

# A novel cerebello-ocular syndrome with abnormal glycosylation due to abnormalities in dolichol metabolism

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**Cerebellar hypoplasia and slowly progressive ophthalmological symptoms are common features in patients with congenital disorders of glycosylation type I. In a group of patients with congenital disorders of glycosylation type I with unknown aetiology, we have previously described a distinct phenotype with severe, early visual impairment and variable eye malformations, including optic nerve hypoplasia, retinal coloboma, congenital cataract and glaucoma. Some of the symptoms overlapped with the phenotype in other congenital disorders of glycosylation type I subtypes, such as vermis hypoplasia, anaemia, ichthyosiform dermatitis, liver**

**dysfunction and coagulation abnormalities. We recently identified pathogenic mutations in the *SRD5A3* gene, encoding steroid 5 $\alpha$ -reductase type 3, in a group of patients who presented with this particular phenotype and a common metabolic pattern. Here, we report on the clinical, genetic and metabolic features of 12 patients from nine families with cerebellar ataxia and congenital eye malformations diagnosed with *SRD5A3*-congenital disorders of glycosylation due to steroid 5 $\alpha$ -reductase type 3 defect. This enzyme is necessary for the reduction of polyprenol to dolichol, the lipid anchor for *N*-glycosylation in the endoplasmic reticulum. Dolichol synthesis is an essential metabolic step in protein glycosylation. The current defect leads to a severely abnormal glycosylation state already in the early phase of the *N*-glycan biosynthesis pathway in the endoplasmic reticulum. We detected high expression of *SRD5A3* in foetal brain tissue, especially in the cerebellum, consistent with the finding of the congenital cerebellar malformations. Based on the overlapping clinical, biochemical and genetic data in this large group of patients with congenital disorders of glycosylation, we define a novel syndrome of cerebellar ataxia associated with congenital eye malformations due to a defect in dolichol metabolism.**

**Keywords:** congenital disorders of glycosylation; *SRD5A3*-CDG; CDG type Iq; glycosylation; dolichol metabolism; polyprenol reductase; *SRD5A3*, vermis hypoplasia; coloboma; cataract; glaucoma

**Abbreviations:** CDG = congenital disorders of glycosylation; CHARGE = coloboma, heart anomaly, choanal atresia, retardation, genital and ear anomalies; PCR = polymerase chain reaction

## Introduction

Congenital disorders of glycosylation (CDG), a group of inborn errors of metabolism due to defects in the biosynthesis of glycans, was first described by Jaeken *et al.* (1987). Since the original description, 16 different CDG type I subtypes have been delineated (Jaeken and Matthijs, 2007; Haeuptle and Hennet, 2009; Rind *et al.*, 2010) with a glycosylation defect in endoplasmic reticulum or cytoplasm. The phenotypic spectrum of this growing group of inborn errors is highly heterogeneous. In addition to the classical symptoms of developmental delay and cerebellar malformations, children with the most common defect, PMM2-CDG (CDG type Ia), frequently have ocular involvement and visual impairment (De Lonlay *et al.*, 2001; Morava *et al.*, 2009). Cataract and visual impairment occur, but relatively late in the course of the disease as a consequence of slowly progressive adolescent retinopathy.

In contrast to variable neurological involvement and late onset ophthalmological symptoms in most patients with PMM2-CDG, a distinct clinical phenotype was observed in a group of patients with CDG with an abnormal type I transferrin isoelectric focusing profile (CDG type Ix, MIM 212067; Assmann *et al.*, 2001; Prietsch *et al.*, 2002; Morava *et al.*, 2008, 2009). In this novel syndrome, besides developmental delay and ataxia, early visual impairment developed due to severe ocular malformations, optic nerve hypoplasia or neuropathy. Some of the children had unique findings, including congenital cataract, retinal (uveal) coloboma and glaucoma. Other overlapping symptoms included a variable degree of vermis hypoplasia and systemic involvement. This syndrome is similar to the combination of symptoms described by Al-Gazali *et al.* (2008) consisting of ocular colobomas, ichthyosis, cardiac anomalies and developmental delay, observed in dysmorphic children from a large inbred Emirati family of Baluchi origin. In this large family, cerebellar vermis malformations were noted in association with polymicrogyria, midline brain malformations and endocrine abnormalities. The underlying glycosylation abnormality (abnormal type I transferrin isoelectric focusing profile; CDG type I) and the genetic defect were not identified in the original publication.

A recent classification of CDGs divides the defects into four major biochemical categories: three involving protein glycosylation (disorders of *N*-linked glycosylation, *O*-linked glycosylation and combined defects in *N*- and *O*-glycosylation) and one involving lipid-glycosylation (Jaeken *et al.*, 2008). To identify defects in the endoplasmic reticulum and Golgi parts of the *N*-glycan biosynthesis pathway, plasma transferrin isoelectric focusing is used as a simple and reliable biochemical screening tool for CDG (Freeze and Aebi, 2005; Babovic-Vuksanovic and O'Brien, 2007). Enzyme measurements and lipid-linked oligosaccharide analysis unravelled the primary defect in most CDG-I cases. Nevertheless, still many patients remained without causative gene defect, the CDG-Ix (MIM 212067) group. Identification of a dolichol kinase deficiency in four patients (Kranz *et al.*, 2007) highlighted the possibility of novel CDG-I gene defects in dolichol metabolism. Dolichol phosphate is the first committed metabolite for synthesis of the lipid-linked oligosaccharide in the endoplasmic reticulum. In search for the long-sought polyprenol reductase, we identified a novel gene in this pathway (Cantagrel *et al.*, 2010). The gene *SRD5A3* encodes steroid 5 $\alpha$ -reductase type 3, an enzyme required for conversion of polyprenol to dolichol (Cantagrel *et al.*, 2010).

