Pediatric RESEARCH

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CLINICAL RESEARCH ARTICLE

Ten years of screening for congenital disorders of glycosylation in Argentina: case studies and pitfalls

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BACKGROUND: Congenital Disorders of Glycosylation (CDG) are genetic diseases caused by hypoglycosylation of glycoproteins and glycolipids. Most CDG are multisystem disorders with mild to severe involvement.
METHODS: We studied 554 patients (2007–2017) with a clinical phenotype compatible with a CDG. Screening was performed by serum transferrin isoelectric focusing. The diagnosis was confirmed by genetic testing (Sanger or exome sequencing).
RESULTS: A confirmed abnormal pattern was found in nine patients. Seven patients showed a type 1 pattern: four with PMM2-CDG, two with ALG2-CDG, and one with classical galactosemia. A type 2 pattern was found in two patients: one with a CDG-IIx and one with a transferrin protein variant. Abnormal transferrin pattern were observed in a patient with a myopathy due to a COL6A2 gene variant.
CONCLUSIONS: CDG screening in Argentina from 2007 to 2017 revealed 4 PMM2-CDG patients, 2 ALG2-CDG patients with a novel homozygous gene variant and 1 CDG-IIx.

Pediatric Research (2018) 84:837–841; https://doi.org/10.1038/s41390-018-0206-6 Dedicated to Dr. Niels Suldrup (March, 2018), an excellent scientist and a very dear friend with whom we shared the effort and dedication to the study of CDG in our country.

INTRODUCTION

Congenital disorders of glycosylation (CDG) are genetic diseases due to deficient glycoprotein and glycolipid glycan synthesis and attachment. Altered protein glycosylation can be divided into N-glycosylation defects, O-glycosylation defects, and combined (reviews in ref.[1] and ref.[2]). Most are multisystem disorders with variable phenotype severity and neurological involvement. This makes diagnosis often challenging.[3,4]

Screening for N-glycosylation disorders is, as a rule, performed by glycan analysis of serum transferrin (Tf) via isoelectric focusing (IEF) or capillary zone electrophoresis.[5,6] A type 1 pattern points to a glycan assembly defect (CDG-I; cytosol and ER), a type 2 pattern to a glycan remodeling defect (CDG-II; Golgi or vesicular defect).[6–9] An abnormal pattern must be checked in a new serum sample for confirmation. Before considering genetic testing, a secondary CDG, such as galactosemia and fructose intolerance, has to be excluded.[9,10] Genetic testing is often performed by analyzing a CDG gene panel. If this is normal, whole exome or whole genome sequencing should be the next step.[11] The most frequent CDG-I is PMM2-CDG (phenotype MIM 212065).[7] Patients with a broad spectrum of clinical phenotypes usually remain undiagnosed in Latin American countries. During recent years, we have been contributing to a CDG Latin American research program to make advances in CDG diagnosis.[12–16] This work presents the results of 10 years of CDG screening in Argentina.

MATERIAL AND METHODS

This study included 554 patients referred by pediatricians from the Center for the Study of Congenital Metabolopathies (CEMECO), the Children's Hospital in Cordoba and other Argentinean medical centers. All patients presented multisystem phenotype and the main clinical manifestations were mild to severe psychomotor disability, hypotonia, seizures, failure to thrive, hormonal anomalies, coagulopathy, and cerebellar hypoplasia. Ethical permissions and informed consents were obtained from the institutional review boards of CIEIS- Ethics Committee, Children's Hospital, Cordoba, Argentina. Whole blood was obtained from donors after informed consent.

Transferrin analysis: Tf IEF, HPLC

Refs.[5,12,17] and Tf neuraminidase digestion[6,9] were performed according to standard methods.

