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1 **GDP-mannose: GlcNAc₂-PP-dolichol mannosyltransferase deficiency (CDG Ik): 5 new**
2 **patients and 7 novel mutations**

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1 **ABSTRACT**

2 **Background:** In type I Congenital Disorders of Glycosylation (CDG I), proteins necessary
3 for the biosynthesis of the lipid-linked oligosaccharide (LLO) required for protein N-
4 glycosylation are defective. A deficiency in GDP-mannose: GlcNAc₂-PP-dolichol
5 mannosyltransferase (MT-1) causes CDG Ik (OMIM 608540), and only five patients, with
6 severe multisystemic clinical presentations, have been described with this disease.

7 **Objective:** To characterise genetic, biochemical and clinical data in 5 new CDG Ik cases and
8 compare these findings with those of the 5 previously described patients.

9 **Methods:** LLO biosynthesis was examined in skin biopsy fibroblasts, mannosyltransferases
10 were assayed in microsomes prepared from these cells, and *ALG1*-encoding MT-1 was
11 sequenced at both the DNA and cDNA levels. Clinical data for the 5 new patients were
12 collated.

13 **Results:** Cells from 5 patients with non-typed CDG I revealed accumulations of GlcNAc₂-PP-
14 dolichol, the second intermediate in the biosynthesis of LLO. Assay of MT-1, 2 and 3, the
15 first three mannosyltransferases required for extension of this intermediate demonstrated only
16 MT-1 to be deficient. DNA sequencing of *ALG1* revealed 9 different mutations, 7 of which
17 have not been previously reported. Clinical presentations are severe with dysmorphias, CNS
18 involvement and ocular disturbances being prevalent.

19 **Conclusions:** 5 patients with CDG Ik are described, and their identification reveals that, in
20 France, this disease and CDG Ib (MPI deficiency: OMIM 602579) are the most frequently
21 diagnosed CDG I after CDG Ia (PMM2 deficiency: OMIM 601785) and substantiates
22 previous observations indicating that this disease presents at the severe end of the CDG I
23 clinical spectrum.

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31 **Key words:** GDP-mannose: GlcNAc₂-PP-dolichol mannosyltransferase (MT-1) deficiency,
32 Congenital Disorders of Glycosylation type Ik (CDG Ik), protein N-glycosylation, ALG1-
33 CDG

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1 INTRODUCTION

2 Type I Congenital Disorders of Glycosylation (CDG I) are rare autosomal recessive metabolic
3 disorders affecting protein N-glycosylation ¹. About 1000 cases have been identified
4 worldwide. The diseases present with multisystemic signs, and their biochemical hallmark is
5 the presence of hypoglycosylated serum glycoproteins ². The underlying deficits in CDG I
6 have been shown to affect steps in the biosynthesis of the lipid-linked oligosaccharide (LLO)
7 precursor that is required for N-glycosylation ³. LLO is generated by the successive additions
8 of 1 residue of GlcNAc-P, 1 residue of GlcNAc, 9 residues of mannose and 3 residues of
9 glucose to dolichol phosphate to generate Glc₃Man₉GlcNAc₂-PP-dolichol. The
10 oligosaccharide moiety of this LLO is transferred onto nascent polypeptides to yield N-
11 glycosyl glycoproteins and dolichol pyrophosphate (dolichol-PP). The so called dolichol
12 cycle is then completed by the regeneration of dolichol-P from dolichol-PP ⁴. Many proteins
13 are required for dolichol recycling. So far, mutations in genes encoding 15 of them have been
14 shown to underly CDG I. CDG I are subtyped according to the defective protein ⁵.
15 Phosphomannomutase 2-deficiency (CDG Ia) is the commonest form of CDG I with more
16 than 600 cases described world wide, and other CDG I subtypes appear to be much rarer ⁶.
17 Identification of the molecular bases of type I CDG is important because first, it enables the
18 generation of antenatal tests for the affected families; second, mannose phosphate isomerase
19 (MPI)-deficiency (CDG Ib) is treatable; and third, the establishment of potential
20 genotype/phenotype relationships for the different CDG I subtypes could potentially facilitate
21 future diagnostic procedures.

22 Here we report that skin biopsy fibroblasts from 5 patients with severe type I CDG-
23 like clinical presentations reveal abnormal accumulations of the immature LLO intermediate
24 GlcNAc₂-PP-dolichol, and display less than 10% normal GDP-mannose: GlcNAc₂-PP-
25 dolichol mannosyltransferase (MT-1) activity. Overall 9 mutations in *ALG1* encoding MT-1
26 were identified. Seven novel mutations are described and the clinical presentations of these 5
27 MT-1 deficient (CDG Ik) patients are compared to those of the 5 previously described cases ⁷⁻
28 ¹⁰

30 MATERIALS, METHODS AND PATIENTS

31 *Patients* – 3 girls (patients P1, P2, P4) and 2 boys (patients P3, P5) were diagnosed with type
32 I CDG of unknown molecular origin at the ages of 4 months, 16 months, 10 months, 18
33 months and 3 years and 7 months respectively. The diagnosis was made after Western Blot of
34 serum proteins using blood samples collected onto paper as previously described ¹¹. After

1 parental consent a skin biopsy was performed on the forearm of each child. For gene studies,
2 signed informed consent protocols were obtained from all parents.

3 *Culture and metabolic radiolabeling of cultured skin biopsy fibroblasts* - Skin biopsy
4 fibroblasts from two control subjects and the 5 patients were prepared and cultivated ¹² as
5 previously described. Cells were metabolically radiolabelled for 30 minutes in RPMI 1640
6 medium containing 0.5 mM glucose and 2% dialysed foetal calf serum with either [2-
7 ³H]mannose (21.5 Ci/mmol, Perkin Life Sciences, FR) or [6-³H]glucosamine (37.7 Ci/mmol,
8 Perkin Life Sciences, FR).