We report on two additional patients with *SRD5A3* mutations and describe the distinctive clinical, biochemical and genetic data from a total of 12 children from nine families with steroid 5 $\alpha$ -reductase type 3 deficiencies. We define a novel autosomal recessive disorder of glycosylation and cerebello-ocular syndrome: *SRD5A3*-CDG. The discovery of *SRD5A3* mutations in such a large group of hitherto unsolved patients with a congenital disorder of glycosylation emphasizes the importance of this novel metabolic syndrome.

## Patients and methods

### Patients with *N*-glycosylation disorder

The first group of patients included in the study were diagnosed with CDG type Ix and a further undefined *N*-glycosylation defect. Based on

the clinical presentation of either ophthalmological malformations or cerebellar involvement associated with CDG type Ix, we evaluated the clinical features of 12 patients from nine families (Tables 1 and 2).

Clinical features of several patients have been described previously as case reports. This concerns the patients of Families 7 (female, Prietsch *et al.*, 2002) and 9 (male, Assman *et al.*, 2001). A cohort of patients with CDG-Ix was described later including patients of Families 3 (two females), 7 and 9 [Patients 24, 25, 26 and 27, respectively, in Morava *et al.* (2009), the same children as Patients 3, 4, 5 and 7 in Morava *et al.* (2008); Tables 1 and 2]. Patients from Families 1 (brother and sister) and 2 (brother and sister) have been described by Al-Gazali *et al.* (2008), but the underlying metabolic defect was not reported in their original publication. In a separate article delineating the role of the *SRD5A3* gene as a polyprenol reductase, we included data on Families 1–6 and 8 (Cantagrel *et al.*, 2010).

The age of the patients varied between 6 months and 12 years, and 7 out of the 12 patients were females. All were born at term to healthy parents. Eight out of the nine families were consanguineous (Assman *et al.*, 2000; Prietsch *et al.*, 2002; Al-Gazali *et al.*, 2008; Morava *et al.*, 2008, 2009; Cantagrel *et al.*, 2010).

## Patients with a clinical suspicion of CHARGE syndrome

Coloboma, heart anomaly, choanal atresia, retardation, genital and ear anomalies (CHARGE) syndrome is a multiple malformation syndrome that shows phenotypic overlap with the here-presented cerebello-ocular syndrome (Sanlaville and Verloes, 2007). Upon informed consent, we selected 25 individuals from a cohort of patients clinically suspected of CHARGE syndrome but negative for *CHD7* mutations (Visser *et al.*, 2004). The patients had colobomas associated with mental retardation and/or growth retardation, with or without other manifestations of the CHARGE syndrome spectrum. Detailed clinical information was available in all individuals. The patients were not screened for glycosylation defects prior to *CHD7* analysis, except for two cases (revealing no abnormalities). *CHD7* sequencing and multiplex ligation-dependent probe amplification analysis (to exclude whole-exon deletions) were performed in the Human Genetics Department of the Radboud University Nijmegen Medical Centre as described in Jongmans *et al.* (2006).

## Congenital disorders of glycosylation diagnostics

Abnormal *N*-glycosylation was identified by isoelectric focusing of serum transferrin (de Jong, 1994) showing a CDG type I profile (Fig. 1). Secondary causes for type I profiles (alcohol abuse, fructosuria or galactosaemia) were excluded. Analyses of lipid-linked oligosaccharides, formation of Dol-PP-GlcNAc<sub>1</sub> and Dol-PP-GlcNAc<sub>2</sub> (Bickel *et al.*, 2005), elongation of Dol-PP-GlcNAc<sub>2</sub> to Dol-PP-GlcNAc<sub>2</sub>Man<sub>5</sub> by cytosolic mannosyltransferases (Schwarz *et al.*, 2004) and oligosaccharyltransferase (Knauer *et al.*, 1994) were performed in fibroblasts of Families 3, 5 and 6.

## Clinical chemistry parameters

Routine diagnostic studies were obtained in patients with *N*-glycosylation disorders and included blood cell count, haemoglobin, creatine kinase, liver enzymes, electrolytes, kidney function, antiprothrombin, activated partial thromboplastin time, cholesterol, triglycerides, thyroid-stimulating hormone, free T4 and albumin level

**Table 1** Laboratory findings and genetic alterations in *SRD5A3* defect

Family (gender)	Patients								
	1 (F and M) <sup>Al-Gazali</sup>	2 (F and M) <sup>Al-Gazali</sup>	3 (F and F) <sup>Morava</sup>	4 (M)	5 (M)	6 (F) <sup>Morava</sup>	7 (F) <sup>Prietsch</sup>	8 (F)	9 (M) <sup>Assmann</sup>
Ethnic background	Baluchi <sup>a</sup>	Baluchi <sup>a</sup>	Polish <sup>a</sup>	Turkish <sup>a</sup>	Polish	Turkish <sup>a</sup>	Turkish <sup>a</sup>	Turkish <sup>a</sup>	Turkish <sup>a</sup>
Laboratory findings									
Elevated liver enzymes	+	NA	+	+	+	+	–	+	+
Abnormal coagulation	NA	NA	+	+	+	+	–	+	+
Decreased AT3/Proteins C and S	NA	NA	+	+	+	+	–	+	+
Microcytic anemia	+	+	–	+	–	–	NA	+	–
Type I TIEF pattern	+	+	+	+	+	+	+	+	+
Normal LLO analysis <sup>b</sup>	+	+	+	+	+	+	+	+	+
Genetic findings									
Mutation	c.286_288 delinsTGAAGTAAAGGC		c.292_293del	c.320G>A	c.424 C>T/ c.489 C>A	c.29 C>A	c.57G>A	Genomic rearrangement	c.57G>A
Mutation at the protein level	p.Gln96delinsX		p.Leu98Val fsX121	p.Trp107X	p.Arg142X p.Tyr163X	p.Ser10X	p.Trp19X	Absent	p.Trp19X
mRNA level (fibroblasts)	Decreased	Normal	Normal	NA	Decreased	NA	Normal	Decreased	NA

<sup>a</sup> Consanguinity.

