard et al., 1999b). R141H and F119L were each associated with a specific haplotype. Our suggestion that the Scandinavian founder mutation was F119L was subsequently confirmed (Kjaergaard et al., 1998b, Bjursell et al., 1998). Haplotype analysis of CDG-Ia patients with the R141H mutation from other Western European countries also revealed a clear linkage disequilibrium for the same haplotype as seen in the Danish patients (Schollen et al., 2000a). These haplotypes indicated that R141H and F119L originated from two ancestral mutations. R141H showed more recombinations with some of the more distant markers than F119L, suggesting that R141H is older than F119L. The frequency of F119L was highest among Scandinavian patients, and it gradually decreased when moving southward in Europe (Mathijs et al., 2000). The F119L mutation may well have arisen in a Scandinavian ancestor. R141H was

### Table 2. PMM2 genotypes identified in 26 unrelated Danish CDG-Ia patients.

<table>
<thead>
<tr>
<th>PMM2 genotype</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>R141H/F119L</td>
<td>20</td>
</tr>
<tr>
<td>R141H/C192G</td>
<td>1</td>
</tr>
<tr>
<td>R141H/V231L</td>
<td>1</td>
</tr>
<tr>
<td>F119L/F119L</td>
<td>1</td>
</tr>
<tr>
<td>F119L/G117R</td>
<td>1</td>
</tr>
<tr>
<td>F119L/V231L</td>
<td>1</td>
</tr>
<tr>
<td>T237R/D223E</td>
<td>1</td>
</tr>
</tbody>
</table>

The recombinant proteins corresponding to the mutations underlined have no detectable PMM activity.
frequent among patients from all Western European countries and the US compatible with an ancient origin (Mathijs et al, 2000).

The lack of patients homozygous for R141H was further explored by determining the frequency of R141H in two normal populations. The carrier frequency in Dutch neonates and in Danish blood donors was 1.79 and 1.66, respectively (Schollen et al, 2000a). These frequencies were not significantly different, and pooled data gave a frequency of 1.72. Under Hardy-Weinberg equilibrium an incidence of R141H homozygotes of approximately 1:20 000 would be expected. These observations further support that R141H in the homozygous state is not compatible with life.

Both populations were also analysed for the second most frequent mutation, F119L. No F119L carriers were identified, indicating a much lower carrier frequency of this mutation than of R141H (Schollen et al, 2000a).

3.7. PRENATAL DIAGNOSIS
Prior to the identification of PMM2 as the gene involved in CDG-Ia, prenatal diagnosis was based on linkage analysis and measurement of PMM activity in amniotic and chorionic villus cells (Bjursell et al, 1998, Charlwood et al, 1998, Mathijs et al, 1998b). Problems with the risk of error due to recombination and with interpretation of enzyme activity were avoided when mutation analysis became available.

Prenatal diagnosis by mutation analysis of chorionic villus cells is now routinely performed and widely requested by affected families in Denmark. So far, we have performed 18 prenatal analyses: seven fetuses were compound heterozygotes, and these pregnancies were terminated. Seven fetuses were heterozygote carriers, and four were homozygous normal (unpublished data). Seven compound heterozygotes out of 18 at-risk pregnancies is a high proportion of affected fetuses compared to the expected 25%, but statistically not significant. We are in the process of pooling results of prenatal mutation analyses with other diagnostic centres to estimate the recurrence risk in a larger sample of CDG-Ia families.

3.8. CLINICAL PRESENTATION
CDG-Ia was first described by Jaeken et al, in 1980. The clinical features vary with age, and usually go through four stages: the infantile alarming multisystem stage, the childhood ataxia-mental retardation stage, the teenage leg atrophy stage and the adult hypogonadotropic stage (Table 3) (Jaeken et al, 1991). The first stage is variable in severity, but often dramatic, requiring frequent hospitalisations. The child becomes more stable with increasing age, and loss of acquired skills is unusual. Most patients are wheelchair-bound, but motor and communicative skills vary. The overall mortality rate is approximately 20%, mostly occurring in the first years of life due to infection, multiple organ failure or heart tamponade (Jaeken et al, 1991 and 2001).

The clinical spectrum of CDG-Ia has expanded since the initial description. Moderate to severe neurological dysfunction, variable dysmorphic features and variable involvement of other organs are now recognised, and a phenotype with only borderline cognitive impairment has been reported recently (Jaeken et al, 2001, Van Ommen et al, 2000, Drouin-Garraud et al, 2001, Enns et al, 2002). Rare presenting features, such as hyperinsulinemic hypoglycemia, congenital nephrotic syndrome and obstructive cardiomyopathy, have also been reported (Van der Knaap et al, 1996, Imtiaz et al, 2000, Böhles et al, 2001).