9 *Recovery of metabolically radiolabeled LLO* – Subsequent to radiolabelling, LLO were
10 extracted from cells as previously described ¹² except that the CHCl₃ fractions from the
11 CHCl₃/methanol/water extracts were pooled with the 10/10/3 fractions before analysis.

12 *Analysis of lipid linked oligosaccharides* – Lipid extracts were dried down, subjected to acid
13 hydrolysis (20 mM HCl) and released oligosaccharides were resolved by TLC using either
14 system A: silica coated plastic sheets (Merck KGaA, DE) developed for 18 h in n-propanol:
15 acetic acid: water, 3/2/1, or system B: cellulose-coated plastic sheets (Merck KGaA, DE)
16 developed in ethyl acetate: pyridine: water: acetic acid, 5/5/3/1 for 8-18 h. Radioactive
17 components were visualised by fluorography after spraying the chromatograms with
18 En³Hance spray (Perkin Life Sciences, FR). Radiolabeled di-N-acetylchitobiose
19 ([³H]GlcNAc₂, BIOTREND GmbH, DE) was used as standard.

20 *Mutation analysis* - Genomic DNA was extracted from blood. RNA was isolated from
21 fibroblasts or from fresh blood cells. Sequencing was performed with the BigDye terminator
22 kit (Applied Biosystems, CA, USA) and analysed on an ABI PRISM 3130 sequencer
23 (Applied Biosystems, CA, USA). The *ALG1* gene (NM_019109.4) was first sequenced on genomic DNA.
24 Primers were designed to amplify all 13 coding exons and flanking intronic sequences, with
25 selected 3'ends matching the correct *ALG1* sequence and not those of the *ALG1* pseudogenes.
26 At the RNA level, primers were designed to amplify cDNA in five fragments from exon 1 to
27 the 3'UTR region. Primer sequences are available on request.

28 To exclude common polymorphisms, 82 unrelated healthy individuals served as control
29 subjects were sequenced in the region of each of the missense mutations (164 alleles). The
30 PolyPhen (<http://coot.embl.de/PolyPhen>), PANTHER
31 (<http://www.pantherdb.org/tools/csnpscoreForm.jsp>), SIFT2 (<http://blocks.fhcrc.org/sift/>
32 SIFT) and SNPs3D (<http://www.snps3d.org/>) algorithms were used to evaluate the potential
33 impact of the missense mutations on protein structure and function. The mutation
34 nomenclature is based on the Human Genome Variation Society recommendations¹³; for

1 cDNA numbering, +1 corresponds to the A of the ATG translation initiation codon in the
2 reference sequence of the GenBank (NM) accession number, and for protein, the initiation
3 codon is codon 1.

4 *Microsatellite analysis* - Haplotype analysis was performed on CDG Ik families with the
5 c.773C>T (p.Ser258Leu) (n=3), c.1263G>A (p.Cys396X) (n=2) and c.826C>T
6 (p.Arg276Trp) (n=2) mutations. Three highly polymorphic microsatellite markers close to
7 *ALG1* were selected: 31GT (UCSC hg18: 5 045 841-5 046 102) at -0.16MB, 16GT (UCSC
8 hg18 : 5 144 906-5 145 138) at +0.67 MB and D1S3134 (UCSC hg18 : 5 164 462-5 164 691)
9 at +0.87 MB. Primer sequences were obtained from the Genome Data Base. Fragments were
10 analyzed on an ABI PRISM 3100 with GeneMapper v4.0 software (Applied Biosystems, CA, USA).

11 *Preparation of GlcNAc₂-PP-dolichol from the mannosyltransferase-1 deficient yeast strain*
12 *alg1-1* - GlcNAc₂-PP-dolichol was generated in the temperature sensitive yeast strain *alg1-1*
13 (kindly donated by Professor L. Lehle, Lehrstuhl für Zellbiologie und Pflanzenphysiologie,
14 Regensburg, Germany) deficient in MT-1 activity¹⁴. After extraction and removing organic
15 solvents, LLO were taken up in CHCl₃/methanol/water, 10/10/3, and subjected to anion
16 exchange chromatography on DE-52 cellulose (acetate form)¹⁵. LLO was washed twice with
17 2 ml H₂O, and GlcNAc₂-PP-dolichol was quantitated by hydrolysing the LLO with 20 mM
18 HCl and assaying the released disaccharide using bovine galactosyltransferase and UDP-
19 [¹⁴C]galactose¹⁵.

20 *Assay of mannosyltransferase-1 activity* – Microsomes prepared from the different fibroblast
21 lines¹⁶ were incubated at 37°C for 20 min in 50 µL 50 mM TrisHCl, pH 8.0 containing:
22 protease inhibitors (Sigma), 1 µM GlcNAc₂-PP-dolichol, 1 µM GDP-[¹⁴C]mannose (275
23 mCi/mmol, GE Healthcare, FR), 10 mM MgCl₂, 0.1% Triton-X100, and 2-20 µg microsomal
24 protein. Reactions were stopped by addition of 150 µL ice cold H₂O, 400 µL methanol and
25 600 µL CHCl₃. After vigorous shaking the tubes were centrifuged to generate two phases. The
26 lower CHCl₃ phase was taken for scintillation counting. Radioactive components recovered
27 from the CHCl₃ phase were further characterised after hydrolysis with 20 mM HCl, and
28 released sugars were examined using TLC system B.

29 *Assay of mannosyltransferase-2 and -3 activities* – Microsomes generated from HepG2 cells
30¹⁶ were incubated with 1 µM GlcNAc₂-PP-dolichol and GDP-[¹⁴C]mannose for 10 min and
31 the resulting [¹⁴C]LLO were extracted and purified as described above then incubated with 20
32 µg microsomal proteins derived from a control subject (Ctrl 2) and the 5 patients in either the
33 absence or presence of 20 µM GDP-Man (Sigma) as described above. After extraction of
34 reaction mixtures with organic solvents, radioactive components recovered from the CHCl₃

1 phase were further characterised after hydrolysis with 20 mM HCl. Released sugars were
2 examined using TLC system A.