<sup>b</sup> Analyses of lipid-linked oligosaccharides (LLOs): normal results for elongation of Dol-PP-GlcNAc<sub>2</sub> to Dol-PP-GlcNAc<sub>2</sub>Man<sub>5</sub> by cytosolic mannosyltransferases (Schwarz *et al.*, 2004) and oligosaccharyltransferase (Knauer *et al.*, 1994).

F = female; M = male; NA = not assessed. TIEF = transferrin isoelectric focusing.

**Table 2** Neurological and ophthalmologic anomalies in SRD5A3 defect

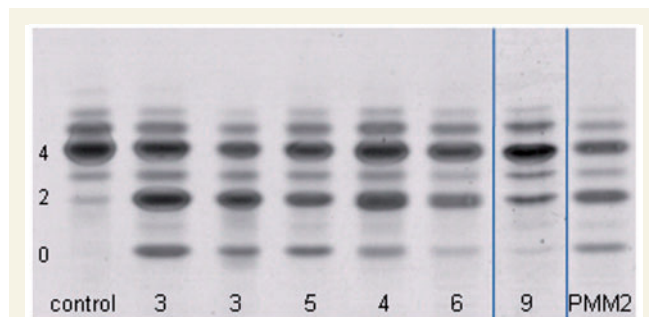
Family	Patients											
	1 <sup>Al-Ghazali</sup>		2 <sup>Al-Ghazali</sup>		3 <sup>Morava</sup>		4	5	6 <sup>Morava</sup>	7 <sup>Prietch</sup>	8 <sup>Assmann</sup>	
	F	M	F	M	F	F	M	M	F	F	F	M
Neurological findings												
Muscle hypotonia	+	–	+	–	+	+	+	+	+	+	+	+
Motor retardation	–	–	–	–	+	+	+	+	+	+	+	+
Intellectual disability	+	+	+	+	+	+	+	+	+	+/–	+	+
Cerebellar vermis atrophy or vermis hypoplasia <sup>a</sup>	– <sup>a</sup>	+	+	–	+	NA	– <sup>a</sup>	–	– <sup>a</sup>	+	+	–
Global cerebellar atrophy or cerebellar hypoplasia	–	+	+	–	–	–	+/NA	–	–	–	–	–
Cerebellar ataxia	+	+	+	+	+	+	–	+	+	NA	+	+
Spasticity	+	–	+	–	+	–	+	–	–	–	–	+
Movement disorder	–	–	–	–	+	–	+	–	–	+	–	–
Stereotypic movements	–	–	–	–	+	+	–	–	–	+	–	–
Ophthalmological findings												
Visual impairment	+	–	+	+	+	+	+	+	+/– <sup>c</sup>	+	+	+
Optic disc hypoplasia	–	–	–	–	+	+	–	+	–	–	–	–
Coloboma (iris) <sup>b</sup>	+	–	+	–	–	–	+	–	–	–	+	–
Coloboma (chorio-retinal) <sup>b</sup>	+	+	+	–	–	–	+	–	–	–	+	–
Nystagmus	+	+	+	+	+	+	+	+	+	+	+	+
Optic nerve hypoplasia/ atrophy	+	+	+	+	+	–	–	+	–	+	–	+
Microphthalmia	–	–	–	+	–	–	–	–	–	–	+	–
Cataract	–	–	–	–	–	–	+	–	–	–	+	–
Glaucoma	–	–	–	–	–	–	–	–	+	–	–	–

a Retrocerebellar cyst.

b Bilateral in all cases.

c Transient visual loss prior to glaucoma operation.

F = female; M = male; NA = not assessed.



**Figure 1** Transferrin isoelectric focusing shows a type I profile with increased levels of asialo- (0) and disialotransferrin (2) in Patients 3–6 and 9.

measurements. Additional coagulation studies included antithrombin 3, factor IX and XI, protein C and S analysis.

## Homozygosity mapping

Genomic DNA was extracted from peripheral blood lymphocytes using standard salting out procedures (Miller *et al.*, 1988). Genotyping was performed by Affymetrix GeneChip Mapping 10K 2.0 array. All single nucleotide polymorphism array experiments were performed and analysed according to the manufacturer's protocols (Affymetrix, Santa Clara, CA, USA). Copy number estimates were determined using the Copy Number Analyser for GeneChip<sup>®</sup> software package (v2.0) (Nannya *et al.*, 2005). Homozygosity mapping was performed

using PLINK v1.06, a tool set for whole-genome association and population-based linkage analyses (Purcell *et al.*, 2007) using a homozygous window of 50 single nucleotide polymorphisms tolerating two heterozygous single nucleotide polymorphisms and 10 missing single nucleotide polymorphisms per window.

## Short-tandem repeat marker analysis

Primers to amplify polymorphic short-tandem repeat markers on 4q12-q21.21 were designed by using the Primer3 program ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)) (Rozen and Skaletsky, 2000). An M13 tail was added to the 5'- and 3'-ends of the primers. Markers were amplified by using an M13 forward primer labelled with one of the fluorophores, FAM, VIC, NED or ROX, at the 5'-end, and a M13 reverse primer with a 5'-gtttctt-3' added to its 5'-end to reduce tailing (Oetting *et al.*, 1995; Brownstein *et al.*, 1996). Primer sequences are shown in Supplementary Table 2. Polymerase chain reaction (PCR) conditions are available on request. Final PCR products were mixed with eight volumes of formamide and half a volume of Genescan<sup>™</sup> 500(-250) LIZ size standard (Applied Biosystems, Foster City, USA), and analysed with the ABI PRISM 3730 DNA analyser (Applied Biosystems, Foster City, USA). The results were evaluated by Genemapper (Applied Biosystems, Foster City, USA).