3.9. BIOCHEMICAL FEATURES
A large number of serum glycoproteins have been shown to have abnormal IEF pattern and/or altered concentration or enzymatic activity, including transport proteins, glycoprotein hormones, coagulation and anticoagulation factors, lysosomal enzymes and enzyme inhibitors (Jaeken et al, 2001). Clinically important biochemical features include:

### Table 3. Clinical stages of CDG-Ia.

<table>
<thead>
<tr>
<th>Infantile alarming multisystem stage 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Failure to thrive</td>
</tr>
<tr>
<td>Floppiness</td>
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<tr>
<td>Psychomotor retardation</td>
</tr>
<tr>
<td>Abnormal subcutaneous fat pads</td>
</tr>
<tr>
<td>Inverted nipples</td>
</tr>
<tr>
<td>Hepatomegaly and liver dysfunction</td>
</tr>
<tr>
<td>Cerebellar atrophy</td>
</tr>
<tr>
<td>Esotropia</td>
</tr>
<tr>
<td>Pericardial effusions</td>
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<tr>
<td>Multisystemic failure</td>
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<table>
<thead>
<tr>
<th>Childhood ataxia mental retardation stage 2</th>
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</thead>
<tbody>
<tr>
<td>Mental retardation (IQ: 40-60)</td>
</tr>
<tr>
<td>Motor disability</td>
</tr>
<tr>
<td>Peripheral neuropathy</td>
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<tr>
<td>Cerebellar ataxia</td>
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<tr>
<td>Tapetoretinal degeneration</td>
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<tr>
<td>Stroke-like episodes</td>
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<td>Seizures</td>
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<th>Teenage leg atrophy stage 3</th>
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<tr>
<td>Atrophy of lower limbs</td>
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<tr>
<td>Stable cerebellar ataxia</td>
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<tr>
<td>Deformity of thorax and spine</td>
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<tr>
<td>Osteopenia</td>
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<tr>
<td>Absence of puberty in females (hypergonadotropic hypogonadism)</td>
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<tr>
<th>Adult stable disability stage 4</th>
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<tr>
<td>Stable neurological impairment</td>
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<tr>
<td>Short stature</td>
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<tr>
<td>Kyphoscoliosis and long thin extremities</td>
</tr>
<tr>
<td>Premature aging of the skin in females</td>
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<tr>
<td>Retinitis pigmentosa</td>
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</table>

1. Decreased activity of factor XI and decreased activities of the coagulation inhibitors ATIII and protein C are present in the vast majority of CDG-Ia patients, and abnormal glycoforms of the latter two have been shown (Van Geet and Jaeken 1993, Stibler et al, 1996, Kjaergaard et al, 2001). A complex coagulopathy may be responsible for stroke-like episodes, thromboembolic events and hemorrhages seen in some patients.

2. Adolescent females have elevated levels of luteinising hormone and follicle-stimulating hormone (De Zegher and Jaeken 1995). Absence of puberty and hypergonadotropic hypogonadism is consistent features of CDG-Ia girls with few exceptions. Kristiansson et al, (1995) were not able to demonstrate hypoglycosylation of gonadotropins. Oestradiol secretion in response to exogenous gonadotropins has suggested biological inactivity of FSH, but the lack of response in one Danish patient confirmed that primary ovarian failure might also be responsible for absence of puberty (De Zegher and Jaeken 1995, Kristiansson et al, 1995, Kjaergaard et al, 2001). Oestradiol therapy induced growth of breast tissue and pubic hair in two Danish females (Kjaergaard et al, 2001).

3. Lysosomal enzyme activities in serum are frequently elevated (e.g., arylsulphatase A, β-galactosidase, β-glucuronidase and β-hexosaminidase). In contrast, lysosomal enzyme activities are frequently low in leukocytes (e.g., α-fucosidase, β-glucuronidase, α-iduronidase, α- and β-mannosidase) (Barone et al, 1998). These observations may be explained by deficiency of the mannose-6-P targeting signal necessary for their uptake in lysosomes. Abnormal glycoforms of N-acetylgalactosaminidase and fucosidase have been demonstrated (Jaeken et al, 1991). Findings of slightly to moderately increased lysosomal enzyme activities in plasma and/or moderately decreased activities in leukocytes from a patient with neurological impairment should alert the clinician to the possibility of CDG.

4. Abnormal glycosylation of serum transferrin, thyroid-binding globulin and α1-antitrypsin are consistently observed (Stibler et
al, 1998). These glycoproteins were used as parameters in our mannose therapy study (see p. 12).

5. Consistent biochemical abnormalities of non-glycosylated proteins include raised liver transaminases and hypoalbuminemia (Jaeck et al, 1991, Kjaergaard et al, 2001). Liver transaminases usually rise during infections, but tend to normalise with increasing age. Hypoalbuminemia may contribute to the extracellular fluid accumulations, e.g., pericardial effusion and ascites, seen in some patients.

3.10. CLINICAL PHENOTYPE IN RELATION TO GENOTYPE

Most reports on the clinical manifestations of CDG-Ia predate the availability of PMM2 mutation analysis (Kristianson et al, 1989, Jaeck et al, 1991, Petersen et al, 1993, Stibler et al, 1994b). A recent mutation update revealed 73 different PMM2 genotypes among 249 CDG-Ia patients, including Danish patients, and the R141H/F119L genotype accounted for 27% of the patients (Matthijs et al, 2000). The extensive genetic heterogeneity has complicated attempts to study genotype-phenotype correlation (Imtiaz et al, 2000, Delonlay et al, 2001). The predominance of the R141H/F119L genotype in our large series of CDG-Ia patients allowed us to study its clinical phenotype and outcome (Kjaergaard et al, 2001). The features of 23 patients are listed in Table 4. All patients had an early, uniform presentation with severe feeding problems, hypotonia, inverted nipples, abnormal subcutaneous fat pads and developmental delay obvious before age 6 months. Motor ability ranged from none to walking with a rooler, and vocabulary ranged from none to comprehensible speech. The overall mortality ascribed to CDG was 18%.