3 4 **RESULTS**

5 *Patients and clinic* – Clinical presentations of the five patients are detailed in the Table.
6 Pregnancies associated with patients P4 and P5 were uneventful whereas pregnancy induced
7 maternal hypertension developed with those associated with P2 and P3, and for P1 and P3
8 foetal growth was retarded. At birth, neurological signs were noted: central hypotonia and
9 psychomotor delay were present. All patients presented with at least one episode of epilepsy;
10 ranging in severity from a unique treatable episode for patient P2 to multiple intractable
11 seizures in patient P4. Exploration of neurological occurrences by EEG revealed more or less
12 serious abnormalities for all children except P2. Using MRI, P2 and P5 presented normally at
13 the time of examination. After MRI examinations at 18 days and 18 months, patient P1
14 revealed a progressive cerebellar hypoplasia. Patients P1, P3 and P4 presented with cortical
15 atrophy. Finally, dysmorphias were found to be present to differing degrees in all children
16 with microcephaly (-2.5 for P5) being noted for 4/5 patients. With the exception of P2 who
17 presented with less severe neurological signs it is notable that the other children presented
18 with ocular problems ranging from simple strabismus to partial blindness. Liver and kidney
19 function appeared normal in all children, and when explored, haematological complications
20 and coagulopathy were absent. Only patient P1 has died (respiratory insufficiency at 4 years
21 and 9 months). These clinical pictures are compatible with those noted for type I CDG in
22 which hypoglycosylation of serum glycoproteins is a hallmark.

23 *Clinical biochemistry* - Western blot analysis of the serum glycoproteins transferrin,
24 haptoglobin, orosomuroid and α 1-antitrypsin revealed the presence hypoglycosylated
25 glycoforms in all patients (Figure 1A). In order to identify the molecular origins of this
26 phenomenon, PMM activity, known to be depressed in CDG Ia, the commonest CDG I
27 subtype¹⁷, was measured in cell extracts from these patients, and in all cases found to be
28 normal.

29 *Metabolic radiolabelling of lipid linked oligosaccharides* – In order to identify potential
30 blocks in the biosynthesis of the LLO precursor required for N-glycosylation, skin biopsy
31 fibroblasts derived from the 5 patients and 2 control subjects were first metabolically
32 radiolabeled with [2-³H]mannose. The use of this radioisotope allows detection of all LLO
33 intermediates containing mannose (Man₁GlcNAc₂-PP-dolichol - Glc₃Man₉GlcNAc₂-PP-
34 dolichol). Analysis of [³H-Man]oligosaccharides released from LLO by mild acid hydrolysis

Table
Comparison of the clinical presentations of five new cases of CDG 1k with those of the previously described cases

	P1	P2	P3	P4	P5	Case Summaries Published^a	
Date of birth	2001	2006	2004	2005	2001		
Age of CDG I diagnosis	4 months	1 year 4 months	1 year 6 months	10 months	3 years 7 months		
Sex	F	F	M	F	M		
Complications during pregnancy	Foetal growth retardation.	Pregnancy-induced hypertension.	Pregnancy-induced hypertension. Foetal growth retardation.	No	Foetal growth retardation.	4/5	1/5
Post delivery complications	Hypotonia.	Low blood pressure. Vomiting.	Hypotonia. Absence of ocular contact.	Hypotonia.	Not reported	4/5	
Feeding difficulties	Yes	Yes	No	Yes	No	3/5	
Central hypotonia	Yes	Yes	Yes	Yes	Yes	5/5	3/5
Psychomotor retardation	Yes	Yes	Yes	Yes	Yes	5/5	
Epilepsy	Multifocal epilepsy.	Once, treatable.	Multifocal epileps.	Intractable seizure.	Multifocal epilepsy.	5/5	5/5
MRI	Evolutionary cerebellar hypoplasia.	Normal	Cortical atrophy. Demyelination.	Cortical and sub cortical atrophy.	Normal	3/5	2/5
EEG	Abnormal	Normal	Abnormal	Abnormal	Abnormal	4/5	
Dysmorphias	Thin lips. Small triangular chin. Turned up nose.	Large cup-shaped ears. Temporal narrowing of forehead. Depressed nasal bridge. Small upturned nose. Thick lower eyelids. Short neck.	No	Triangular face. Almond-shaped eyes.	Thin lips. Large forehead. Large mouth. Epicanthus. Long smooth filtrum.	4/5	4/5
Microcephaly	Yes		Yes		Yes	3/5	2/5
Ocular manifestations	Abnormal VEP ^b test Poor visual contact	Normal	Abnormal VEP test. Partially blind.	Abnormal VEP test. Absence of ocular pursuit.	Abnormal VEP test. Strabism.	4/5	3/5
Fatal outcome	Yes					1/5	4/5
Maternal allele	p.Cys396X/p.Arg438Trp ^c	p.Met377Val	p.Ser150Arg	p.Gly145Asp	p.Cys396X		
Paternal allele		p.Met377Val	p.Ala211_Arg247del	p.Ser258Leu	p.Arg276Trp		

^aData taken from references 7,8,9,17 ;

^bVisually Evoked Potential test;

^cDeduced from mRNA

1 revealed no significant differences between the distribution of these components generated in
2 cells from either the patients or control subjects (Figure 1B, lower panel)

3 Blocks in the second and third steps in LLO production potentially lead to the accumulation
4 of GlcNAc-PP-dolichol and GlcNAc₂-PP-dolichol and these intermediates can be detected
5 after metabolic radiolabeling of cells with [³H]glucosamine. Examination of [³H]GlcNAc-
6 labelled oligosaccharides released from LLO by mild acid hydrolysis reveals a disaccharide
7 that comigrates with standard di-N-acetylchitobiose (GlcNAc₂) in pathological but not normal
8 cells (Figure 1B, upper panel). An accumulation of GlcNAc₂-PP-dolichol has previously been
9 shown to be indicative of a deficiency in GDP-mannose: GlcNAc₂-PP-dolichol
10 mannosyltransferase (MT-1) that adds the first mannose residue onto LLO⁸⁻¹⁰.