## SRD5A3 expression analysis in fibroblasts and different brain areas

SYBR Green-based real-time quantitative PCR expression analysis was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems,



Foster City, CA, USA) by using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Primers were developed by the primer3 programme ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)) (Rozen and Skaletsky, 2000) and validated as described previously (de Brouwer *et al.*, 2006). Primer sequences are given in Supplementary Table 2. PCR products encompassed at least one boundary between two exons. *GUSB* was used as a reference gene (de Brouwer *et al.*, 2006). Total RNA from fibroblasts was isolated by using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. To remove residual traces of genomic DNA, the RNA was treated with DNase I (Invitrogen, Leek, Netherlands) while bound to the RNA binding column. The integrity of the RNA was assessed on 1.2% agarose gel, and the concentration and purity determined by optical densitometry. Total RNA from different human brain areas was purchased from Stratagene (La Jolla, CA, USA), except for hippocampus, thalamus and spinal cord total RNA that was ordered from Biochain (Hayward, CA, USA). Total RNA from different human adult tissues was ordered from Stratagene Europe (Amsterdam, Netherlands). Total RNA was transcribed into complementary DNA by using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. Complementary DNA was purified by using the NucleoSpin extract II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Quantitative PCR quantifications were performed in duplicate on the equivalent of 12.5 ng total RNA input. Experimental threshold cycle (Ct) values were within the range of complementary DNA dilutions used to validate the primers. The melt curves of all PCR products showed a single PCR product. All controls were negative. Differences in expression of a gene of interest between two samples were calculated by the comparative threshold cycle or  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001). *SRD5A3* expression in patient fibroblasts was compared to the average expression in eight separately measured control fibroblasts and the *P*-value derived from the standard score (*Z*-value).

## Sequence analysis

The sequence of the *SRD5A3* gene was analysed using primers flanking the coding exons to generate PCR products. After purification, the sequence reaction was performed using the bigDyeTerminator kit (Life Sciences, Krimpen aan de IJssel) according to the manufacturer's instructions. The resulting fragments were run on an ABI 3730 machine. Primers and conditions are available in Supplementary Table 2.

## Results

### Biochemical analysis

Based on the increased asialo- and disialo-transferrin isoforms (Fig. 1), a glycosylation anomaly consistent with a defect in cytoplasm or endoplasmic reticulum was present in 12 patients. Analyses of lipid-linked oligosaccharides, elongation of Dol-PP-GlcNAc<sub>2</sub> to Dol-PP-GlcNAc<sub>2</sub>Man<sub>5</sub> by cytosolic mannosyltransferases (Schwarz *et al.*, 2004) and oligosaccharyltransferase (Knauer *et al.*, 1994) were unremarkable. Based on the biochemical pattern, the patients were diagnosed with an unsolved type of congenital disorder of glycosylation, CDG type Ix. The genetic

defect in this group of patients was shown to reside in the polyprenol reductase *SRD5A3* (Cantagrel *et al.*, 2010). A slight increase in the levels of plasma polyprenols (chain length 18–20) was detected by tandem-mass spectrometry for all patients investigated.

### Molecular findings

In Families 6 and 3 (Table 1), the largest homozygous region, consisting of 181 (57 Mb) and 97 (26 Mb) homozygous single nucleotide polymorphisms, respectively, was found on chromosome 4 and these two regions overlapped at 4q12–q21.21. Short tandem repeat marker analysis confirmed homozygosity of 4q12 between markers D4S2916 and D4S1569. In Family 7, 16 consecutive single nucleotide polymorphisms were found to be homozygous in this region, pointing to a 2.9 Mb region on chromosome 4 delimited by D4S2916 and rs951232. There were no other overlapping homozygous regions in these three families. The overlapping homozygous region contained 21 genes, none of which was known to be involved in the metabolism of dolichol until the recent identification of *SRD5A3*.

### Sequence analysis of *SRD5A3*

Direct DNA sequencing of the five exons, intron–exon boundaries and branch sites in the affected family members of Families 3 and 6, revealed homozygous changes (c.292\_293del; p.Leu98ValfsX121 in Family 3, c.29 C>A; p.Ser10X in Family 6, also described by Cantagrel *et al.*, 2010; Table 1). We could identify an additional homozygous mutation for Family 7 (c.57G>A; p.Trp19X). All three changes result in a premature termination codon and are thus predicted to result in nonsense-mediated decay.

An additional homozygous c.57G>A (p.Trp19X) mutation was identified for Family 9, identical to the mutation in Family 7. In three unrelated patients with CDG type Ix with a similar phenotype, three additional changes were identified. One patient was compound heterozygous [c.(424C>T; 489 C>A); p.(Arg142X; p.Tyr163X)]. Further homozygous mutations were detected in Family 4 (c.320G>A; p.Trp107X) and the original family of Al-Gazali *et al.* (2008) (Families 1 and 2, c.402\_404 delinsTGAGTAAGGC; p.Gln96delinsX). A complex rearrangement was shown in exon 5 in a patient from Family 8 (Cantagrel *et al.*, 2010).

Sequencing of the *SRD5A3* gene did not reveal any mutations in the 25 patients with a clinical suspicion of CHARGE syndrome, despite the significant overlap in clinical presentation of the two patient groups.

### Expression of *SRD5A3* in patient fibroblasts

Expression analysis of *SRD5A3* expression in fibroblasts by quantitative PCR in Families 1 and 2, and in the patients from Families 5 and 8 showed a significant reduction (Cantagrel *et al.*, 2001). Additional studies in the two patients from Family 3 and the one from Family 7, however, did not show a significant reduction in *SRD5A3* expression [the expression level was 149% (*P*=0.20),

91% ( $P=0.69$ ) and 97% ( $P=0.86$ ) of normal, respectively (Table 1)].