Brain imaging showed cerebellar atrophy in all but two patients. Cerebral atrophy was frequently present, and serial imaging indicated that the cerebellar and cerebral atrophy ran a progressive course in some patients, as previously observed (Jensen et al, 1995).

Table 4. Clinical features of CDG-Ia patients with R141H/F119L genotype.

<table>
<thead>
<tr>
<th>Consistent features (present in &gt;90% of the patients)</th>
<th>Neontal presentation</th>
<th>Severe feeding difficulties</th>
<th>Severe failure to thrive</th>
<th>Severe hypotonia</th>
<th>Developmental delay obvious before age 6 months</th>
<th>Hepatic dysfunction (raised liver transaminases and coagulation abnormalities)</th>
<th>Inverted nipples</th>
<th>Abnormal subcutaneous fat pads</th>
<th>Esotropia</th>
<th>Delayed visual maturation</th>
<th>Mental retardation</th>
<th>Cerebellar atrophy</th>
<th>Ataxia</th>
<th>Muscular atrophy</th>
<th>Peripheral neuropathy</th>
<th>Febrile seizures</th>
<th>Absence of puberty in females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequent manifestations (present in ≥50% of the patients)</td>
<td>Pericardial effusions (asymptomatic)</td>
<td>Afebrile seizures</td>
<td>Stroke-like episode</td>
<td>Acquired microcephaly</td>
<td>Congenital joint restriction</td>
<td>Hernia/hydrocele/retroto testis in males</td>
<td>Thorax deformity</td>
<td>Myopia</td>
<td>Decreased vision</td>
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<tr>
<td>Less frequent (present in &lt;50% of the patients)</td>
<td>Neurogenic hearing impairment</td>
<td>Thromboembolic event</td>
<td>Episode of prolonged bleeding</td>
<td>Episode of ascites</td>
<td>Kyphoscoliosis</td>
<td>Fracture due to minimal trauma</td>
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The mean head circumference standard deviation score declined gradually from normal at birth to −1.9 at age 5 (Kjaergaard et al, 2002).

Eye manifestations were evaluated during another Danish survey (Jensen et al, 2003). Patients with the R141H/F119L genotype had congenital esotropia, delayed visual maturation and a reduced rod response by electroretinography. The vast majority also had low visual acuity and progressive myopia.

Failure to thrive is a frequent feature of CDG-Ia, although the onset and degree in individual patients have been variable (Jaeck et al, 1991, Petersen et al, 1993, Kristianson et al, 1998). Feeding problems and failure to thrive were prominent in all our patients, and we analysed longitudinal data of weight and length to delineate the pattern of prepubertal growth (Kjaergaard et al, 2002). Anthropometric data of patients with identical CDG genotypes were remarkably similar. Measurements of weight and length were within normal range at birth. During the first 7 months of life, standard deviation scores of mean weight and length declined markedly, 2.7 and 2.4, respectively, and there was no prepubertal catch up. Our patients fed poorly, and the majority had frequent vomiting and diarrhea during the first year of life. Although data on their protein and caloric intake and gastrointestinal losses were not available, inadequate nutrition is the most likely major explanation of the severe failure to thrive. Hormones and other factors involved in growth are likely to be affected by defective glycosylation, but previous studies have not been able to hold growth hormone or thyroid hormones responsible (Macchia et al, 1995, De Zegher and Jaeck, 1995). Controlled dietary and hormonal interventional studies as well as studies of intestinal protein loss are required to further explore the causes of growth failure.

Patients with the R141H/F119L genotype have an early “classical” presentation, including severe failure to thrive, but with variable functional outcome, and they may well represent the severe end of the broad clinical spectrum (Kjaergaard et al, 2001, Grønwald et al, 2001). Although few patients share other genotypes, some of these seem to be associated with severe disease and high mortality (R141H/D188G, R141H/V231M) or mild disease (R141H/C251S, R141H/F183S, F157S/C241S) (Matthijs et al, 2000, Grønwald et al, 2001, Erlandson et al, 2001, Drouin-Garraud et al, 2001). In general, the PM 2 genotype does not seem to be a good predictor of clinical functional outcome in individual CDG-Ia patients.