11 *Mutation analysis of ALG1* – MT-1 is encoded by *ALG1*, and the 13 exons and intron-exon
12 boundaries of genomic *ALG1* were sequenced using primers designed to discriminate between
13 this gene and its documented pseudogenes. Where appropriate, cDNA was also sequenced.
14 Nine mutations were encountered in the genomic DNA prepared from the five patients
15 (Figure 2 and Table). Patient P3 harbours a previously described disease causing missense
16 mutation c.450C>G (p.Ser150Arg⁸) and a 36 amino acid deletion (p.Ala211_Arg247del)
17 caused by skipping of exon 6. Exon skipping is potentially caused by one or both of two
18 mutations on the same allele: the last base of exon 6 and +5 from the donor site (c.740G>T;
19 c.740+5G>A). Patient P4 revealed compound heterozygosity with respect to the false sense
20 mutations c.434G>A (p.Gly145Asp) and c.773C>T (p.Ser258Leu). Whereas the latter
21 mutation has been demonstrated to be deleterious⁸⁻¹⁰, the former is predicted to be deleterious
22 to protein function by four *in silico* software packages that were used to evaluate the impact
23 of the missense mutations on protein structure and function (see Supplementary Table). In
24 addition, a synonymous mutation (c.765G>A, p.Thr255Thr: not found in the NCBI SNP
25 database (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?locusId=56052) in exon 7
26 was found to be associated with the maternally inherited c.434G>A (p.Gly145Asp) mutation
27 in patient 4. Patient P1 and P5 have the same splicing mutation of the last base of exon 12
28 (c.1263G>A) resulting in a premature stop codon (p.Cys396X) and loss of exons 12 and 13
29 on one allele. In conjunction with this mutation, patients P1 and P5, harbour the c.1312C>T
30 (p.Arg438Trp) and c.826C>T (p.Arg276Trp) mutations, respectively, on the other allele.
31 Patient P2 is homozygous with respect to the c.1129A>G (p.Met377Val) mutation that is
32 predicted *in silico* to be deleterious. Allelic inheritance was confirmed in all cases by analysis
33 of DNA obtained from the parents, or RNA in the case of patient P1.

1 Haplotype studies revealed a possible founder effect for the frequent p.Ser258Leu mutation
2 observed in the three non-related families. In contrast, no specific common haplotype was
3 associated with p.Arg276Trp and the c.1263G>A splicing mutation, that were each observed
4 in two families (data not shown).

5 *In vitro assays of microsomal GDP-Man mannosyltransferases* – The mutations described
6 above are not all predicted to perturb protein function so MT-1 activity was measured in the
7 fibroblast cell lines. To this end, microsomes were prepared from cells from the 5 patients and
8 incubated with GDP-[¹⁴C]Man in either the absence or presence of GlcNAc₂-PP-dolichol.
9 Microsomes derived from the control subject are able to incorporate substantial amounts of
10 radioactivity into lipid components only when GlcNAc₂-PP-dolichol is added to the
11 incubation mixtures (Figure 3). By contrast, the microsome preparations generated from cells
12 derived from the patients manifested less than 10% of control GlcNAc₂-PP-dolichol-
13 dependent synthesis of radioactive lipid components (Figure 3A, B). In order to examine the
14 radioactive products generated by microsomes derived from the 5 patients under our assay
15 conditions, LLO-derived oligo-, and monosaccharides were resolved by TLC as shown in
16 Figure 4A-C. When incubated with GlcNAc₂-PP-dolichol and GDP-[¹⁴C]Man, control
17 microsomes yielded substantial quantities of components migrating as Man₁₋₅GlcNAc₂
18 (Figure 4A), consistent with the capacity of MT-1, GDP-mannose: Man₁GlcNAc₂-PP-
19 dolichol mannosyltransferase (MT-2) and GDP-mannose: Man₃GlcNAc₂-PP-dolichol
20 mannosyltransferase (MT-3) to generate [¹⁴C]Man₁₋₅GlcNAc₂-PP-dolichol in a GlcNAc₂-PP-
21 dolichol-dependent manner. Microsomes derived from cells of the 5 patients generate reduced
22 amounts of all the [¹⁴C]Man₁₋₅GlcNAc₂-PP-dolichol species (Figure 4A). These results are
23 compatible with but do not prove MT-1 deficiency. In order to rule out a general reduction in
24 GDP-Man requiring mannosyltransferase activity in these microsome preparations, MT-2 and
25 MT-3 that act subsequent to MT-1 in the dolichol cycle were examined (Figure 4B).
26 Microsomes were incubated with exogenously added [¹⁴C]Man₁₋₂GlcNAc₂-PP-dolichol in
27 either the absence or presence of GDP-Man. Results demonstrate that control and patient
28 microsome preparations are similarly capable of elongating exogenously added [¹⁴C]Man<sub>1-
29 2</sub>GlcNAc₂-PP-dolichol to yield predominantly [¹⁴C]Man₅GlcNAc₂-PP-dolichol (Figure 4B).
30 The ensemble of these assays indicates that only MT-1 is defective in the 5 patients.
31 Finally, the origin of the high GlcNAc₂-PP-dolichol-independent incorporation of
32 radioactivity into lipids by microsomes originating from patient P4 (Figure 3A) was
33 investigated. Data shown in Figure 4C demonstrate that these microsomes generate

1 substantially more dolichol-P-[¹⁴C]Man than the other microsome preparations, and further
2 experiments are being conducted in order to examine the origin of this phenomenon.

