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MOLECULAR AND FUNCTIONAL STUDY OF PEDIATRIC PATIENTS WITH NIEMANN-PICK C IN ARGENTINA

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Abstract Niemann-Pick type C (NP-C) is a rare, autosomal recessive disorder. At least 95% of all the cases with this disease are due to mutations in the *NPC1* gene. The clinical signs and symptoms of NP-C are classified into visceral, neurological and psychiatric. Our aim is to report the clinical findings, molecular results and filipin staining of 4 patients. The age of onset, expressed as median and range, was 0.2 (0.08-4.0) years and the age of diagnosis was 4.0 (2.5-8.9) years. Neurological and/or visceral manifestations were presented in our patients. Foamy cells in bone marrow biopsy were found in two patients. Through a molecular analysis of *NPC1* gene, one non-reported (novel) and 4 previously described mutations were found. The filipin staining showed a positive pattern in all the patients. The diagnostic confirmation of these pediatric patients means a contribution to the casuistry of this disease in Argentina.

Key words: Niemann-Pick disease type C, Filipin, sequence analysis

Resumen ***Estudio molecular y funcional de pacientes pediátricos con Niemann-Pick C en Argentina***. Niemann-Pick tipo C (NP-C) es una enfermedad poco frecuente, con un patrón de herencia autosómico recesivo. Al menos el 95% de los casos se producen por mutaciones en el gen *NPC1*. Los signos y síntomas clínicos de NP-C se clasifican en viscerales, neurológicos y psiquiátricos. En este trabajo presentamos los hallazgos clínicos, los resultados moleculares y la tinción con filipina de 4 pacientes con NP-C. La edad de presentación de los primeros síntomas, expresada como mediana y rango, fue de 0.2 años (0.08-4.0) años y la edad del diagnóstico fue 4.0 (2.5-8.9) años. Los pacientes presentaron manifestaciones neurológicas y / o viscerales. Se encontraron células espumosas en la biopsia de médula ósea en 2 pacientes. El análisis molecular del gen *NPC1* encontró 1 variante nueva y 4 previamente publicadas. La tinción de filipina mostró un patrón positivo en todos los pacientes. La confirmación diagnóstica de este grupo de pacientes pediátricos significa un aporte a la casuística de esta enfermedad en Argentina.

Palabras clave: enfermedad de Niemann-Pick C, Filipina, análisis de secuencias

Niemann-Pick type C (NP-C) is a progressive autosomal recessive disorder caused by mutations in the *NPC1* or the *NPC2* genes. An abnormal endosomal-lysosomal trafficking is described, resulting in multiple lipid accumulation. NP-C onset might appear from prenatal life to adulthood. NP-C is a rare disease, with an estimated incidence of 1 case per 100 000 live births. It is pan-ethnic, and at least 95% of the cases are due to mutations in the *NPC1* gene[1].

The clinical signs of NP-C can be classified into visceral, neurological and psychiatric. Because of the phenotypic heterogeneity, they could overlap with other metabolic diseases. Therefore, in order to confirm the diagnosis, it is mandatory to carry out specific laboratory tests[2].

Previously, the diagnostic confirmation of NP-C in our hospital required sending the patient samples to be analyzed abroad, which implied a consequent delay in the diagnosis. Our following aim was to develop the NP-C sequence analysis and the filipin staining as functional study to confirm the diagnosis. Coding sequence (exons 1-25) and the flanking intronic regions of *NPC1* gene (RefSeq NM_000271.5) were amplified by PCR using specific

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primers as already reported[3]. The PCR products were sequenced on a capillary DNA sequencer and the variants found were classified according to American College of Medical Genetics (ACMG) guidelines[4]. Filipin staining was performed on fibroblast cell culture from skin biopsies. The assay included, simultaneously, the cell culture of the patient, as well as one positive and one negative control. Cells with fluorescent perinuclear vesicles, after incubation with *Streptomyces filipensis* complex, were considered "positive", as described by Vanier et al[5]. The filipin test was always performed in separate cultures in duplicate.

Find below a series of reported cases with infantile onset diagnosed in our hospital. The age at onset, expressed as median and range, was 0.2 (0.08-4.0) years and the age of diagnosis was 4.0 (2.5-8.9) years.