Clinical variability, especially of functional outcome, was evident among patients with identical PM 2 genotypes and even among affected sibs and monozygotic twins (Kjaergaard et al, 2001). The lack of any clear genotype-phenotype correlation parallels other diseases, e.g., Gaucher disease (Dipple and McCabe, 2000). The inter- and intrafamilial variability in CDG-Ia suggests that additional environmental factors and/or genes that are inherited independently of PM 2 may have an impact on the clinical phenotype. Apart from unknown environmental stresses, polymorphisms/mutations in other genes, e.g., of the N-glycosylation pathway, may contribute to the severity of the disease. In fact, severely affected patients have an increased frequency of a polymorphism (F304S) in the ALG6 gene compared to moderately and mildly affected CDG-Ia patients (Westphal et al, 2002). ALG6 encodes the glycosyltransferase that adds the first glucose to the growing LLO in the rER (Figure 1) (Körner et al, 1998c). ALG6 is the gene mutated in CDG type Ic (Table 1), and the glucosyl transferasene glycosylation when expressed in yeast under certain conditions (Westphal et al, 2002). Clearly, this is not an explanation of clinical variability in a highly complex disorder. Several biochemical steps with possible modifying influences occur between a mutated gene and its clinical outcome. The importance of the genetic background has been demonstrated in animal models in which phenotypic differences among strains were obvious, e.g., the survival of the CDG type IIa (CDG-Ia) mice. Wang et al, (2001) introduced a deletion into the mouse germline that reproduced the glycansynthetic defect responsible.
for human CDG-IIa (see p. 13). Mice lacking the Mgat2 gene had deficiency of N-acetylglucosaminyltransferase II activity and severe gastrointestinal, hematologic, and skeletal abnormalities. All Mgat2-null mice died in early postnatal life. However, crossing the Mgat2 mutation into another genetic background (strain) resulted in Mgat2-null mice surviving for several months.

3.1. MANNOSE THERAPY
Mannose is presumed to enter human fibroblasts by a mannose-specific transporter, supplying most of the mannose for N-glycosylation of proteins (Figure 1). Previously, glucose was thought to be the major source of mannose in this process (Panneerselvam and Freeze 1996b, Panneerselvam et al, 1997a). In vitro studies have demonstrated that addition of exogenous mannose (≥0.25 mM) to the culture medium of CDG-Ia fibroblasts can correct both the deficient mannose incorporation and the size of lipid- and protein-linked oligosaccharides (Panneerselvam and Freeze 1996a, Körner et al, 1998b).

Furthermore, CDG-Ia patients had low blood mannose levels (average: 25 µM) compared to those of normal subjects (average: 55 µM) (Panneerselvam et al, 1997b).

These observations encouraged us to perform a short-term study of dietary mannose therapy in CDG-Ia patients. Information about mannose absorption, clearance and tolerance in man was sparse. We demonstrated that both normal controls and CDG-Ia patients had at least a two- or threefold elevation in blood mannose at a dose of 0.1 g/kg body weight given at 3 h intervals (Alton et al, 1997). Mannose in serum was determined by an enzymatic assay described by Ethchison and Freeze (1997): glucose was selectively removed from the sample by gluco kinase and glucose-6-P-dehydrogenase, whereupon anionic reaction products and excess substrates were removed by anion-exchange chromatography. Mannose in the effluent was then assayed spectrophotometrically by the reduction of NADP to NADPH following coupled enzymatic reactions catalysed by hexokinase, phosphoglucosamine and glucose-6-P-dehydrogenase in the presence or absence of PMI (Figure 3).

Our study established the feasibility of testing mannose as a therapy of CDG-Ia patients. Next step was to determine whether short-term supplementation of mannose in CDG-Ia patients could improve their abnormal glycosylation of circulating glycoproteins (Kjaergaard et al, 1998b). Five CDG-Ia patients completed the protocol of enteral supplementation with mannose 0.1 g/kg body weight every 3 h for 9 days. A higher dose had caused gastrointestinal distress (Alton et al, 1997). Unfortunately, the mannose levels obtained in the patients were lower than the concentration shown to correct abnormal glycosylation in fibroblasts. However, since the in vitro correction was dose-dependent, a lower concentration therefore could be at least partial beneficial. Surprisingly, the mean concentrations of the glycoproteins tended to decline during continued mannose supplementation, and CDT increased as did the abnormal glycoforms, with the most pronounced changes observed after cessation of mannose supplementation. Physiological fluctuation of blood levels of glycoproteins or methodological variations could explain these observations. However, it seemed unlikely that the same trend in glycosylation of all glycoproteins analysed in five patients should be caused by random variation. Since the aim of our study was to test for correction of glycosylation in order to justify long-term mannose supplementation, we did not attempt to include a hard-to-obtain group of normal children or a group of CDG-Ia patients given another substance than mannose, which might have been able to illuminate the unexpected observations. Our results suggest an effect of mannose on the glycosylation of circulating glycoproteins in CDG-Ia, but it does not seem to be a beneficial one in the short-term. More knowledge of mannose metabolism in man is required to interpret the results correctly.

Mannose supplementation has not been effective biochemically or clinically in other studies of CDG-Ia. Continuous intravenous mannose infusion for three weeks with serum levels up to 2 mM lowered by oral mannose for six months did not improve the glycosylation of various glycoproteins (Mayatepek et al, 1997, Mayatepek and Kohlmüller 1998).

Apparently, low PMM activity cannot be rescued by increasing the amount of its substrate in CDG-Ia patients in vivo. The biochemical basis for the in vitro correction of protein glycosylation is still not clear. So far the trials on mannose supplementation in CDG-Ia have been small and uncontrolled, and a long-term trial has not been reported.