4 **DISCUSSION**

5 Here we report upon 5 CDG I patients whose skin biopsy fibroblasts manifest less than 10%
6 normal MT-1 activity. Sequencing of *ALG1* revealed 9 mutations, 2 of which (p.Ser150Arg⁸,
7 p.Ser258Leu⁸⁻¹⁰) have been demonstrated to be pathogenic. The c.1263G>A mutation leads
8 to loss of exons 12 and 13 and a premature stop codon that causes a 69 amino acid truncation
9 of the MT-1 protein. Interestingly, the underlying mutation in *ALG1* of the temperature
10 sensitive *alg1-1 S. cerevisiae* strain¹⁸ also causes a C-terminal truncation, and it has been
11 suggested that this region of the protein is important for its interactions with yeast MT-2 and
12 MT-3¹⁸. One or both of two novel mutations (c.740G>A/c.740+5G>A) at the exon6/intron6
13 splice site junction leads to skipping of exon 6 and generation of an enzyme with a 36 amino
14 acid deletion. This deleted region contains two amino acids (Asp 216, Phe 222) that are
15 conserved in yeast and mammals. Finally, 4 novel point mutations have been identified. The
16 p.Met377Val mutation is predicted to be deleterious by all four of the pathogenicity software
17 packages (supplemental online table). Met 377 is conserved in yeast and mammals and lies
18 between 2 similarly conserved aspartic acid residues whose substitution affects yeast MT-1
19 activity¹⁸. Pathogenicity predictions for the p.Arg438Trp substitution are ambiguous but Arg
20 438 is adjacent to the C-terminal region that, in the yeast enzyme, affects interactions with
21 MT-2 and MT-3 as described above. Likewise, pathogenicity predictions for the p.Arg276Trp
22 and p.Gly145Asp substitutions are ambiguous but these residues are situated adjacent to the
23 previously described p.Ser258Leu and p.Ser150Arg pathogenic mutations, respectively.

24 Five patients with CDG Ik were described in 2004⁷⁻¹⁰, and because no further case reports
25 have appeared, the 5 patients described here double the descriptions of patients with this
26 disease. CDG Ik seems to be associated with a severe clinical picture⁷⁻¹⁰. Four of the five
27 originally described patients died in the first year of life. Of the five patients described here,
28 only one has died. By contrast, serious neurological complications are a constant feature with
29 central hypotonia (4/5 our patients and 5/5 originally described patients) and epilepsy (5/5 and
30 5/5 cases, respectively) being particularly prominent. Ocular complications ranging from
31 absence of visual contact (2/5 and 1/4) to blindness (1/5 and 1/4) are noteworthy. With the
32 exception of microcephaly described in 3/5 of the patients described here, dysmorphias are
33 not constant and when present they appear to be variable. Recurrent infections and/or
34 complications of the immune system were only noted in one of the presently described

1 patients compared to 4/5 of the originally described cases. Another notable difference
2 between the originally described cases and those described herein is the absence of hepatic
3 and renal complications in the latter patients. The 773 C>T (p.Ser258Leu) mutation is present
4 in all the previously described cases, and in two, homozygosity is associated with fatal
5 outcome. In our study, this mutation was only found in patient P4 and occurred in the
6 heterozygous state. All other factors being equal, it might be that the p.Ser258Leu mutation
7 may be more severe than the p.Met377Val mutation, present in the homozygous state in P2.
8 Despite these observations, with only 9 patients harbouring 11 different mutations it is
9 difficult to establish potential genotype/phenotype relationships. All CDG Ik descriptions
10 include severe neurological presentations that are also observed in other CDG I subtypes with
11 the exception of CDG Ib where only visceral hepato-intestinal manifestations are observed ¹⁹.
12 In CDG Ih (ALG8 deficiency: OMIM 608104) and CDG Im (DK1 deficiency: OMIM
13 610768) neurological signs are accompanied by hepato-intestinal ²⁰ and skin/heart ²¹
14 complications, respectively.

15 In our French experience of CDG diagnosis, 131 families have now been demonstrated to be
16 affected by CDG I. The majority of patients (81 families, 62%) are affected by CDG Ia and
17 the next commonest forms of the disease are CDG Ib and CDG Ik with 8 families each, (6%),
18 closely followed by CDG Ic (ALG6 deficiency: OMIM 603147; 5 families, 4%). We have
19 also diagnosed 3 families with CDG Ie (DPM1 deficiency: OMIM 603503), 3 families with
20 CDG Ig (ALG12 deficiency: OMIM 607143), and one family each with CDG Ih and CDG
21 Im. The 20 other families remain to be subtyped (CDG Ix: 15%). These statistics suggest that
22 CDG Ik should be given more consideration when diagnostic strategies are prioritised based
23 on apparent CDG I subtype frequencies.

24 To summarise, 5 patients with severe type I CDG-like clinical presentations possessing an
25 underlying MT-1 deficiency are described. The identification of these patients reveals that, in
26 France, CDG Ik and CDG Ib are the most frequently diagnosed type I CDG after CDG Ia.