Genetic studies

gDNA was obtained using a commercially available kit (Qiagen, Hilden, Germany). The primers for *PMM2* used followed the ENSEMBL database sequence (http://www.ensembl.org/index.html ENSG00000140650) and GenBank accession number NM_000303.2 and were analyzed by direct sequencing in an AB3130 system (Applied Biosystems).[7] The exome sequencing studies of the AR04 and AR05 patients were performed in collaboration with research centers using NimbleGen or Agilent

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Capture Arrays, in combination with sequencing on an Illumina HiSEQ sequencer. The bioinformatic analysis was made by bioinformaticians and the evaluation of variants by biochemists with a strong basis in cell biology.

RESULTS

Patients with N-glycosylation disorders

Patient AR01 was the first child of healthy non-consanguineous parents. The pregnancy was complicated by a HELLP syndrome. The patient was born at 36 weeks by caesarean section. His weight was 3.15 kg and he had difficulties establishing oral feeds. He was a floppy baby and initial investigation included a brain CT showing a cerebellum and corpus callosum hypoplasia. He presented with failure to thrive and vomiting. A gastrostomy was performed at 2 months of age. The post-surgery episode was complicated by hemorrhage. He presented hemorrhage and abnormal coagulation factors. He presented hypogammaglobulinemia (IgG 116 mg/dL (n.v. 700–1.700 mg/dL) and IgM 25 mg/dl (n.v. 50–300 mg/dL) and T lymphopenia with CD3 30% (n.v. 65–80%), CD4 22% (n.v. 30–50%), CD8 4% (n.v. 20–40%), elevated alanine transaminase (ALT) 277 U/L (n.v. 5–45 U/L), and decreased coagulation factors (proteins S and C, factors IX and XI). He presented decreased primary and absent secondary platelet aggregation. A severe multisystem phenotype with pericardial effusion, hypothyroidism, hypertension, and tubulopathy were observed in the neonatal period. Clinical examination showed macrocephaly, severe axial hypotonia with hyporeflexia, strabismus with optic nerve atrophy, and macular hypoplasia. At age 1 year, his weight was 10.6 kg and his height was 78.4 cm, blood pressure 126/82 with normal heart auscultation, and no hepatosplenomegaly. He had pronounced truncal hypotonia with normal peripheral reflexes. An echocardiography and an ECG showed hypertrophic cardiomyopathy. He presented an inguinal hernia and inverted nipples with increased gluteal fat pads (Fig. 1a). Platelet function (collaboration with the Institute of Hematology, National Academy of Medicine, Buenos Aires, Argentina), showed decreased primary aggregation and absent secondary aggregation in platelets stimulated with ADP, adrenalin, and collagen. Serum transferrin IEF (Fig. 2 line B) and HPLC (not shown) showed a type 1 pattern. Molecular diagnosis by Sanger sequencing revealed two known pathogenic missense variants in the *PMM2* gene, in exon 5 (c.G422A; p.Arg141His) and in exon 8 (c.G691A; p.Val231Met).[18] His parents were found be carriers.

At 21 months he presented a crisis with pneumonia, edema, ascites, pericardial and pleural effusion, coagulopathy, liver failure, and diarrhea with steatorrhea. He died due to heart failure and pulmonary edema.

Patient AR02 is the second child of healthy non-consanguineous parents. Clinical examination at 6 years showed developmental disability, hypotonia, hyporeflexia strabismus with optic nerve atrophy and macular hypoplasia, macrocephaly, inverted nipples, and increased gluteal fat pads. He had tonic-clonic seizures and decreased primary platelet aggregation. Brain MRI showed cerebellar and corpus callosum hypoplasia (Fig. 1b, c). Serum Tf IEF showed a type 1 pattern (Fig. 2, line C). He presented known pathogenic *PMM2* gene missense variants in exon 5 (c.G422A, p.Arg141His)[18] and a second variant (c.G415A; p.Glu139Lys).[19] His parents were found to be carriers. The patient, now aged 9 years, is a happy boy making developmental progress. He improved oral communication expressing ideas clearly and he can walk with help.