Providing PMM-deficient cells with mannose-1-P may be a way to increase the GDP-mannose pool, but mannose-1-P is not able to penetrate cell membranes due to its high polarity. Attempts to develop membrane-permeant derivatives of mannose-1-P are in progress (Rutschow et al, 2002). However, therapy of CDG-Ia remains problematic. One reason is the prenatal onset of CDG-Ia demonstrated by the presence of dysmorphic features and neurological dysfunction at birth. On the other hand, the normal fetal growth and the failure to detect hypoglycosylation of transferrin in CDG-Ia prematurely (see p. 6) suggest that maternal compensation and/or a developmentally regulated alternate pathway may bypass PMM deficiency in utero. Presently, treatment offered patients with CDG-Ia remains supportive.

4. CONGENITAL DISORDER OF GLYCOSYLATION TYPE IB
4.1. PHOSPHOMANNOSIDASE DEFICIENCY
CDG-Ib is due to mutations in the MPI gene resulting in deficiency of PMI (Niehues et al, 1998, Jaeken et al, 1998, De Koning et al, 1998a). PMI is a cytosolic Zn²⁺-dependent enzyme catalysing the interconversion of fructose-6-P and mannose-6-P (Figure 1). As previously outlined, mannose-6-P is the precursor of GDP-mannose, which is required for glycosylation. PMI deficiency leads to a reduction of the GDP-mannose pool, and hence to incompletely glycosylated glycoproteins. PMI is widely distributed in human tissues, and low PMI activities, ranging from 3% to 18% of controls, have been demonstrated in leukocytes, cultured fibroblasts and liver tissue from CDG-Ib patients (Niehues et al, 1998, Jaeken et al, 1998, De Koning et al, 1998a, De Lonlay et al, 1999, Babovic-Vuksanovic et al, 1999, Westphal et al, 2001b).

Human PMI has been cloned based on its homology to the gene in yeast. MPI, located on chromosome 15q22, consists of eight exons encoding a protein of 423 amino acids (Proudfoot et al, 1994, Schollen et al, 2000b). Fourteen different MPI mutations in 13 families have been reported, and 12 of these mutations are of the missense type, mostly affecting conserved amino acids (Schollen et al, 2000b, Westphal et al, 2001b, Vuillaume-Barrot et al, 2002).

4.2. CLINICAL PRESENTATION
Clinical manifestations of CDG-Ib include hepatic fibrosis, protein-losing enteropathy, cyclic vomiting, thromboses, bleeding episodes and hypoglycemia, the latter sometimes due to hyperinsulinemia (Niehues et al, 1998, Jaeken et al, 1998, De Koning et al, 1998a). De Lonlay et al, 1999, Babovic-Vuksanovic et al, 1999, Westphal et al, 2001b). The absence of both neurological involvement and dysmorphic features sets the clinical phenotype quite apart from those of other types of CDG. Transferase IEF pattern is identical to that of patients with other type I CDG (Figure 2). Albumin is low, liver transaminases are frequently raised, and the coagulopathy seen in CDG-Ib is indistinguishable from that of CDG-Ia with low levels of factor XI, ATIII and protein C.

Two Danish sibs with CDG-Ib have been diagnosed by detection of "type I" transferase IEF pattern, decreased PMI activity in cultured fibroblasts and identification of missense mutations in the MPI gene (Westphal et al, 2001b). The patients were originally reported in 1980 by Pedersen and Tytgard because of their unusual clinical manifestations of recurrent venous thromboses, protein-losing enteropathy and hepatic fibrosis. No diagnosis was reached at the
time, and one sibling died due to complications of the disease at 5 years of age. The good long-term outcome of the surviving sibling, now age 35, demonstrates that serious manifestations in childhood may subside and allow for a normal adult life. Higher metabolic demands in infancy and childhood may overwhelm the cellular capacity for glycosylation, but with increasing age the patients become more stable as observed in other metabolic diseases, e.g., defects in branched chain amino acid catabolism. Symptoms, age at onset, severity of disease and outcome of CDG-Ib vary among reported patients. The Danish siblings presented later (2½ y) than previously described CDG-Ib patients (0–12 months), and their clinical manifestations were clearly episodic and occurred in association with fever. Environmental stresses (e.g., infections) and genetic background may be important factors in severity and outcome of the disease.

4.3. MANNOSE THERAPY
Mannose-6-P is normally derived from two sources: from fructose-6-P via PM1 or from free mannose entering the fibroblasts by a specific transporter, onto which mannose is converted to mannose-6-P by a hexokinase (Figure 1). The latter pathway bypasses PM1 deficiency, and oral mannose supplementation has been beneficial both clinically and biochemically to PM1-deficient patients. Daily oral mannose supplements have substantially reversed their protein-losing enteropathy, coagulopathy, and hypoglycemia as well as improved their glycosylation of transferrin (Niewies et al., 1998, De Lonlay et al., 1999, Babovic-Vuksanovic et al., 1999). Mannose supplementation in our patient normalised the ATIII level and improved her glycosylation of transferrin, which, as expected, returned to the presupplementation state after cessation (Westphal et al., 2001b). Cellular import of mannose appeared to be much more efficient in our patient than in controls since her non-endogenous serum mannose was lower and blood clearance of oral mannose threefold higher. These observations may be explained by upregulation of the mannose-specific transporter. Mannose clearance studies have not been reported in other CDG-Ib patients. Free mannose in man presumably comes from endogenous glycoprotein catabolism and dietary sources, but little is known about the availability of mannose in the human diet. Differences in dietary mannose intake may also contribute to the variable and episodic manifestations of CDG-Ib.

