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

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Abstract

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 previous 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 monitoringization 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...
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 1/19,000/36,000. (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 intracerebral 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 Wüsten, 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 cholesteryl-β5a,6β-triol (3β,5a,6-β-triol) and 7-ketosterol (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,5a,6-β-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 (GC/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,5a,6β-triol measurement for NP-C therapy monitoring and also as a biomarker for NP-C diagnosis, in this work we analyzed 3β,5a,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. Cholesterol-3β,5a,6β-triol analysis

Levels of triol were determined by LC-MS/MS in EDTA-plasma, using cholestane-3β,5a,6β-triol D7 as internal standard and derivatization with dimethylglycine, 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 colll 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-methylumbelliferyl-N,N,N'-triacetylcitritoside 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/l/
2.4. Filipin staining

Skin biopsy samples were used for fibroblasts culture with HAM-F-10 medium and 10% of Fetal Calf Serum. After the cells reached confluence, Low Density Lipoprotein (LDL) was added to the culture. After two days, cells were transferred to slides and stained with Filipin reagent for histological examination in a fluorescent microscope. Intracellular lipid accumulation was determined as described in Blanchette-Mackie et al., (1988); ‘classical’ pattern of cholesterol accumulation showed a strong fluorescence in perinuclear vesicles (positive). The pattern of cells samples was categorized in normal (clear, no fluorescence), inconclusive or variant (moderated fluorescence) or typical or “classical” (high fluorescence).

2.5. Molecular analysis

Mutation analysis was performed using DNA isolated by standard method from patients’ blood samples. Coding sequences and flanking regions of the NPC1 and the NPC2 genes were amplified with PCR, purified and submitted to direct DNA sequencing using the BigDye1 Terminator Cycle Sequencing kit v. 3.1 (Applied Biosystems, Foster City, CA, USA), following the manufacturer’s instructions. Products were then submitted to capillary electrophoresis in an ABI PRISM1 3130x1 Genetic Analyzer, and sequences were analyzed with DNA Sequencing Analysis software v. 5.2 (Applied Biosystems). Mutations were confirmed by sequencing an independent DNA sample with both forward and reverse primers. Molecular analyses were conducted in blood of all patients of this study.

2.6. Statistical analysis

All results were expressed as mean ± standard deviation (SD). Unpaired Student’s t-test was used for groups comparison, and difference was considered significant when p < 0.05. All analysis and graphs were performed using the software GraphPad Prism® (GraphPad Software Inc., San Diego, CA, USA – version 7.0 for Windows®) in a compatible PC.

3. Results

Subjects were segregated in 2 different groups: patients with 3β,5α,6β-triol levels higher than 100 ng/mL (group A) and lower than 100 ng/mL (group B). This separation was established according to the cut-off value founded for this analyte in our laboratory. Table 1 shows that individuals from group A also presented higher chitotriosidase activity compared to group B, although this biomarker is not specific for NP-C, and also can be found increased in others LSD, such as Gaucher disease and NP-A/B (Sheth et al., 2010). Molecular analysis showed that 2 patients from group A did not have any mutation in NPC gene, excluding the existence of the disease. On the other hand, 3 individuals from group B presented positive molecular analysis for NPC, despite low levels of 3β,5α,6β-triol. Results of molecular analysis are summarized in Table 2. Considering these data and applying MedCalc software, the analysis of 3β,5α,6β-triol levels presented 88% of...
sensitivity, 96.08% of specificity, a positive and negative predictive value calculated in 91.67% and 94.23%, respectively. For these calculations, it was used molecular analysis as gold standard, since Filipin staining could not be performed in all individuals.

The therapy with miglustat and its effect in 3β,5α,6β-triol levels was also evaluated. It can be observed in Table 3 and Fig. 2 that 3β,5α,6β-triol levels are significantly lower in treated patients when compared to non-treated individuals, showing that this metabolite could be used not only for screening but also for therapy monitoring in NP-C.

4. Discussion

NP-C is a LSD currently conceived as a lipid trafficking disorder. Impaired egress of cholesterol from the late endosomal/lysosomal compartments is a specific key element of the pathogenesis (Vanier, 2015). Accumulated cholesterol in viscera and CNS can be oxidized by reactive oxygen species (ROS) in a nonenzymatic reaction forming oxysterols, mainly 3β,5α,6β-triol and 7-KC, which can be measured in plasma, contributing therefore for the disease investigation (Jiang et al., 2011). In this work, we determined 3β,5α,6β-triol levels, chitotriosidase activity, Filipin staining and NPC gene mutations from 76 individuals with NP-C suspicion, in order to investigate the potential of 3β,5α,6β-triol analysis for NP-C screening. The current method used for diagnosis in NP-C is Filipin staining, although this analysis shows a difficult interpretation and a variant presentation that can confuse the analyst (Vanier and Latour, 2015). For Filipin analysis, it was found 1 false positive, 7 false negative and 24 inconclusive cases, showing the limitations of this assay. The sensitivity (88%) and specificity (96.08%) of 3β,5α,6β-triol analysis verified by this work is consistent with previous studies (Jiang et al., 2011; Ribas et al., 2016; Reunert et al., 2016), and reaffirm the high potential of this metabolite for screening and its importance in NP-C diagnosis, especially when Filipin cannot be performed or is inconclusive. Even so, considering that the predictive positive value of 3β,5α,6β-triol was 91%, these data reinforce the crucial role of molecular analysis for definitive diagnosis, that should be performed in all individuals with a strong clinical suspicion, independent of 3β,5α,6β-triol concentrations.