Clinical cases

Case 1

Female child who debuted at 0.08 years old with splenomegaly as a visceral sign, and having presented fetal hydrops. Hepatomegaly was diagnosed at the age of 3.00. As neurological signs it was described a progressive vertical supranuclear gaze palsy and gelastic cataplexy. Frequent seizure episodes appeared at 12.5 years.

Her mother and father were aged 23.0 and 24.0, respectively. Consanguinity was not reported. She had two siblings, a brother who was 3.0 years older and a two years younger sister. No family member presented symptoms.

Liver biopsy performed at 2 months of age described fatty vacuoles in hepatocytes and macrophages, canalicular thrombi, cholestasis, perisinusoidal fibrosis, mixed inflammatory infiltrate and ductular proliferation. Skin biopsy reported a histiocytic proliferation with large foamy cytoplasm. Bone marrow biopsy showed hypercellularity with increased phagocytes (Table 1). *NPC1* gene sequencing informed two known missense variations: c.3419G>T and c.3182T>C[6]. Both variants were classified as likely pathogenic (PS3, PM2, PP2, PP3, PP5). NP-C was confirmed by the filipin test on fibroblasts cell culture at the age of 3.00 (Fig. 1).

Case 2

Male child, whose first hospitalization in the intensive care unit was at 0.08 years old because of respiratory distress. At 1.0 year of age, he presented vertical supranuclear gaze palsy. His parents were 25.0 years old. No more data about his family was available.

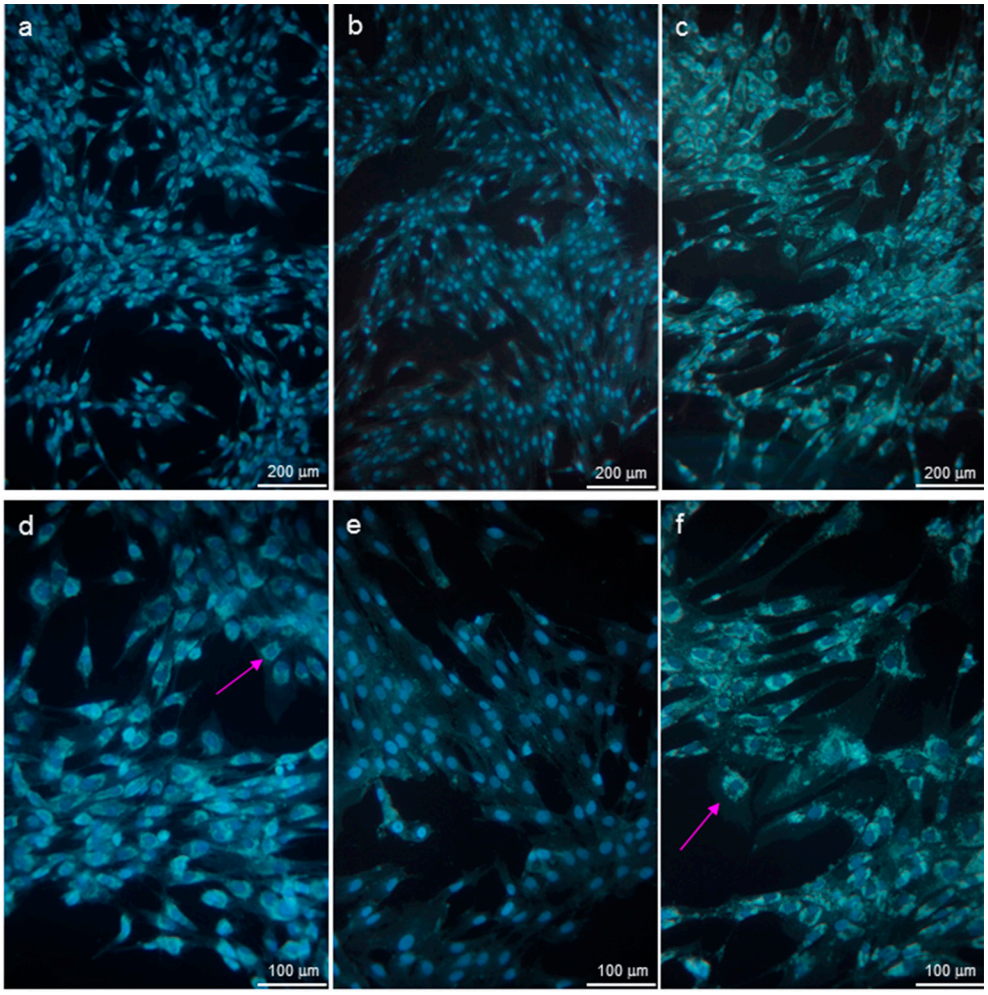
TABLE 1.– *Clinical findings, complementary studies and molecular results in Niemann-Pick disease type C patients*