Although the metabolic defect in CDG-Ib is localised in the same biosynthetic pathway and in the step immediately preceding the enzymatic block in CDG-Ia, the clinical manifestations of the two disorders are quite dissimilar. Why is the clinical presentation of PM1 deficiency mainly hepato-intestinal? Why is the central nervous system spared in PM1 deficiency as opposed to other types of CDG? One possible explanation is that hexokinase I expressed in brain has a high affinity for mannose (Km 5 mM), thereby bypassing PM1 and its deficiency. In contrast, glucokinase, the major hexokinase of hepatocytes, has a very low affinity for mannose (Km 33 mM), which may explain the liver involvement in CDG-Ib (Jaeken et al., 1998). Other possible explanations include the existence of different PM1 isozymes and differences in expression of the mannose-specific transporter.

5. CONGENITAL DISORDERS OF GLYCOSYLATION - OTHER TYPES
Several other defects in N-glycosylation have recently been elucidated (Table 1 and Figure 1). Comprehensive descriptions are not yet available as few patients of each type have been reported. In general, clinical manifestations are less specific than in CDG-Ia and Ib and include variable psychomotor retardation, hypotonia, epilepsy, variable dysmorphic features, and, frequently, coagulopathy and raised liver transaminases. It is important to emphasize that patients with other CDG types do not have the characteristics of CDG-Ia, i.e., inverted nipples, abnormal subcutaneous fat pads or cerebellar atrophy. Descriptions beyond the following summary of other CDG types are outside the scope of this thesis.

CDG-Ic is due to deficiency of the glycosyltransferase, which adds the first glucose to the growing LLO in the ER (Figure 1) (Körner et al., 1998c, Imbach et al., 2000). The clinical presentation includes moderate psychomotor retardation, hypotonia, epilepsy, feeding problems, esotropia and coagulopathy (Grünwald et al., 2000). The IEF of serum transferrin shows a "type I" pattern.

CDG-Ib is due to deficiency of themannosyltransferase, which adds the sixth mannose to the growing LLO in the ER (Figure 1) (Körner et al., 1999). Clinical features are spastic paraparesis, severe psychomotor retardation, epilepsy, microcephaly and dysmorphism. ATIII may be reduced, and serum transferrin IEF shows a "type I" pattern.

CDG-Ie is due to deficiency of the cGPS subunit of dolichol-P-mannose synthetase, the enzyme required to generate dolichol-P-mannose, which is the donor of mannose for the growing LLO on the luminal side of the ER (Figure 1) (Imbach et al., 2000, Kim et al., 2000). Clinical manifestations include severe psychomotor retardation, hypotonia, epilepsy, microcephaly, cortical blindness, hepatospplenomegaly, dysmorphic features and coagulopathy. Liver transaminases are raised, and serum transferrin IEF pattern shows a "type I" pattern.

CDG-Ig (Figure 2) is a Danish patient with psychomotor retardation, hypotonia, failure to thrive, low immunoglobulins and low ATIII led to the identification of a novel type of CDG, type Ig (Grubenmann et al., 2002). CDG-Ig is due to mutations in the ALG12 gene encoding the mannosyltransferase, which adds the eighth mannose to the growing LLO in the ER (Figure 1) (Chantret et al., 2002a, Grubenmann et al., 2002).

CDG-Ih, the most recently characterised type of CDG, is due to deficiency of the glycosyltransferase adding the second glucose onto the growing LLO in the ER (Figure 1) (Chantret et al., 2003). The only reported patient had hypoalbuminemia, protein-losing enteropathy, hepatomegaly and coagulopathy, but no CNS involvement – a clinical presentation close to that of CDG-Ib. Serum transferrin IEF showed a "type I" pattern.

CDG-Ia is caused by deficiency of N-acetylglucosaminyltransferase II, the enzyme adding a glucosamine residue to the olsigosaccharide of the newly synthesised glycoprotein in the Golgi apparatus (Figure 1) (Jaeken et al., 1994). Clinical features are severe psychomotor retardation, seizures and variable skin affection. ATIII may be reduced, but liver transaminases are normal. The serum transferrin IEF shows a "type I" pattern.

CDG-Ia is caused by deficiency of N-acetylglucosaminyltransferase II, the enzyme adding a glucosamine residue to the olsigosaccharide of the newly synthesised glycoprotein in the Golgi apparatus (Figure 1) (Jaeken et al., 1994). Clinical features are severe psychomotor retardation, dysmorphism, hypotonia, epilepsy and stereotyped gestures. Raised liver transaminases and reduced ATIII activity are present. The serum transferrin IEF pattern is different from that seen in CDG-I with markedly decreased or absent tetrasialotransferrin and increased mono-, di- and trisialotransferrin ("type II" pattern).