Patient AR03: The parents of an 8 months-old female who died ten years ago at 2 years, requested a genetic diagnosis. Genomic DNA was extracted from dry blood samples and analyzed. She presented divergent strabismus, generalized hypotrophy, decreased muscle mass of limbs and buttocks, significant lipodystrophy, and psychomotor disability (Fig. 1d). The abdomen was globular and showed a collateral circulation and an umbilical hernia. Osteotendinous reflexes were almost absent in the lower limbs. Neurological features were hypotonia, diminished active movements and reactivity, and hyporeflexia in the lower limbs. Brain MRI showed cerebellar hypoplasia. Complementary exams showed moderate pericardial effusion, decreased visual acuity with bilateral papillary hypoplasia, and bilateral hearing loss. Both kidneys were enlarged, with decreased corticomedullary diameter and moderate hepatic cytolysis was noted. Sanger sequencing of the PMM2 gene on genomic DNA detected two known pathogenic gene missense variants in exon 5 (c.G422A, p.Arg141His) and in exon 8 (c.G691A; p.Val231Met).[18] Her parents were found to be carriers.

Patient AR04 was born at 40 weeks by caesarean section with birth weight 3.75 Kg. He is the second child of healthy non-consanguineous parents. At 1- year of age, he showed inverted nipples, abnormal fat pads, cerebellar hypoplasia, stunted growth, and gastrointestinal involvement. Episodes of bacterial sepsis required hospitalization. Serum Tf IEF showed a type 1 pattern (Fig. 2, line F). Analysis of PMM2 gDNA showed two pathogenic missense variants in exon 5(c.G422A; p.Arg141His)[18] and in exon 7 (c.G623C; p.Gly208Ala).[18]

Patient AR05 and Patient AR06 are children of healthy non-consanguineous parents, male and female siblings, 8 and 10 years old, respectively, at diagnosis. They presented severe neurological manifestations in their early years. Both showed refractory epilepsy, developmental and intellectual disability (from the fourth month of life), dimorphism dysmorphism, (severe microcephaly, mongoloid palpebral fissures, broad nasal bridge, bilateral epicanthus), and bilateral convergent strabismus. A brain MRI (AR05) at 8 months showed hyperintensity in the basal ganglia and periventricular white matter and electroencephalography indicated hypsarrhythmia. In AR06, brain MRI showed an increased supratentorial ventricular system, thinning of the corpus callosum and increased frontotemporal subarachnoid spaces. At 24 months slight changes were observed in the periventricular white matter, hyperintense cerebellar hemispheres, relative hypotrophy of the anterior pole of the temporal lobes, and moderate increase in the lateral ventricles. The EEG showed modified hypsarrhythmia. Both patients presented severe developmental disability, bilateral iris colobomas, and unilateral cataract. Serum Tf IEF showed a type 1 pattern (Fig. 2 AR05 line D and AR06 line E). In collaboration with Prof. Hudson Freeze's Lab a homozygous unreported pathogenic missense variant c.G752T, p.Arg251Leu was observed in exon 2 of the *ALG2* gene. It affects the mannosyltransferase enzyme during the first step of the N-glycosylation pathway in RE and causes ALG2-CDG (OMIM # 607906).

Patient AR07 is a 26-year-old male patient with healthy consanguineous parents. He has no siblings. He presented severe developmental disability, hepatic involvement from birth with subsequent cirrhosis, and hemostatic alterations (decreased protein S, AT-III, and factor IX). Serum Tf IEF showed a type 2 pattern (Fig. 2, line G). Glycogenoses, galactosemia, fructosemia, organic aciduria, and mitochondrial diseases were ruled out. Whole exome sequencing is underway.