27

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33

34

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18 COMPETING INTERESTS

19 None
20

21 ABBREVIATIONS

22 Man, mannose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GlcNH₂, glucosamine; Dol-P,
23 dolichol-phosphate; ER, endoplasmic reticulum; LLO, lipid-linked oligosaccharide; SDS-
24 PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis.
25

26 FIGURE LEGENDS

27 Figure Legend 1

28 *Serum glycoprotein hypoglycosylation and defective lipid-linked oligosaccharide biosynthesis*
29 *in 5 patients with type I CDG* – A. Western blot analysis of serum proteins derived from
30 control subjects (Controls), patients P1-5, and a patient diagnosed with CDG Ia (Ia).
31 Migration positions of normal transferrin (TFR), haptoglobin (HAP), orosomucoid (ORO)
32 and alpha 1 antitrypsin (AAT) are indicated by arrowheads whereas hypoglycosylated
33 isoforms of these glycoproteins are indicated by lines. B. Fibroblasts derived from control
34 subjects 1 and 2 (different from above control subjects) and the 5 patients (P1-5) were
35 radiolabeled with either [6-³H]glucosamine ([³H]GlcNH₂, upper panel) or [2-³H]mannose
36 ([³H]Man, lower panel) and oligosaccharides released from LLO by mild acid hydrolysis
37 were examined by TLC using system B (upper panel) or system A (lower panel). The

1 radioactive components that migrate faster than di-N-acetylchitobiose were not further
2 characterised. The abbreviations used are: $G_{1-3}M_9$, $Glc_{1-3}Man_9GlcNAc_2$; GN_2 , di-N-
3 acetylchitobiose.

4

5 Figure Legend 2

6 *Mutations found in genomic DNA corresponding to the exons and intron/exon junctions of the*
7 *human ALG1 gene derived from patients.* Genomic and or cDNA were prepared from blood
8 leukocytes or cultured skin biopsy fibroblasts as described in Materials, Methods and Patients.
9 Apart from the maternally inherited synonymous mutation in exon 7 detected in patient 4, the
10 genotypes of patients P1-5 are given in the Table. Where possible (P2-P5), allelic inheritance
11 is indicated.

12

13 Figure Legend 3

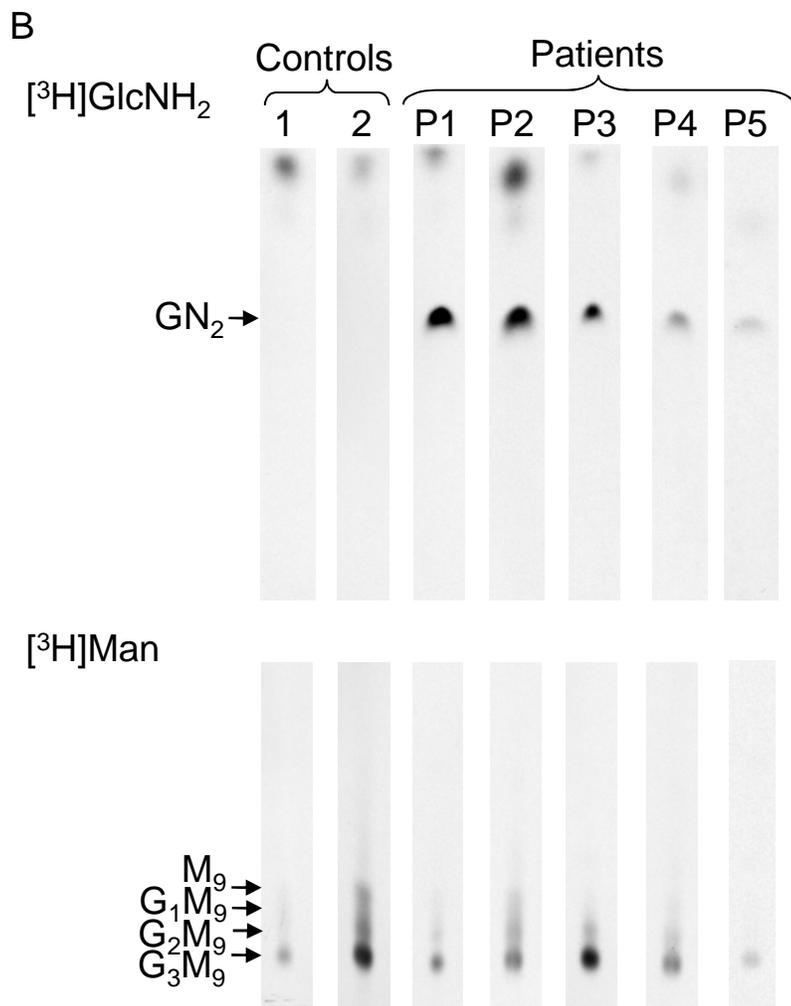
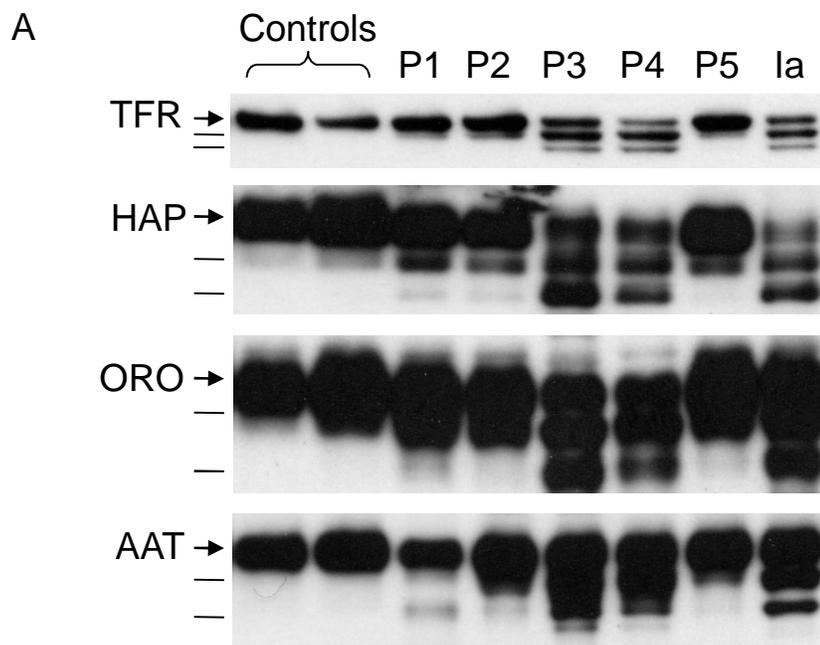
14 *In vitro LLO biosynthesis in microsomes derived from fibroblasts of a control subject and*
15 *patients* – A. Increasing amounts of microsomes derived from control subject 2 (Ctrl 2) and
16 the 5 patients (P1-5) were incubated with GDP- $[^{14}C]$ Man in either the absence (-) or presence
17 (+) of $GlcNAc_2$ -PP-dolichol (GN_2 -PP-dol) for 20 minutes. After stopping the reactions by the
18 addition of organic solvents $[^{14}C]$ LLO was quantitated by scintillation counting. The results
19 were obtained from a single experiment. B. The capacity of microsomes to incorporate
20 radioactivity into lipid components in a $GlcNAc_2$ -PP-dolichol-dependent manner was
21 calculated from the 20 μ g data point shown in A and expressed as a percentage of the control
22 (Ctrl2).