CDG-Ib is caused by deficiency of glucosidase I, the enzyme removing the terminal glucose from the olsigosaccharide after its transfer to the polypeptide in the ER (Figure 1) (De Praetet al., 2000). The only reported patient had severe hypotonia, dysmorphic features, hypoventilation, seizures, severe infections, hepatomegaly and coagulopathy. Serum transferrin IEF pattern was normal. The diagnosis was based on structural analysis of an abnormal tetrasaccharide found by thin layer chromatography of urine from the patient in question.

CDG-Ic is usually called Leukocyte Adhesion Deficiency Type II, is caused by reduced transport of GDP-fucose into the Golgi, resulting in hypofucosylation of glycoconjugates, including selectin ligands (Lübke et al., 2001). Features of CDG-Ic are psychomotor retardation
and dysmorphic features combined with recurrent severe non-suppurative bacterial infections and high blood leukocyte counts. The immunodeficiency is due to defective selectin-dependent leukocyte trafficking to the sites of infection. IEF of serum transferrin is normal. Notably, oral fucose supplementation has proven beneficial biochemically and clinically in one CDG-IIc patient (Marquardt et al, 1999).

CDG-Ig is due to deficiency of galactosyltransferase, the enzyme adding galactose to the oligosaccharide of the newly synthesised glycoprotein in the Golgi apparatus (Figure 1) (Hansske et al, 2002). The only reported patient had myopathy, Dandy-Walker malformation, psychomotor retardation, raised liver transaminases and coagulopathy. IEF of serum transferrin showed a “type II” pattern.

CDG-x. Several cases with not yet characterised glycosylation defects detected by transferrin IEF have been reported. Their diverse clinical presentations included psychomotor retardation, hypotonia, dysmorphic features and seizures as well as hydrops fetalis, severe neonatal diarrhoea and Budd-Chiari syndrome (Huemer at al. 2000, Menton et al, 2001, De Koning et al, 1998b).

Within the last few years defects in proteoglycan synthesis and α-dystroglycan synthesis have been identified, and the range of phenotypes related to glycosylation defects has been extended to previously known clinical diagnoses such as hereditary multiple exostoses, a progeroid variant of Ehlers-Danlos syndrome, muscle-eye-brain disease and Walker-Warburg syndrome (Duncan et al, 2001, Okajima et al, 1999, Yoshida et al, 2001, Beltran-Valero De Bernabe et al, 2002). I-cell disease (or mucolipidosis II), which is due to deficiency of N-acetylgalcosamine-1-phosphotransferase, required for synthesis of the mannose-6-P targeting signal, is usually assigned to the group of lysosomal storage diseases, but it is in fact a glycosylation defect. These disorders are not detected by IEF screening of serum transferrin.

6. DIAGNOSTIC STRATEGY
CDG (except type Ib) should be considered in a patient with unexplained psychomotor retardation, isolated or associated with the clinical and biochemical features mentioned in Table 3 and p. 10. Inverted nipples, abnormal subcutaneous fat pads, cerebellar atrophy, failure to thrive, pericardial effusions and liver dysfunction suggest CDG-Ia.

Early diagnosis of CDG-Ib is crucial as the disorder is potentially lethal, but curable with mannose supplementation. CDG-Ib should be considered in unexplained liver disease and in protein-losing enteropathy, isolated or in association with thrombosis, recurrent vomiting and hypoglycemia in otherwise normal children. Low AT III activity and raised liver transaminases are common but not consistent findings in CDG.

IEF of serum transferrin is a useful screening test, and it will identify the vast majority of patients with CDG (N-glycosylation defects), but not all (e.g., CDG-IIb and CDG-IIc). Protein variants of transferrin and causes not related to CDG should be excluded when an abnormal IEF pattern is detected (see p. 6). IEF analysis cannot distinguish between different types of CDG, and further analyses are needed to reach a specific diagnosis (Figure 5). Patients of Danish origin with a “type II” transferrin IEF pattern are analysed for the two most frequent PMM 2 mutations, R141H and F119L. If not found, all exons of PMM 2 are sequenced. If normal, the next step is to assay PMM activity in cultured fibroblasts or leukocytes, since PMM deficiency could be due to large deletions or mutations in the promoter not detected by routine sequencing. When CDG-Ia is excluded, an oligosaccharide profile of cultured fibroblasts is done to detect accumulation and/or depletion of oligosaccharide structures suggesting possible enzymatic defects and responsible genes. Profiling of oligosaccharides is based on HPLC analysis of LLO extracted from cultured fibroblasts following incubation with [3H] mannose (Powell et al, 1994). Next, the suspected enzyme is assayed and the responsible gene screened for mutations.

The N-glycosylation pathway in the rER is highly conserved among eukaryotes, and yeast mutants have been valuable in characterisation of novel types of CDG-I. For example, fibroblasts from the Danish patient who led to the identification of CDG-Ig accumulated lipid-linked GlcNAc1Man5 oligosaccharides, as do alg12 mutant yeast cells (Grubenmann et al, 2002). The sequence information obtained from the yeast alg12 locus made it possible to identify the orthologous human ALG12 gene and, mutations at the locus were subsequently detected in patient DNA. In order to know if the mutations compromised the function of the ALG12-encoded mannosyltransferase, normal and mutant CDNA were expressed in alg12-deficient yeast. Indeed the mutant human ALG12 protein was unable to complement alg12 deficiency in yeast, as opposed to the normal ALG12. The same approach has been used to characterise CDG-Ic, CDG-IId and CDG-Ie.