Case	1	2	3	4
Gender	Female	Male	Male	Male
Age at onset (years)	0.08	0.08	0.33	4.0
Age at diagnosis (years)	3.0	2.5	4.5	8.9
Neurological manifestations:				
Delayed early development		+	+	
Epilepsy	+			+
Dysphagia				+
Weakness	+			
Vertical supranuclear gaze palsy	+	+		
Cataplexy	+			
Encefalopathy				+
Ataxia-Telangiectasia				+
Visceral manifestations:				
Hepatomegaly	+		+	
Splenomegaly	+		+	
Cholestasis			+	
Respiratory distress			+	
Foetal hydrops	+			
Foamy cells in bone marrow	+		+	
Filipin staining*	+	+	+	+
Molecular analysis: *NPC1* gene				
Variant 1	c.3419G>T (p.Gly1140Val)	c.2872C>T (p.Arg958Ter)	c.3557G>A (p.Arg1186His)	c.3557G>A (p.Arg1186His)
Variant 2	c.3182T>C (p.Ile1061Thr)	– –	c.287+1G>A (splicing)	

+: present or typical; -: absent or negative

*Cells of the fibroblast culture (skin biopsy) show the presence of intracellular lipid accumulation.

Fig. 1.– Filipin test results: Niemann-Pick type C (NP-C) positive control (a, d). NPC negative control (b, e), and NP-C suspected sample with a filipin positive pattern (c, f). Arrow shows a filipin positive cell, with fluorescent perinuclear vesicles. Panel c and f, represent a 90% of filipin positive cells



A molecular study of the *NPC1* gene revealed a nonsense variant in homozygous or hemizygous state, c.2872C>T[6]. It was classified as pathogenic (PVS1, PS3, PM2, PP3, PP5). The filipin test confirmed the diagnosis of NP-C at 2.5 years old (Table 1).

Case 3

Male child, hospitalized for respiratory distress syndrome as newborn and for cholestasis at 0.3 years old. At the age of 4.0, he presented growth retardation, maturation delay and hepatosplenomegaly. As neurological manifestations, he presented development delay. The age of his parents was not reported, nor was a consanguinity. His family background also included three older sisters, one of them died at the age of 5.0, presumably because of a metabolic disease.

A bone marrow biopsy showed micro-megakaryocytes grouped in clusters. A *NPC1* gene sequencing revealed a missense variant, c.3557G>A, present in the global database[6]. It was classified as likely pathogenic (PS3, PM2, PP2, PP3, PP5). A novel intronic variant c.287+1G>A was located at the 5' donor splice site of intron 3. This location has a 0.98 score prediction as a splice site (Neural Network), and a confidence of 0.76 as donor splice site (NetGene2). This variant has not been reported, at least in ClinVar, The Human Gene Mutation Database, Ensembl, gnomAD, SIFT or in PROVEAN databases, nor has it been published in Pubmed or Mastermind. *In silico* analysis predicts that this variant is one of the main causes of this disease, since it affects the splice site. In other words, as it is likely to disturb normal splicing, it alters the protein features (Mutation Taster). According to recommendations of the ACMG, this variant was classified as pathogenic (PVS1, PS3, PM2, PP3). NP-C was confirmed by a filipin test on fibroblasts cell culture at 4.5 years old (Table 1).

Case 4

A male child who presented ataxia and telangiectasia with dysphagia at the age of 4.0. A progressive encephalopathy was detected at 8.0 years old, with a generalized tonic-clonic seizure. His mother was 24.0 years old while his father was 20.0. Although consanguinity was not revealed, they had the same last name and lived in a small village. This patient had a 2.0-year-younger brother without symptoms. Further data

about patient's family involves a 28.0 years old maternal aunt's death by a stroke and a cousin of his mother's with congenital hydrocephalus.