## Expression of *SRD5A3* in different human adult tissues and brain areas

Expression levels of *SRD5A3* were analysed by quantitative PCR in a selection of normal human adult tissues. *SRD5A3* showed highest expression in the brain (Fig. 2). Medium expression levels were found in the retina and heart and low expression levels in other tissues tested including the liver. Comparing the *SRD5A3* expression levels between several adult brain areas showed highest expression levels in the hippocampus and cerebellum. In addition, we show that the expression of *SRD5A3* in the foetal brain is much higher than in adult whole brain, indicating a function for *SRD5A3* in brain development, perhaps specifically for the cerebellum and hippocampus.

## General clinical findings

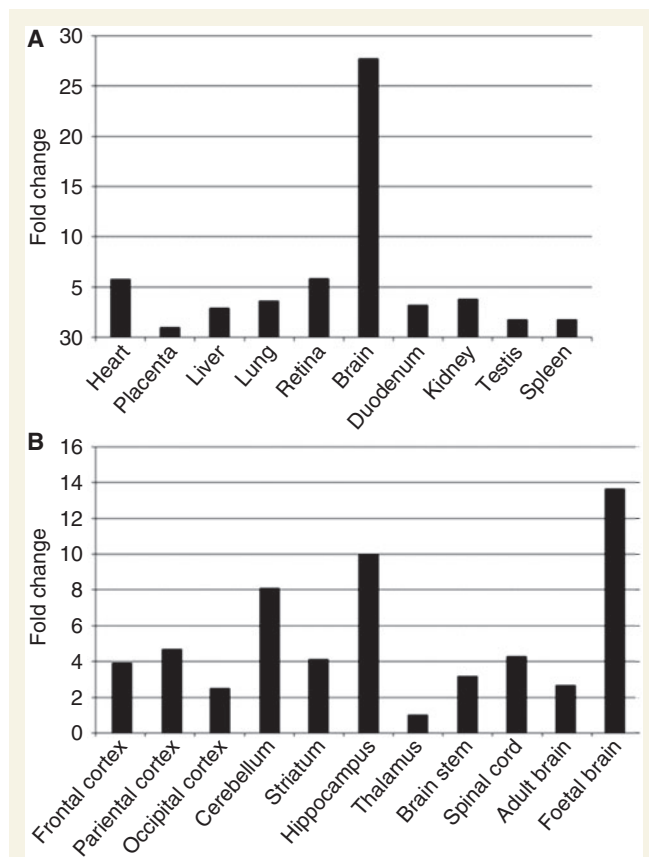
The 12 patients included in the study all came early to medical attention due to developmental delay and suspected visual problems. Most were initially seen by the clinical geneticist. The majority had deep-set eyes, hypertelorism and malformed

and/or prominent ears. No abnormal fat distribution was noted and inverted nipples were present only in the index patient from Family 3. All patients had variable degree of visual impairment and nystagmus detectable from the neonatal-infantile period. Muscle hypotonia and motor developmental delay was characteristic. Growth delay was present in five patients, including all from Family 1. Dysmorphic features were variable and mostly subtle (Fig. 3). None of the patients showed kidney, bowel or liver involvement or skeletal malformations. Only three had congenital cardiac malformations (atrial septal defect, transposition of the great arteries and pulmonary valve defect). Midline malformations, genital anomalies (micropenis, undescended testis), corneal opacities and endocrine anomalies were present in only the index patients reported by Al-Gazali *et al.* (2008). Microcytic anaemia was mostly transient. Ichthyosiform erythroderma was strikingly common (5 out of 12) and therapy resistant. Skin abnormalities were the most severe in Patient 8, showing fluctuating severity of ichthyosiform erythroderma with severe scaling, itching and irritability (Fig. 2, Table 2 and Supplementary Table 1). The most severe multisystem involvement was present in one of the siblings from Family 1 and one sibling from Family 2 and in the patient from Family 8. There was a high intra-familial variation in Families 1 and 2 (Supplementary Table 1).

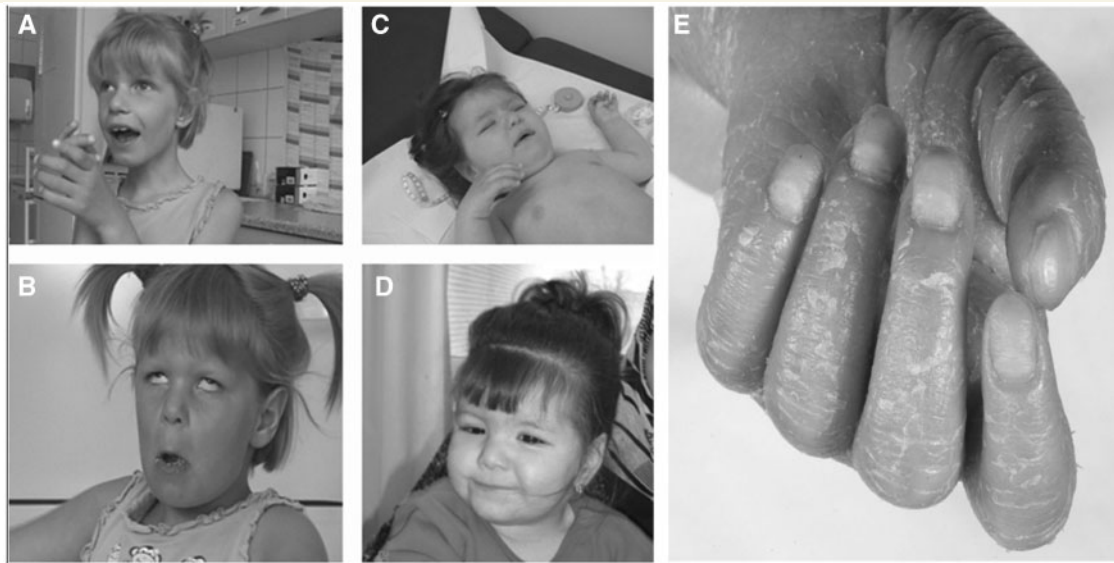
## Development, neurological findings and progression