23

24 Figure Legend 4

25 *Further evaluation of the mannosyltransferase activities in microsomes derived from cells of*
26 *patients with CDG I* - A. Microsomal MT-1, MT-2 and MT-3 mannosyltransferases generate
27 $[^{14}C]$ Man $_5$ GlcNAc $_2$ -PP-dolichol using exogenously added $GlcNAc_2$ -PP-dolichol and GDP-
28 $[^{14}C]$ Man. Using lipid fractions derived from the incubations described in Figure 3, LLO were
29 subjected to mild acid hydrolysis, and liberated oligosaccharides were resolved by TLC using
30 system B. The abbreviations used are M_1GN_2 , $Man_1GlcNAc_2$; M_5GN_2 , $Man_5GlcNAc_2$. B. To
31 assay MT-2 and MT-3, a preparation of $[^{14}C]$ Man-labelled LLO enriched in the MT-1 product
32 $Man_1GlcNAc_2$ -PP-dolichol was generated, and incubated with the different microsome
33 preparations in the presence or absence of unlabelled GDP-Man. Oligosaccharides liberated
34 from LLO were resolved using TLC system A. The abbreviations used are $M_{1-5}GN_2$, Man_1 -

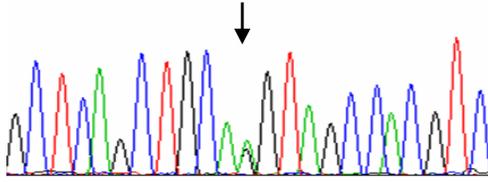
1 $_5\text{GlcNAc}_2$. C. Dolichol-P-mannose synthase (*DPM synthase*) generates dolichol-P-
2 $[^{14}\text{C}]$ mannose (dol-P- $[^{14}\text{C}]$ Man) from GDP- $[^{14}\text{C}]$ Man. The different microsome preparations
3 were incubated with GDP- $[^{14}\text{C}]$ Man in either the absence or presence of GlcNAc_2 -PP-
4 dolichol (GN_2 -PP-dol) as described for Figure 3. The resulting radiolabeled lipid linked
5 sugars were examined using TLC system B. The region of the chromatogram corresponding
6 to the migration position of mannose (Man) is shown.



Patient 1

Exon 12

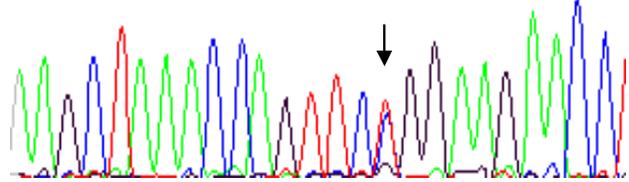
GCTCAGCTGCAAGTAGCCACGTC



c.1263G>A (p.Cys396X)

Exon 13

AAGCTAAACCAGTTCYGGAAAGAACT

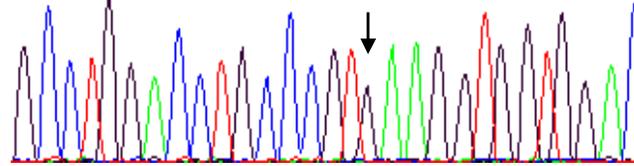


c.1312C>T (p.Arg438Trp)

Patient 2

Exon 11

GGCCTGGACCTGCCCGTGAAGGTGGTGGAC



c.1129A>G (p.Met377Val)

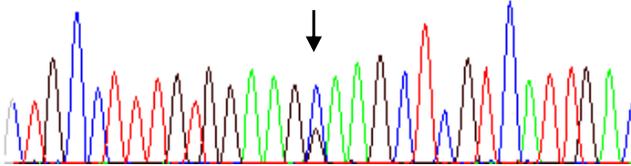
Maternal allele

Paternal allele

Patient 3

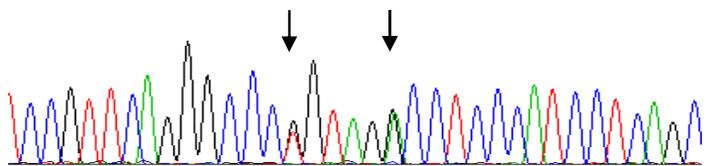
Exon 4

CTGCCCTTTGTTGGAAAGSAAAGCTCGTCATTGAC



c.450C>G (p.Ser150Arg)