Patients with secondary protein hypoglycosylation

Patient GALT-BE is the second female child of healthy consanguineous Argentinean parents. The patient presented severe neurological involvement (microcephaly, psychomotor disability, axial hypotonia, seizures, hyperreflexia, and cerebellar hypoplasia). Feeding problems and malnutrition, hepatomegaly, dysmorphism, and ophthalmological abnormalities (strabismus, palpebral ptosis, and nuclear-cortical cataracts) were also observed. Biochemical analysis showed increased serum transaminases, hyperinsulinemia, hypoglycemia, and coagulopathy with a normal metabolic screening (amino acids, urinary organic acids and galactitol,

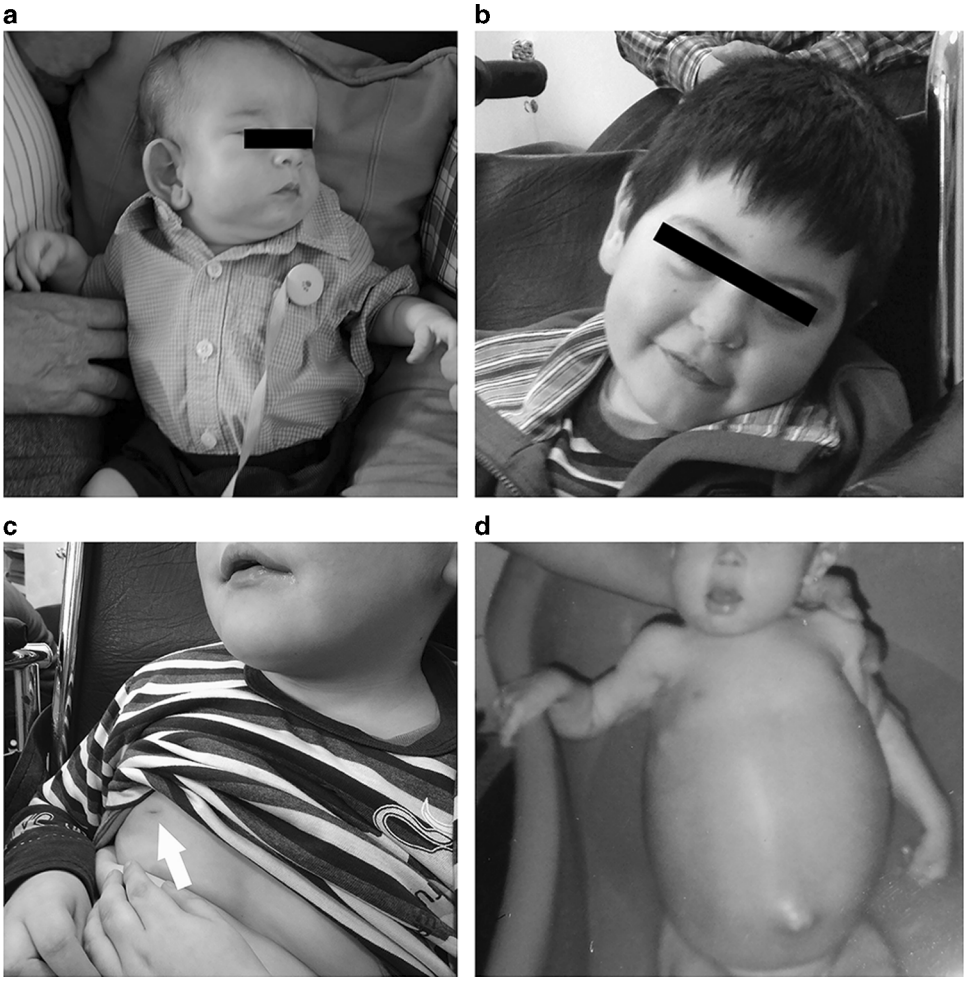

Fig. 1 Clinical features of PMM2-CDG patients. **a** Patient AR01 (1-year-old male) with a severe multisystem phenotype; **b**, **c** Patient AR02 (6-year-old male) with a mild neurologic phenotype. Note the subcutaneous fat pad and inverted nipples, divergent strabismus, and generalized hypotony. **d** Patient AR03 (8-month-old female), note the marked decrease in muscle mass with predominance in limbs and significant lipodystrophy and hepatomegaly