The absence of psychomotor retardation in CDG-Ib sets the clinical phenotype apart from those of other CDG types. The diagnosis is confirmed by demonstration of PM1 deficiency in fibroblasts or leukocytes and identification of MP1 mutations.

“Type II” serum transferrin IEF pattern points to a N-glycosylation defect localised after the transfer of oligosaccharides to the protein, and a structural analysis of protein-bound glycans is performed to suggest an affected enzymatic step. Then, the suspected enzyme is assayed and the responsible gene screened for mutations.

CDG-Ib may be picked up by analysis of urine oligosaccharides as described in the only case reported so far (De Praetere et al, 2000). CDG-Ic is detected by determination of the blood groups as the fucose-containing blood groups A, B, O and Lewis A are absent in these patients, followed by mutation screening of the responsible gene (Lübké et al, 2001).

7. CONCLUSIONS
CDG-Ia was diagnosed in 32 Danish patients by detection of abnormal serum transferrin IEF pattern, profoundly decreased PMM activity in fibroblasts and mutations in the PMM 2 gene. The incidence was 1:41 000 newborns, the highest reported so far. Two of seven missense mutations predominated, R141H and F119L, and 83% of the patients were compound heterozygous for these two mutations. Unexpectedly, we did not observe homozygosity for the R141H mutation.

In vitro expression studies on the impact of mutations on PMM activity suggested the presence of two types of mutations. One type resulted in virtually null activity, and the other type resulted in PMM 2 with decreased activity and/or altered properties. Each of our patients had at least one mutation that retained residual PMM 2 activity when expressed in E. coli. The studies supported that homozygosity for R141H (or another “severe” mutation) impairs PMM 2 activity to a degree incompatible with life, whilst F119L (or another...
“mild” mutation) conveys a phenotype compatible with survival due to residual PMM2 activity. R141H and F119L were associated with separate specific haplotypes suggesting that each is derived from an ancestral mutation. R141H is by far the most frequent mutation in Caucasian patients with a carrier frequency of 1/60 in Denmark. F119L is a Scandinavica founder mutation with a much lower carrier frequency.

Patients with the R141H/F119L genotype have an early uniform presentation with severe feeding problems, severe growth failure, hypotonia, inverted nipples, abnormal subcutaneous fat pads and developmental delay obvious before age 6 months but variable functional outcome. Patients with this genotype present with the “classical” phenotype and belong to the severe end of the broad clinical spectrum of CDG-Ia.

Short-term mannose supplementation was not able to improve glycosylation in CDG-Ia patients. In contrast, mannose supplementation in an adult CDG-Ib patient proved beneficial biochemically.

The reasons for the clinical variability seen among both CDG-Ia and CDG-Ib patients with identical molecular defect are unknown, but environmental factors and genetic background may have an impact on the clinical phenotype. The genotype does not seem to be a good predictor of clinical outcome in the individual patient. Prenatal diagnosis by mutation analysis of chorion villus sample has been performed in 18 at-risk pregnancies.

8. FUTURE PERSPECTIVES

Efforts should be made to raise awareness among physicians of CDG as a large group of highly variable multi-system disorders, some of which are treatable. Increased awareness and broader serum trans-ferrin IEF screening will increase the number of CDG patients with less typical presentations, and novel CDG types will be identified. The many biochemical steps/genes involved in the glycosylation pathways suggest a large number of potential defects leading to pathological conditions. More detailed clinical characterisations by compiling clinical data of novel CDG types is another way to promote their diagnosis and to improve their management.

How abnormal glycosylation leads to the plethora of symptoms and signs in CDG-Ia and Ib is currently unexplained. Animal models may be helpful in unravelling the pathophysiology and, ultimately, in exploring possible treatments. The availability of the sequence and structure of each of the mouse Pmm1, Pmm2 and Mpi genes is the initial step in development of mouse models with deficiency of one of the enzymes (Heykants et al, 2001, Davis et al, 2002). Based on the lethality of R141H homozygotes in humans, it is unlikely that mice with complete ablation of PMM2 will produce viable offspring. Introducing appropriate mutations in the gene conveying residual PMM2 activity is more likely to have a viable outcome.

Patients and the pharmaceutical industries will continue to benefit from increased knowledge of the impact of glycans on the stability, targeting, action and clearance of bioactive molecules. One example is Gaucher disease due to deficiency of the lysosomal enzyme glucocerebrosidase resulting in accumulation of glucocerebroside. During normal biosynthesis of lysosomal enzymes N-glycans become modified with man-6-P residues to target them to the man-6-P receptor of lysosomes. Consequently, recombinant glucocerebrosidase produced for enzyme replacement therapy in Gaucher disease is modified to contain the targeting signal. The recombinant enzyme is thereby targeted to the correct site of action.

Collaboration with other centres dedicated to CDG is necessary for several reasons. The diagnosis and of basic research in CDG require diverse technologies, and gathering of patients is needed to increase the number required for clinical studies. EUROGLYCAN, a European collaboration financed by the EU, is an excellent forum for these purposes. Research in CDG will most likely provide more knowledge of the roles of N-glycans in physiology.