---Exon 6 ---><--- Intron 6 -----
TCCGTTCAGGGCCCRGTAGRCCTCCCATCCTCAGC

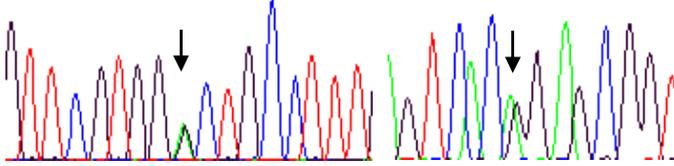


c.740G>T; c.740+5G>A (p.Ala211_Arg247del)

Patient 4

Exon 4

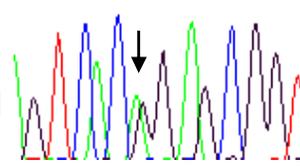
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c.434G>A (p.Gly145Asp)

Exon 7

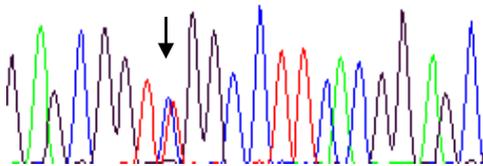
AGTTCACRGAAGCGGT



c.765G>A (p.Thr255Thr)

Exon 7

GAGCGGTYGGCCCTTCACGGAGCG

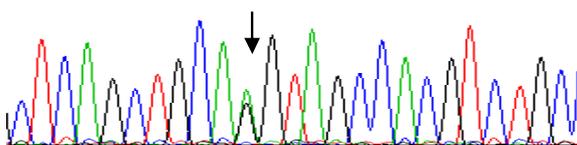


c.773C>T (p.Ser258Leu)

Patient 5

Exon 12

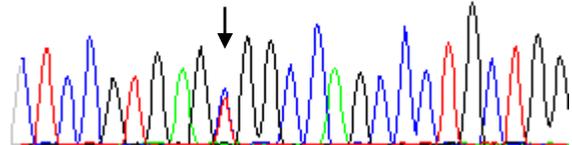
CTCAGCTGCARGTAGCCACGTCTGC



c.1263G>A (p.Cys396X)

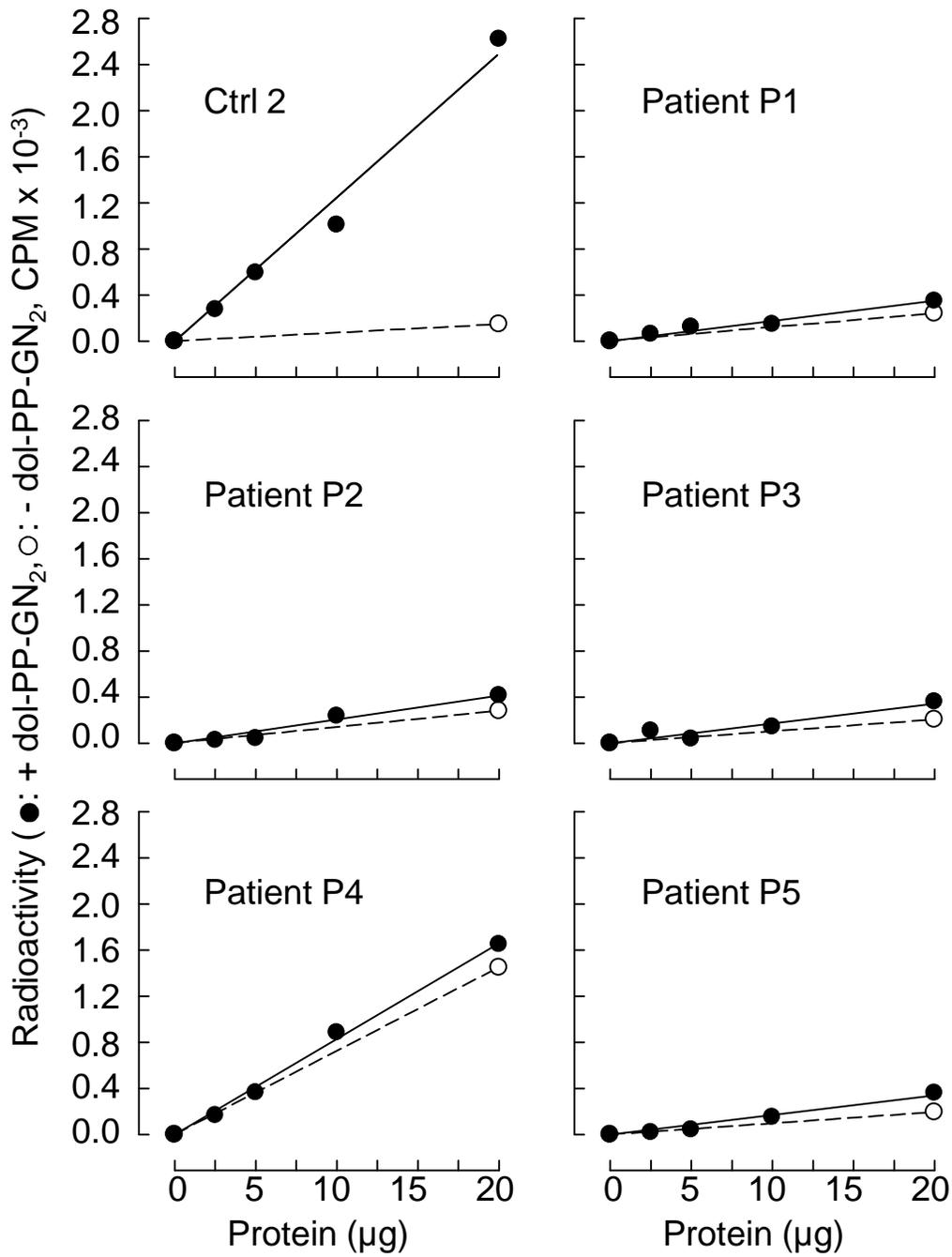
Exon 7

CTCCGTGAGYGGCCAGCCCTGCTGG



c.826C>T (p.Arg276Trp)

A



B