By the molecular study of the *NPC1* gene, the missense variant c.3557G>A was detected in homozygous or hemizygous state. This was the same variant detected in the case 3 and it was classified as likely pathogenic (PS3, PM2, PP2, PP3, PP5). NP-C was confirmed by a filipin test at 8.9 years old (Table 1).

None of the cases facilitated parental samples for molecular analysis.

Discussion

A series of cases of Argentinian pediatric patients was presented, with 4.0 years old as median age at diagnosis. It is already known that the clinical manifestations markedly vary according to the age of the disease onset[1]. An early infantile form, characterized by neurological manifestations and hepatosplenomegaly, was found in cases 1 and 3. While case 2 only presented neurological signs, the remaining patient exhibited a late infantile neurodegenerative form.

Molecular analysis of the *NPC1* gene in the four patients identified the following variants: p.Gly1140Val, p.Ile1061Thr, p.Arg958Ter, p.Arg1186His and c.287+1G>A. Unfortunately, parental samples were not available at the moment of this study. Having access to them would have allowed us to assign each variable to a specific allele (cases 1 and 3) and to understand if the single variable was homozygous or hemizygous (cases 2 and 4). Even though the same variant was found in our patients 3 and 4, they presented different signs, mainly visceral in the former while neurological in last one. Case 3 not only had this variant, but also another mutation that affected the splicing site. In this case, a longitudinal follow-up would be recommended, taking into account that the clinical manifestations may change as he grows.

Case 4, on the other hand, had this variant in hemicigosis (combined with a deletion) or in homocigosis, and the clinical picture included neurological damages.

To confirm the molecular diagnosis, our group developed the filipin staining as a functional study to detect intracellular lipid accumulation[7]. The filipin test resulted positive in the four cases, which corroborated a disorder in the intracellular cholesterol trafficking.

According to the Consensus of 2018 of the International Niemann-Pick Disease Registry, once NP-C is clinically suspected, a laboratory diagnosis algorithm proposes to test biochemical markers (as lyso-sphingomyelin) as screening tools. As a second step, a molecular analysis of *NPC1* and *NPC2* genes is recommended if the biochemical markers profile is compatible with NP-C. In the presence of inconclusive results, and to assess the pathogenicity of novel genetic variants, a filipin test must be performed[1].

In fact, the accuracy of these procedures tended our institution to offer both the molecular sequencing and the filipin staining, as tools for the NP-C diagnosis in pediatric patients all around Argentina.

Until this moment, there haven't been any reports on the incidence of NP-C in Argentinian pediatric patients. Only one paper described two adult siblings with a choreic phenotype and diagnosed as NP-C through the measurement of lyso-SM-509 biomarker using high-performance liquid chromatography/tandem mass spectrometry and molecular analysis on *NPC1* gene that were analyzed in a German company specialized in study of rare diseases[8].

Many reports from Brazil discuss this topic. One of them is a study in a large cohort of 265 patients with a wide distribution of age[9]. Interestingly, among the variants they found in *NPC1* gene, three of them match with ours: p.Gly1140Val; p.Ile1061Thr; p.Arg1186His. Furthermore, a review from Colombia focused on the link between psychiatric disorders and neurometabolic diseases[10]. Although no psychiatric disorder has been found in our patients, probably because of their young age, we do not discard an evolution to psychiatric disorders in adulthood. On their part, a group of Mexico reported three juvenile NP-C patients, but they do not coincide with ours in terms of molecular mutations[11]. Another Mexican group reported the case of a male patient with a juvenile form of NP-C that involved a "variant" filipin staining (different to the classical pattern found in our series) and a paternal germline mosaicism[12].

In summary, there is a limited offer of reports about patients with NP-C in Latin-American countries, mainly published in Brazil. The aim of this paper is to enhance the knowledge about NP-C, which is quite difficult to diagnose.

Thus, our analysis and confirmation of this illness in four pediatric patients contribute to the characterization of this disease in Argentina, and consequently increase its casuistry.

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