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The impact of biomarkers analysis in the diagnosis of Niemann-Pick C disease and acid sphingomyelinase deficiency

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ABSTRACT

Background: Although representing two distinct disease entities, Niemann-Pick disease type C (NP-C) disease and acid sphingomyelinase deficiency (ASMD) share several phenotypic features. The lack of biomarkers was responsible in the past of diagnostic delay. Recently, plasma oxysterols, cholestan-3β,5α,6β-triol (Triol) and 7-ketocholesterol (7-KC) and lysosphingolipids, Lyso-sphingomyelin (Lyso-SM) and Lysosphingomyelin-509 (Lyso-SM-509), have been proposed as diagnostic biomarkers. We aimed to assess the diagnostic power of the two biomarkers categories and to evaluate possible correlations with patients' age and clinical phenotypes.

Patients and methods: We analyzed plasma oxysterols and lysosphingolipids in patients affected by NP-C and ASMD, and compared with healthy controls.

Results: Oxysterols were always increased in both NP-C and ASMD. In NP-C, Lyso-SM and Lyso-SM-509 were increased in 70%, and 100% of patients, respectively. Biomarkers negatively correlated with patients' age, with highest levels in early-infantile, intermediate in the late-infantile and lowest in the juvenile phenotype. In ASMD, lysosphingolipids were both increased, with a greater order of magnitude than in NP-C, with highest levels in chronic-neurovisceral vs visceral phenotype.

Conclusions: Lysosphingolipids are useful biomarkers for a rapid and precise diagnosis, allowing clear distinction between NP-C and ASMD. They are more reliable biomarkers than oxysterols and correlate with patients' age and clinical phenotype.

1. Introduction

At the beginning of the last century, the eponym "Niemann-Pick disease" referred to a heterogeneous group of lysosomal disorders characterized by lipid storage in tissues and overlapping clinical features, which mainly included hepatosplenomegaly with or without central nervous system (CNS) involvement. The original classification into three types (A, B, C) was based on age of onset, disease severity and sphingomyelin accumulation [1]. Today, two distinct recessively inherited disease entities exist. Niemann-Pick disease type C (NP-C), due to mutations in *NPC1* (MIM *607623) and *NPC*2 (MIM *601015) genes, accounting respectively for 95% and to 5% of patients, and acid sphingomyelinase deficiency (ASMD), former Niemann-Pick disease type A and B, due to mutations in sphingomyelin phosphodiesterase 1 (*SMPD1)* gene (MIM *607608).

Both genetic subtypes of NP-C disease lead to impaired intracellular cholesterol trafficking, with accumulation of cholesterol and glycosphingolipids in the brain and visceral organs. NP-C is a heterogeneous disease, in which visceral and neurological symptoms appear at different ages and progress independently in the course of life. Visceral symptoms, when present, anticipate neurological signs, sometimes appearing years or decades before. Visceral involvement may be mild or

Abbreviations: NP-C, Niemann-Pick type C; ASMD, Acid Sphingomielinase Deficiency; Triol, Cholestan-3β,5α,6β-triol; 7-KC, 7-ketocholesterol; LC-MS/MS, Liquid chromatography tandem mass spectrometry; ESI, Electrospray Ionization; Lyso-SLs, Lyso-Sphingolipids; Lyso-SM, Lyso-sphingomyelin; Lyso-SM-509, Lysosphingomyelin-509

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absent in late-onset cases, and virtually all patients experience a progressive involvement of the CNS, which ultimately determines the prognosis and life expectancy. In patients with pre/perinatal onset, manifesting with a severe systemic involvement - which include fetal hydrops, ascites, neonatal cholestasis, hepatosplenomegaly and pulmonary disease - liver and or respiratory failure may lead to death within the firsts months of life, even before the appearance of overt neurological signs. Based on the age at onset of neurological manifestations, four disease subgroups have been defined: early-infantile (before 2 years), late-infantile (2–6 years), juvenile (6–15 years) and adolescent/adult onset [2]. Although there are overlaps between the various neurological phenotypes, the current classification is still useful for the clinician to predict disease severity and prognosis. NP-C is still lacking a definitive therapy. Miglustat is available in some countries for the treatment of neurological symptoms in NP-C, and it has been shown to stabilize or slow disease progression, especially in patients with the juvenile form [3–5]. New therapeutic approaches are under evaluation (ie. hydroxypropyl-β-cyclodextrin, ClinicalTrial.gov Identifier NCT01747135; arimoclomol, ClinicalTrial.gov Identifier NCT02612129). Until a few years ago, the diagnosis of NP-C was based on the demonstration of free cholesterol accumulation in cultured skin fibroblasts by filipin staining, followed by confirmatory genetic analysis. More recently, new biomarkers have been proposed for its diagnosis. Analysis of plasma oxysterols, cholestan-3β,5α,6β-triol (Triol) and 7- ketocholesterol (7-KC), represents a sensitive and reliable diagnostic tool [6,7]. However, plasma oxysterols have been shown to be non-specific disease biomarkers, since they are also elevated in ASMD, as well as in other inherited disorders of lipid metabolism, such as lysosomal acid lipase deficiency, cerebrotendinous xanthomatosis, and Smith-Lemli-Opitz syndrome [8–11]. Furthermore, specific bile acid derivatives, produced by accumulated Triol and 7-KC, have been found increased in plasma and dried blood spot from NP-C patients [12,13]. More recent researches, led to the identification of lyso-sphingolipids (Lyso-SLs), the N-deacetylated lyso-forms of glycosphingolipids, for the diagnosis of lysosomal disorders affecting sphingolipids metabolism. Elevation of plasma lyso-sphingomyelin (Lyso-SM), has been reported both in ASMD and in NP-C [14,15]. Lysosphingomyelin-509 (Lyso-SM-509), an analogue of Lyso-SM, which exact structure has not yet been defined, was then identified and proposed as a more specific biomarker for NP-C [16]. Finally, new multiplex LC-MS/MS methods, allowing the simultaneous measurement of different Lyso-SLs, have been proposed for the rapid diagnosis of different lysosomal storage diseases affecting sphingolipid metabolism [17–19].