The patients showed no significant progression of their clinical features throughout the course of their disease follow-up. All patients presented with early clinical symptoms, mostly muscle hypotonia and visual problems in the first 6 months of life. Several had a severe delay in early motor development. The visual impairment played a significant role in the psychomotor developmental delay, especially in the two sisters of Family 3. The patient with a severe truncating mutation (homozygous c.29 C>A; p.Ser10X) has a developmental delay, but continuously gains skills. She showed improvement in motor development after her glaucoma operation. At the age of 4 years, she attends a special school, feeds herself and walks without support. However, she has a severe speech delay and uses only two words. The oldest patient under regular follow-up is currently 11 years old. Despite her severe genetic alteration (c.292\_293del; p.Leu98ValfsX121) and a severe visual impairment, she is able to walk, she can make short sentences, attends special education and feeds normally. Most patients walk unaided despite significant cerebellar vermis atrophy. Patient 8 does not sit without support, does not crawl or walk at age 5.5 years. She vocalizes, but remains non-verbal.

Neurological involvement showed interindividual variability, also within families. This was observed in Families 1 and 2, and also in Family 3. Learning problems varied from mental retardation to low normal intellect (IQ: 56–72), and motor development was generally delayed. All patients had delayed speech development, and 4 developed no speech at all. Cerebellar ataxia was found in 10 patients, following the initial features of hypotonia, nystagmus and motor developmental delay. Ataxia involved the trunk more than the extremities and appeared non-progressive during



**Figure 2** Expression of *SRD5A3* in adult tissues (A) and selected brain areas (B). Expression levels are indicated as the fold change in comparison to the tissue or area with the lowest expression level.



**Figure 3** (A–D) Facial features of patients from Family 3, and patient from Family 6 (at 6 months and 3 years). Note deep-set eyes and strabismus. Short nose in all patients, somewhat larger, posterior-rotated ears (A and B) in patients from Family 3, no significant dysmorphic features. (E) Severe ichthyosiform abnormalities of the hand in Patient 8.

follow-up. Five patients also suffered from mild spastic diplegia. Other movement abnormalities, mainly stereotypic or dystonic movements, were only sporadically encountered. Epilepsy and hearing loss were both uncommon. One of the sisters from Family 3 showed absence seizures, which improved significantly in time, and one patient had sensorineural hearing loss, documented by abnormal auditory evoked potentials, but not requiring hearing-aid use (Table 2).

## Cerebral imaging

The CNS imaging abnormalities were highly variable, including variable cerebellar hypoplasia/atrophy in several cases, in combination with cerebellar vermis hypoplasia in five patients (Table 2 and Supplementary Table 1). Two patients (from Families 6 and 9) had completely normal MRI results at the age of 2 and 4 years. Eight patients had clinical features of cerebellar ataxia. Only the index patients reported by Al-Gazali (2008) had midline malformations. Three patients had a retrocerebellar cyst without cerebellar vermis hypoplasia (Table 2). Cortical malformations were observed in 1 out of 11 evaluated cases (one male patient from Family 1, Al-Gazali *et al.*, 2008). None of the patients had white matter abnormalities. Somewhat delayed myelination, possibly within the normal range for the age, in association with spasticity was found in one patient from Family 5. Unfortunately, this patient was not available for follow-up (Table 2, Fig. 4).

## Ophthalmological findings

All patients showed early signs of visual impairment with late development of following objects and focusing and variable degree of nystagmus. Visual evoked potentials were performed in the index patient of Families 3 and 6, both showing a mixed

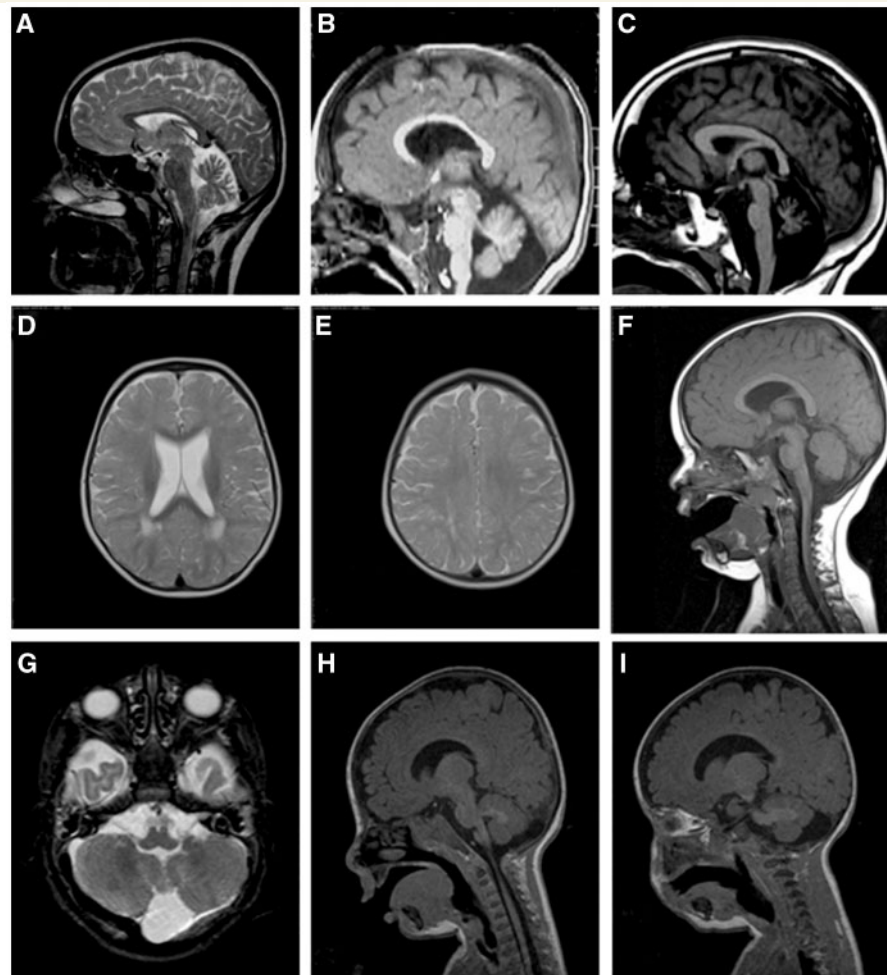
abnormality with peripheral and central visual impairment. Detailed ophthalmological evaluation was requested in all patients. Coloboma of the iris in association with retina or choroid coloboma was present in five patients. In all but three patients coloboma and/or hypoplasia or atrophy of the optic disc and nerve were present. Unique findings, including microphthalmia in two patients and infantile cataract in two patients (as an additional finding to colobomas) and a congenital glaucoma in the index patient from Family 6 were discovered during infancy and successfully treated surgically. No progression of the ophthalmological findings has been observed in any of the patients (Table 2 and Supplementary Table 1).

