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Molecular and biochemical biomarkers for diagnosis and therapy monitorization of Niemann-Pick type C patients

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A B S T R A C T

Background: Niemann-Pick type C (NP-C), one of 50 inherited lysosomal storage disorders, is caused by NPC protein impairment that leads to unesterified cholesterol accumulation in late endosomal/lysosomal compartments. The clinical manifestations of NP-C include hepatosplenomegaly, neurological and psychiatric symptoms. Current diagnosis for NP-C is based on observation of the accumulated cholesterol in fibroblasts of affected individuals, using an invasive and time expensive test, called Filipin staining. Lately, two metabolites that are markedly increased in NP-C patients are arising as biomarkers for this disease screening: 7-ketocholesterol and cholestane-3β,5α,6β-triol, both oxidized cholesterol products.
Objective: In this work, we aimed to evaluate the performance of cholestane-3β,5α,6β-triol analysis for the screening and monitoring of NPC patients, correlating it with chitotriosidase levels, Filipin staining and molecular analysis. It was investigated 76 non-treated individuals with NP-C suspicion and also 7 patients with previous NP-C diagnosis under treatment with miglustat, in order to verify the cholestane-3β,5α,6β-triol value as a tool for therapy monitoring.
Results: Considering molecular assay as golden standard, it was verified that cholestane-3β,5α,6β-triol analysis presented 88% of sensitivity, 96.08% of specificity, a positive and negative predictive value calculated in 91.67% and 94.23%, respectively, for the diagnosis of NP-C. Chitotriosidase levels were increased in patients with positive molecular analysis for NP-C. For Filipin staining, it was found 1 false positive, 7 false negative and 24 inconclusive cases, showing that this assay has important limitations for NP-C diagnosis. Besides, we found a significant decrease in cholestane-3β,5α,6β-triol concentrations in NP-C patients under therapy with miglustat when compared to non-treated patients.
Conclusion: Taken together, the present data show that cholestane-3β,5α,6β-triol analysis has a high potential to be an important NP-C screening assay, and also can be used for therapy monitorization with miglustat in NP-C patients.

1. Introduction

Niemann-Pick type C (NP-C) is a lysosomal lipid storage disease (LSD) with autosomal recessive inheritance, caused by mutations in NPC1 or NPC2 genes and mainly characterized by unesterified cholesterol accumulation in late endosomal/lysosomal (LE/L) compartments (Vanier and Millat, 2003). As a result of this genetic defect, there is an accumulation of other lipids, such as glucosylceramide, GM1 and GM2

Abbreviations: NP-C, Niemann-Pick type C; LSD, lysosomal storage disorder; CNS, central nervous system; 7-KC, 7-ketocholesterol; GC/MS, gas chromatography/mass spectrometry; LC/MS-MS, liquid chromatography/tandem mass spectrometry; LDL, low density lipoprotein; LE, late endosomal; ROS, reactive oxygen species; CSF, cerebrospinal fluid; HSEM, horizontal saccadic eye movement

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gangliosides in peripheral tissues (liver, spleen and lungs) and in central nervous system (CNS) of the affected individuals (Patterson, 2003). In this way, clinical presentation is extremely heterogenous and includes hepatosplenomegaly, neonatal jaundice, dysarthria, dysphagia, vertical supranuclear gaze palsy, psychiatric and/or cognitive dysfunction and it may vary between patients in terms of age-onset and disease severity, delaying the recognition of the disease (Patterson, 2003). Regarding its rarity, NP-C incidence is estimated in 1/89,000, but this data may be significant uncertain because of a late-onset NPC1 phenotype, with a markedly higher incidence, on the order of 1/19,0001/36,000. (Wassif et al., 2016).

Despite there is no cure for NP-C, the management of symptoms is an important goal in therapy for these patients (Patterson et al., 2012). Miglustat, a small iminosugar molecule able to cross the blood-brain barrier and to reversibly inhibit glucosylceramide synthase (the first enzyme in glycosphingolipid synthesis) was proposed for the treatment of the disease (Fecarotta et al., 2015). The efficacy of miglustat on neurological manifestations progression has been studied in NPC patients enrolled in international clinical trials and observational studies. Data from one-year treatment of juvenile and adult NPC patients suggested that miglustat improves or stabilizes several neurological manifestations (Fecarotta et al., 2015; Patterson et al., 2007). Cyclodextrins are also showing some promising results in several studies, but the mechanisms are not yet completely established (Atger et al., 1997; Aqul et al., 2011). Treatment with subcutaneously hydroxypropyl-β-cyclodextrin (HPβCD) of a NP-C murine model shows an improvement in cholesterol metabolism in the liver and the most other organs, as well as ameliorates cerebellar neurodegeneration (Ramirez et al., 2010; Nusca et al., 2014). Administration of intracisternal HPβCD to NP-C cats with ongoing cerebellar dysfunction slowed disease progression, increased survival time, and decreased the accumulation of brain gangliosides (Vite et al., 2015). Recent phase I/II clinical trial showed that patients with NPC1 treated with intrathecal HPβCD had slowed disease progression with an acceptable safety profile (Ory et al., 2017).