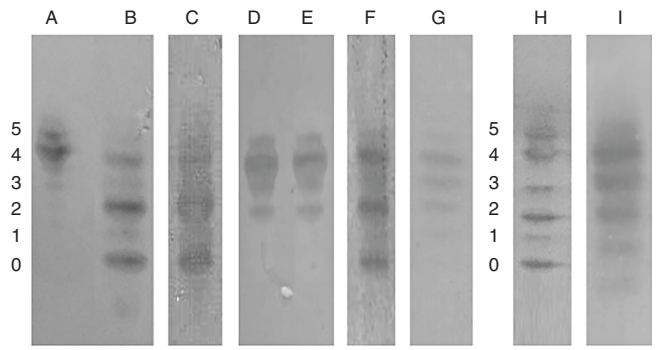

Fig. 2 Serum transferrin isoelectric focusing (Tf IEF).The numbers of terminal sialic acids of serum transferrin are indicated (0–5). Line A is a healthy control subject with normal IEF transferrin pattern. From Line B to F, a CDG type I pattern, decreased tetrasialotransferrin (except lane D a heavily prominent perhaps due to overloading of serum or overstaining), elevated asialo- and disialotransferrin bands (except in lanes D and E the asialotransferrin band is not present) due to PMM2-CDG (AR01 line B; AR02 line C, AR04 line F) and ALG2-CDG (AR05 line D and AR06 line E). Line G is a CDG type IIx patient (AR07) with increase of mono-, di-, and trisialotransferrin bands). The Tf-IEF pattern of patients with altered secondary protein glycosylation are GALT-BE (galactosemia) (lane H) and COL6A2-DI (lane I)

ammonia, lactate, blood pH, and very-long-chain fatty acids). Serum arylsulfatase A and beta-glucuronidase activities were increased. The serum Tf IEF showed a type 1 pattern (CDG-Ix) (Fig. 2, lane H) and lipid-linked oligosaccharides in fibroblasts were normal.[12] In collaboration with Prof. Gert Matthijs' Lab homozygosity mapping and whole exome sequencing revealed two known missense variants in the galactose-1-phosphate uridylyltransferase gene, one in exon 9 (c.G855T; p.Lys285Asn)[20] and the other in exon 7 (c.T584C; p.Leu195Pro).[21] Her sister (who died in the third month of life) was also compound heterozygous for these variants. Galactose-1-phosphate uridylyltransferase activity in red blood cells was strongly decreased (< 0.4 U/g Hg; normal range GALT activity > 2,3 U/g Hb) (IACA Labs, Bahía Blanca).

Patient COL6A2-DI is the 10-year-old son of healthy non-consanguineous parents. His mother suffered a threatened abortion due to gestational insulin-dependent diabetes in the 7th month. He presented severe hypoglycemia at birth, apneas, and progressive skeletal dysplasia including hand deformities and macrocephaly. Thickening of joints (especially knees, wrists and fingers) has increased in recent years. He cannot close his hands. Congenital cataracts were operated at 1-year of age, without intraocular lenses. He showed macrocephaly (PC 52 (75-95th centile) with height at 52th centile). There was a globular abdomen with an umbilical hernia. Skeletal radiology showed osteosclerosis and osteopetrosis-like abnormalities.

Nephrocalcinosis and chronic kidney failure were observed together with vesicoureteral reflux grade III. Serum Tf IEF showed a type 2 pattern (Fig. 2 lane I). By exome sequencing, we identified two pathogenic missense variants in *COL6A2*, exon 26 (c.2371G > A; p.Asp791Asn) and an unreported variant in exon 29 (c.2765T > C; p.Val922Ala) (collaboration with Prof. Dr. Hudson Freeze's Lab). Thus the diagnosis was made of COL6A2 deficiency, an autosomal recessive myosclerotic myopathy (phenotype MIM255600). The *COL6A2* gene encodes the alpha-2 subunit of type VI collagen, an ubiquitously expressed extracellular matrix protein.[22]