## Laboratory findings

Liver enzyme activities and coagulation factors have not been assessed in all patients. However, all nine evaluated patients had elevated levels of liver enzymes (aspartate amino transferase 75–220 U/l, C:<50 U/l). Decreased antithrombin 3 levels were present in all six patients studied for coagulation abnormalities. Mild microcytic anaemia ranging from 67–79 fl (mean corpuscular volume controls: 85–100 fl) was also a characteristic symptom.

## Discussion

Based on an evolving distinct phenotype in 12 children (out of nine families) with a consistent biochemical phenotype, we define a novel cerebello-ocular syndrome. This novel inborn error of glycosylation presents with psychomotor retardation, cerebellar ataxia, nystagmus, visual impairment, variable congenital eye malformations, ichthyosiform skin disorder and abnormal coagulation parameters.



**Figure 4** (A–C) T<sub>1</sub>-weighted sagittal MRI images indicating variable degree of cerebellar and vermian hypoplasia/atrophy in patients from Families 6, 3 and 1, a spectrum from mild to severe vermian abnormality. (D and E) T<sub>2</sub>-weighted axial MRI brain images showing increased signal intensity in the parieto-occipital white matter and a small lesion with increased signal intensity left, frontal at the age of 18 months. (F) Slightly enlarged cisterna magna, normal infra-tentorial structures, including normal cerebellum on a T<sub>1</sub>-weighted sagittal image in Patient 5. (G) T<sub>2</sub>-weighted axial MRI image indicating retrocerebellar arachnoidal cyst, otherwise normal images of the brain, including white matter signal intensities and gross anatomy of the cerebellum at the age of 4 months in Patient 4. (H and I) T<sub>1</sub>-weighted para-sagittal MRI image of a retrocerebellar arachnoidal cyst at 2.8 years in Patient 6.

Cerebellar ataxia, cerebellar atrophy and congenital vermian malformation are characteristic features of CDG, especially the glycosylation defects localized in the endoplasmic reticulum. The cerebellar atrophy is usually slowly progressive; however, vermian hypoplasia might lead to severe restriction in motor development, especially of independent movement. Ataxia in CDG syndrome presents early in life and several patients demonstrate nystagmus as the first clinical feature. This feature was present in most of the children in our patient group, which in combination with visual disturbance initiated genetic and metabolic investigations.

The striking ophthalmological malformations are unique, even within the CDG group. Optic atrophy and progressive visual impairment have been observed in patients with PMM2-CDG (CDG-Ia), ALG3-CDG (CDG-Ic) and DMP1-CDG (CDG-Ie), and in so far unsolved endoplasmic-associated defects (Garcia-Silva *et al.*, 2004; Schollen *et al.*, 2005; Morava *et al.*, 2009).

Cataract has been described in ALG8-CDG (CDG-Ih) and as a later presentation in PMM2-CDG. Congenital colobomas, however, are extremely rare in CDG syndromes (De Lonlay *et al.*, 2001; Thiel *et al.*, 2003; Eklund *et al.*, 2005; Morava *et al.*, 2008). Glaucoma has not yet been described in other types of CDG defects (Morava *et al.*, 2008). Discriminating features are of diagnostic importance in patients with a suspected endoplasmic reticulum-associated CDG defect; based on eye symptoms, the abnormal transferrin isoelectric focusing profile, plasma polyprenols and clinical grounds, direct molecular analysis will lead to the diagnosis.

The dolichol biosynthesis pathway contains many unknown enzymatic activities. The identification of novel defects in this part of the *N*-glycan biosynthesis pathway in patients with CDG-Ix has been hampered by the complexity of the pathway and the technical challenge to measure several dolichol-related components.