Table 1 Results of Filipin staining, chitotriosidase activity and molecular analysis in patients with cholestane-3β,5α,6β-triol levels higher than 100 ng/mL (Group A) and lower than 100 ng/mL (Group B).

|                       | 3β,5α,6β-triol concentration (ng/mL; mean ± SD) | CT activity (nmol/h/mL; mean ± SD) | Positive cases by Filipin staining | Negative cases by Filipin staining | Inconclusive cases by Filipin staining | Positive cases by molecular analysis | Negative cases by molecular analysis |
|-----------------------|-----------------------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|---------------------------------------|--------------------------------------|--------------------------------------|
| Group A               | 164.6 ± 10.67                                 | 929.3 ± 346.5                     | 10                                | 0                                 | 2                                     | 22                                   | 20                                   |
| (n = 24)              |                                               |                                   |                                   |                                   |                                       |                                      |                                      |
| Group B               | 34.7 ± 3.803                                  | 174.5 ± 49.73                     | 1                                 | 7                                 | 24                                    | 3                                    | 49                                   |
| (n = 52)              |                                               |                                   |                                   |                                   |                                       |                                      |                                      |

* α Chitotriosidase.
* β Patients later diagnosed as Niemann-Pick A/B by measurement of sphingomyelinase activity using a radioactive method adapted from Pentchev et al. (Pentchev et al., 1980).

Table 2 Results for molecular analysis.

| Molecular analysis | Patients |
|--------------------|----------|
| Heterozygous for mutations p.D945N and p.F1221SfsX20 | 1 |
| Homozygous for mutation p.A1035 V | 5 |
| Heterozygous for mutations p.I923 V and p.E1166 K | 1 |
| Homozygous for mutations p.G992R | 1 |
| Heterozygous for mutations p.G992W | 2 |
| Homozygous for mutation p.R518 M | 1 |
| Heterozygous for mutation p.G1140 V | 1 |
| Homozygous for mutations p.A1035 V and p.E1166 K | 1 |
| Heterozygous for mutations p.Q710Rfs*27 | 1 |
| Homozygous for mutation p.P1007 V | 1 |
| Homozygous for mutation p.P1007A | 1 |
| Homozygous for mutation p.R518 M | 1 |
| Heterozygous for variation p.G992R and heterozygous for mutation p.A1035 V | 1 |
| Heterozygous for mutations p.P1007 A and p.E1166 K | 1 |
| No pathogenic mutations in the NPC1/NPC2 genes | 51 |

Table 3 Cholestane-3β,5α,6β-triol concentration, age of NP-C patients and therapy duration.

|                       | 3β,5α,6β-triol concentration (ng/mL; mean ± SD) | Age (years; mean ± SD) | Treatment duration (years; mean ± SD) |
|-----------------------|-----------------------------------------------|------------------------|--------------------------------------|
| Treated (n = 7)       | 44.47 ± 13.3                                  | 23.44 ± 11.95          | 4.29 ± 1.80                          |
| Non-treated (n = 25)  | 148.5 ± 10.0                                  | 12.5 ± 13.63           | –                                    |
Progressive neurological manifestations in NP-C have a profound effect on life’s quality of patients and their families. The correct and early identification of NP-C, as well as the appropriate use of symptomatic and disease-specific therapies can dramatically improve life quality for all those affected. Currently therapy for NP-C patients consists in a symptomatic treatment together with the use of miglustat to reduce neurodegenerative impairment. Miglustat is a small molecule that can cross the blood-brain barrier and acts as an inhibitor for the glucosylceramide synthase enzyme, decreasing glycosphingolipids, GM2 and GM3 levels in NP-C patients (Lyseng-Williamson, 2014). Besides, miglustat improves the traffic ligands in lymphocytes type B of NP-C patients (Lachmann et al., 2004), and also decreases lipid peroxidation and increases antioxidant status in NP-C1 patients (Ribas et al., 2012).

In this context, our study found significantly $\beta$-3,5a,6b-triol decreased levels in patients treated with miglustat when compared to non-treated patients, probably caused by reduced cholesterol availability for oxidation (Lachmann et al., 2004), and also providing a less oxidative environment at a cellular level of these patients. Currently, monitoring of miglustat efficacy in NP-C patients consists in clinical evaluation of neurologic symptoms (e.g. ambulation, manipulation, language and swallowing) as well as horizontal saccadic eye movement velocity (HSEM) (Lyseng-Williamson, 2014). In this way, our data shows that miglustat provides an improvement in biochemical status in treated individuals by reducing $\beta$-3,5a,6b-triol levels, what reinforces the potential use of this metabolite for the therapy monitoring of NP-C patients.

Porter et al. found an increase of $\beta$-3,5a,6b-triol levels in mice brain tissue and in NP-C patients cerebrospinal fluid (CSF) (Porter et al., 2010), showing a possible role of this metabolite in neurological disorders related to cholesterol metabolism. J. Lipid Res. 57, 361–367. http://dx.doi.org/10.1194/jlr.M061978.

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Declaration of interest

The authors declare that there is no conflict of interests.

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