DISCUSSION

In Argentina, we have genetic import due to the European migration process at the beginning of the 20th century.[23] The four PMM2-CDG patients carried the ancestral European disease-causing variant in exon 5 (c.G422A; p.Arg141His). The two classical clinical presentations were observed in our patients: a non-fatal neurological phenotype (AR02 and AR04 patients) and the neurological/multi-visceral form with severe developmental disability, hypotonia, liver involvement, decreased coagulation factors and near 20% mortality in the first years (patients AR01 and AR03).[24] We did not dispose of all clinical findings because the patients were referred from different medical centers. We also detected ALG2-CDG siblings. To date, nine patients with ALG2-CDG have been reported.[25–27] Mutations in the *ALG2* gene affect the early steps of dolichol-linked oligosaccharide biosynthesis. ALG2-CDG is a deficiency of alpha-1,3-mannosyltransferase, an enzyme that catalyzes the elongation of Man1GlcNac2-PP-dolichol. This emerging CDG is a miasthenic myopathic syndrome, as well as a multisystem disorder with mental disability, iris coloboma, hepatomegaly, coagulation abnormalities, and defective myelination.[28] Our patients carried a new variant (c.G752T; p.Arg251Leu) that on silico analysis was moderately pathogenic with different prediction tools (DANN score 0.9955; Mutation Taster 0.999; FATHMM-MKL 0.8844; Provean -3.33). Functional tests are underway. Patients with secondary alterations in protein glycosylation were observed, one with classic galactosemia (GALT-BE), one with COL6A2 deficiency, as well as a Tf protein gene variant. Whether the abnormal Tf IEF in COL6A2 deficiency is a coincidence or part of the syndrome deserves further study.

Galactosemia has been referred to as a secondary disorder of glycosylation.[29,30] The affected individual (patient GALT-BE) shared many clinical similarities with CDG.

In summary, this 10 years' CDG study in Argentina resulted in the diagnosis of 4 PMM2-CDG patients, 2 ALG2-CDG siblings, a CDG-IIx patient, 1 galactosemia patient, and 1 patient with a COL6A2 deficiency.

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AUTHOR CONTRIBUTION

B.M.M.B. and P.M.: as CONICET fellows, were responsible for preparatory studies in terms of IEF, HPLC, molecular studies, data gathering, and contributed in critically reviewing the manuscript. A.N.B. collaborated with the molecular studies and contributed critically reviewing the manuscript. P.M.F., G.N.B., P.M.: are physicians specialized in metabolic diseases and were responsible for the evaluation and follow-up of patients with suspect CDG, and also critically reviewed the manuscript. S.N.: contributed with transferrin studies and critically reviewed the manuscript. R.d.D.K.: is a physician responsible for the evaluation and follow-up of patients and coordinates the Center for Study of Congenital Metabolopathies (CEMECO). She participated in the interpretation of data and reviewed it critically for important intellectual content. C.G.A.: was responsible for the research, conception and design of the idea for the article and contributed to preparing and drafting the manuscript. She participated in the critical interpretation of data. She coordinated the CDG research program in CEMECO, Children´s Hospital, in Cordoba Argentina. All the co-authors contributed to the final draft of the manuscript and they agree to this submission. This work was supported by grants from CONICET (PIP6338; PIP0038), (ANCyT-FONCyT) PICT2007-2350/PICT2010-2824, and Catholic University of Córdoba (2010–2017).

ADDITIONAL INFORMATION

Ethical approval: Procedures employed were reviewed and approved by the appropriate institutional review committees. Written informed consent was obtained from the patients and their parents to participate in this study, and they allowed us to submit this manuscript with images for publication. Copies of the written consents are available for review. The study was approved by the Ethics Committee of the Children's Hospital of Córdoba (CIEIS) Act Nº 95/2005/2007/2016. All studies were carried out in accordance with the World Medical Association Declaration of Helsinki.

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