Identification of *SRD5A3*, essential for the conversion of polyprenol to dolichol (Cantagrel *et al.*, 2010), elucidated the defect in a large cohort of patients with unsolved CDG-Ix with specific clinical symptoms. Specific clinical features may relate to the various roles of dolichol in a cell. Dolichol is a general constituent of membranes (Rip *et al.*, 1985). Furthermore, dolichol is required to form dolichol phosphate, the precursor of four different glycosylation routes. Besides *N*-glycosylation, glycosylphosphatidylinositol-anchor formation, *C*- and *O*-mannosylation depend on the availability of dolichol phosphate mannose. *O*-mannosylation defects are known in a different group of disorders, the dystroglycanopathies (Walker–Warburg syndrome, muscle–eye–brain syndrome), with a defect in the *O*-mannosylation of  $\alpha$ -dystroglycan. These disorders present with congenital muscular dystrophy, congenital brain and eye malformations and glycosylation defects. Patients with Walker–Warburg or muscle–eye–brain syndromes present with microphthalmia, cataract and frequently also with colobomas, and show cerebral cortex and cerebellar developmental defects (García-Silva *et al.*, 2004; van Reeuwijk *et al.*, 2006; Lefeber *et al.*, 2009). It is important to note that none of these patients express glycosylation abnormalities in blood. Furthermore, in our study, none of the patients showed the third diagnostic sign of muscular dystrophy. No progressive muscular atrophy was noted and creatine kinase levels were normal in all investigated cases (6 out of 12). No muscle biopsies were, therefore, performed in the patients. Cobblestone lissencephaly was not observed in our patients. Synthesis of glycosylphosphatidylinositol-anchored proteins also requires Dol-P-Man. No clinical overlap was observed with the known Phosphatidylinositol glycan, class M gene (MIM 610273) defect in the glycosylphosphatidylinositol-biosynthesis pathway and analysis of leucocyte glycosylphosphatidylinositol-anchored proteins (CD59 and CD24) in two patients showed normal results (Patients 3 and 6; data not shown).

The phenotype of the patients showed an interesting overlap with CHARGE syndrome. Important characteristics of both syndromes are coloboma, balance problems, swallowing difficulties and growth and developmental delay. In the current group of patients with CDG none had choanal atresia, and in the investigated group of patients with a suspicion of CHARGE syndrome, only 5 of the 25 children showed choanal abnormalities. Furthermore, several patients had prominent dermatological manifestations in the form of ichthyosiform erythroderma, which is not a common feature in CHARGE syndrome. Mutations in the *CHD7* gene, usually found in 60–70% of patients suspected for CHARGE syndrome, were excluded, making the patients good candidates for a *SRD5A3* defect. However, none of the 25 *CHD7* negative patients, evaluated in the current study, carried a *SRD5A3* mutation. This finding emphasizes the importance of metabolic screening prior to mutation analysis.

Uveal colobomas can arise between the fifth and seventh week of gestation due to incomplete closure of the optic or choroidal fissures. Depending on the position where the fissure does not close, an iris coloboma or a chorio-retinal coloboma will occur. Coloboma is sometimes found in association with microphthalmia, cataract, nystagmus or glaucoma. This combination occurred in Families 1, 2, 4 and 8. The occurrence of uveal colobomas in patients with a defect in *SRD5A3* suggests important roles for

polyprenol reductase and for dolichol-dependant biochemical pathways in the early development of the eye, and more specifically in the closure of the choroidal fissure. A broad spectrum of cerebello-ocular syndromes is known, demonstrating variable eye malformations, and a different degree of cerebellar involvement, such as cerebellar vermis hypoplasia, oligophrenia, congenital ataxia, ocular coloboma, hepatic fibrosis (COACH; MIM 216360) and Joubert syndrome (MIM 213300). Based on the similar cerebello-ocular involvement in Joubert-like patients (Kroes *et al.*, 2005) and the very severe cerebellar vermis malformation in one of the *SRD5A3*-CDG patients, Joubert syndrome could theoretically be a possible clinical presentation of the *SRD5A3* defect. One should note, however, that the so-called molar tooth sign (the clinical hallmark of Joubert syndrome) was not present on the brain MRI of any of the patients. The association of abnormal glycosylation and a Joubert-like phenotype is also unique (Morava *et al.*, 2004).

Three siblings have been reported with a novel mental retardation syndrome, juvenile cataract, iris coloboma and joint contractures (Kahziri *et al.*, 2009). Linkage analysis revealed a 10.4 Mb interval of homozygosity in the pericentromeric region of chromosome 4 flanked by single nucleotide polymorphisms rs728293 (4p12) and rs1105434 (4q12). This interval also contains the *SRD5A3* gene, suggesting that these patients might have the same disease as the patients in our study.

In our unique group of patients with a novel biochemical defect of the dolichol pathway, the underlying inborn error of a polyprenol reductase leads to a defect of protein glycosylation. In this long searched for metabolic route of dolichol synthesis, all patients were diagnosed with functional 'null-mutations'. This implies that a milder phenotype may be anticipated in patients with less severe *SRD5A3* mutations. The full clinical spectrum of this disease has yet to be explored. Our patients showed a substantial phenotypic variability despite the presence of truncating mutations in all of them. Some of the patients had severe multisystem disease, while others only had mild neurological symptoms, learned to walk and survived to adolescence. No genotype–phenotype correlation was found with respect to the clinical or the biochemical findings. An alternative pathway for dolichol biosynthesis as suggested by Cantagrel *et al.* (2010) could help to explain these findings. Also a second disease may have aggravated the clinical presentation in patients from the eight consanguineous families.

We suggest screening patients with the combination of congenital coloboma, cerebellar abnormalities, ichthyosis and developmental or growth delay first via glycosylation studies. Analysis of plasma polyprenols and subsequent molecular genetic analysis of the *SRD5A3* gene may lead to the diagnosis. The diagnostic criteria of this novel inborn error of glycosylation, *SRD5A3*-CDG, are psychomotor retardation, nystagmus, visual impairment due to variable eye malformations, vermis anomalies and abnormal coagulation. Ichthyosiform skin lesions may support the clinical suspicion.

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## Supplementary material

Supplementary material is available at *Brain* online.

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