ASMD is due to deficiency of the lysosomal enzyme catalyzing of the hydrolysis of sphingomyelin to ceramide and phosphocholine, which leads to the progressive accumulation of sphingomyelin in tissues, i.e. spleen, liver, lung, bone marrow, and neurons. The clinical phenotypes of ASMD have been recently reviewed [20]. The infantile neurovisceral phenotype, historically known as Niemann Pick disease type A, is the most severe and rapidly progressive form, leading to death in infancy. The chronic neurovisceral phenotype, known as Niemann Pick disease type A/B, is an intermediate form, characterized by multisystemic involvement and slowly progressive psychomotor regression, with prolonged survival. In the chronic visceral ASMD, known as Niemann Pick disease type B, symptoms may appear at different ages, from infancy to adulthood, with exclusive visceral involvement and with absence of neurological symptoms. No treatment is still available for ASMD, but a therapeutic trial with enzyme replacement therapy is ongoing (ClinicalTrial.gov Identifier: NCT 00410566). The diagnosis of ASMD relies on the enzymatic assay of acid sphingomyelinase activity in peripheral white blood cells, dried blood spot or cultured skin fibroblasts followed by gene sequencing [20]. Lyso-SM and Lyso-SM-509 have been found elevated also in ASMD [17–19].

Although NP-C and ASMD represent distinct disease entities, they share several phenotypic features. Clinical similarities, combined with the lack of reliable biomarkers were responsible, at least in the past, of a diagnostic delay, often of years, making the diagnosis of these two rare lysosomal storage diseases challenging for physicians [5,20].

In this study, we compared the two categories of plasma biomarkers (i.e. oxysterols and Lyso-SLs) in patients with NP-C disease and ASMD, to establish their diagnostic power and eventual correlations with patients' age, and clinical phenotypes.

2. Material and methods

2.1. Ethical approval and patients consents

This study was performed in compliance with the Declaration of Helsinki. The study was approved from Bambino Gesù Children's Hospital Ethic Committee (no. 1002_OPBG_2015). Blood samples were collected after obtaining informed consent during routine clinical evaluation.

2.2. Analysis by LC-MS/MS

Triol and 7-KC were analyzed as dimethylaminobutyrate esters (DMAB-derivatives) by LC-MS/MS, as previously described [7]. The column for chromatographic separation was a Phenomenex Synergi Fusion C18–50 mm × 2.0 mm id, 4 μm (Phenomenex Inc. Torrance, CA, US). Chromatographic separation of metabolites was obtained with gradient elution of two solutions as described. Total run was 5.1 min. Elution times were 1.54 min for Triol and $^{2}H_{7}$-Triol and 1.77 min for 7-KC and $^{2}H_{7}$–7-KC. The monitored MRM transitions for Triol-DMAB and 7-KC-DMAB derivatives were 534/132 *m/z* and 514/132 m/z, respectively.

Lyso-SLs were analyzed by LC-MS/MS, according to Polo et al. [17]. Lyso-SM and Lyso-SM-509 separation was performed by reverse-phase liquid chromatography using a Waters BEH C18 column, 2.1 × 50 mm with 1.7-μm particle size (Waters Corporation, Milford, MA, US) and a gradient elution of two solutions. The monitored MRM transition for Lyso-SM and $^{2}H_{7}$-Lyso-SM were 465.4/184.1 m/z and 472.4/191.1 m/z respectively, at the retention time 1.42 min; for Lyso-SM-509 MRM transition was 509.4/184.1, and the retention time 1.50 min. Since the structure of Lyso-SM 509 is unknown, and a reference standard is not available [16,17], Lyso-SM-509 was quantified using $^{2}H_{7}$-Lyso-SM as internal standard and the value was expressed as multiple of the median (MoM).

For both analyses, liquid chromatography was performed on an Agilent series 1290 pump equipped with auto-sampler (Agilent Technologies Inc., Wilmington, DE, USA). Tandem mass spectrometry experiments were carried out on a 4000-QTrap mass spectrometer (Sciex, Toronto, Canada), equipped with a Turbo Ion Spray Source operating in positive ion mode.

2.3. Sample treatment procedure

Plasma was obtained from blood samples after an overnight fast, collected in EDTA K2 tubes immediately centrifuged for 5 min at 2000*g* and separated from red cells. Plasma was stored at −80 °C. Briefly, for oxysterols analysis, 5.0 μL of 500 ng/mL $^{2}H_{7}$-Triol and $^{2}H_{7}$–7KC standard solution were mixed with 50 μL of plasma sample in eppendorf tube. Liquid-liquid extraction was performed adding 500 μL of ethyl acetate and mixing by vortex for 2 min. After 5 min centrifugation at 16,200*g*, the supernatant was transferred into a clean glass vial and dried under nitrogen steam. Derivatization was performed adding 20 μL of derivatizing solution (100 mmol/L dimethyl-aminobutyric acid imidazolide) at 65 °C for 15 min. Finally, the sample was dried again, reconstituted with 200 μL solvent and 5 μL were injected for oxysterols detection and quantification, as dimethyl-aminobutyrate mono-derivatives [7].

For Lyso-SLs analysis, 500 μL of precipitating solution (methanol/acetone/water 45:45:10, v/v) was added to 100 μL of plasma. Samples

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