Due to its heterogeneity in symptoms and clinical nature, prompt diagnosis for NP-C is a challenge. Once considered standard gold assay for NP-C diagnosis, Filipin staining is based in a coloration using a fluorescent antibiotic, which binds to cholesterol accumulated in fibroblasts from NP-C patients. However, a variant profile in fluorescent pattern can cause doubts in assay interpretation. Besides, Filipin test is an invasive and expensive procedure, requiring a specialized center to perform it (Vanier et al., 2016). Fluorescence microscopy is a valuable tool for studying intracellular transport processes, but this method can be challenging for lipid molecules, such as cholesterol (Maxfield and Wüstner, 2012). Alternatively, accumulated cholesterol can be also visualized by immunofluorescence using a cholesterol-binding bacterial toxin, perfringolysin O (Kwiatkowska et al., 2014).

Determination of chitotriosidase is also used as a general and potential indicator of LSD, including NP-A, NP-B and NP-C. However, normal levels of this enzyme may occur in these patients, showing a lack of sensitivity and specificity of this assay (Vanier et al., 2016). Therefore, definitive diagnosis depends on molecular analysis of NPC1 and NPC2 genes for most cases.

In NPC deficient cells, there is an association between oxidative stress and accumulated cholesterol by increased production of reactive oxygen species and oxidative damage (Ribas et al., 2012). Cholesterol can suffer oxidation in different ways, what could be mediated by enzymes or through non-enzymatic reactions (Fig. 1). Oxidized cholesterol products, specifically cholestane-3β,5α,6β-triol (3β,5α,6β-triol) and 7-ketocholesterol (7-KC), are markedly increased in plasma of NP-C patients and in animal models, whereas remain normal in other LSD (Jiang et al., 2011). These findings indicate that 3β,5α,6β-triol and 7-KC are NPC1 disease-specific biochemical markers and suggest a possible utility of these markers in diagnosis and therapeutic evaluation of NPC1 disease (Jiang et al., 2011). Determination of these metabolites can be performed using gas chromatography/mass spectrometry (CG/MS) or by liquid chromatography/tandem mass spectrometry (LC–MS/MS) methods (Boenzi et al., 2016; Porter et al., 2010). Therefore, oxysterols analysis by LC–MS/MS became an alternative and non-invasive assay to screen potential NP-C patients, as well as a tool for treatment monitoring. However, its correlation with tests currently used for NP-C diagnosis must be better investigated.

In order to evaluate the 3β,5α,6β-triol measurement for NP-C therapy monitorization and also as a biomarker for NP-C diagnosis, in this work we analyzed 3β,5α,6β-triol and chitotriosidase levels, Filipin staining and mutations in NPC genes in biological samples from patients with NP-C suspicious and in treated NP-C patients referred to our specialized center in South Brazil.

2. Materials and methods

2.1. Samples

Skin biopsy and blood samples were obtained from 76 individuals with suspicious of NP-C disease in Medical Genetics Service of *Hospital de Clínicas de Porto Alegre*, Brazil. Additionally, 7 blood samples from patients with previous diagnosis of NP-C under miglustat therapy (therapeutic regime: 200 mg thrice a day) were collected. These blood samples were collected in tubes with EDTA as anticoagulant, centrifuged for five minutes at 3000 rpm and plasma was frozen at −80 °C. The clinical features presented by these patients included dystonia, dysphagia, seizures, vertical supranuclear palsy and psychiatric disorders.

This work was carried out according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). All subjects in this study signed an informed consent, and this project was approved by the Ethics Committee of *Hospital de Clínicas de Porto Alegre* (HCPA), RS, Brazil under the registration number 13-0239.

2.2. Cholestane-3β,5α,6β-triol analysis

Levels of triol were determined by LC–MS/MS in EDTA-plasma, using cholestane-3β,5α,6β-triol D7 as internal standard and derivatization with dimetylglycine, according to Jiang et al. (2011), with some modifications. The chromatographic separation was performed on a column ACE 3 C18 (4.6 × 150 mm, 3 μm) using a gradient of mobile phase A (0.1% formic acid + 1 mM ammonium acetate in water) and mobile phase B (0.1% formic acid + 1 mM ammonium acetate in methanol). Detection was performed with a Waters Quattro Micro API tandem mass spectrometer in positive atmospheric-pressure chemical ionization (APCI) and multiple reaction monitoring (MRM) mode. The optimized MS/MS conditions were as follows. APCI probe temperature and source temperature were 500 °C and 120 °C, respectively; cone voltage and coll energy were 30 V and 20 eV, respectively; desolvation gas flow and cone gas flow were 600 L/h and 50 L/h, respectively; monitored mass transitions were 591.5 → 104 for the triol and 598.8 → 103.8 for the internal standard; retention time was 5.5 min and quantification was based on standard curve ranging from 2 to 400 ng/mL for the triol (Ribas et al., 2016).

2.3. Chitotriosidase assay

Plasma enzyme determination was performed according to Hollak et al. (Hollak et al., 1994), using 4-methylumbelliferylβ-DN,N′,N″-triacetylchitotrioside as reaction substrate. The mixture for the enzyme assay was composed by 5 μL of acidified plasma and 26 μM of substrate dissolved in 100 mM citrate plus 200 mM phosphate buffer (pH 5.2), obtaining a total volume of 105 μL. This mixture was incubated for 15′ at 37 °C. Glycine-sodium hydroxide buffer (0.5 M, pH 10.3) was used as stop solution for the reaction and the fluorescence was determined with a Hitachi F2000 spectrofluorometer (λ excitation 365 nm and emission 450 nm). Normal range was considered between 8.8 and 132.0 nmol/h/

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