UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE FARMÁCIA

DISCIPLINA DE TRABALHO DE CONCLUSÃO DE CURSO DE FARMÁCIA

MOLECULAR AND BIOCHEMICAL BIOMARKERS FOR DIAGNOSIS AND THERAPY MONITORIZATION OF NIEMANN-PICK TYPE C PATIENTS

TATIANE GRAZIELI HAMMERSCHMIDT

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Trabalho apresentado como requisito parcial para aprovação na Disciplina de Trabalho de Conclusão do Curso de Farmácia da Universidade Federal do Rio Grande do Sul

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MOLECULAR AND BIOCHEMICAL BIOMARKERS FOR DIAGNOSIS AND THERAPY MONITORIZATION OF NIEMANN-PICK TYPE C PATIENTS

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Abstract

Niemann-Pick type C (NP-C), one of 50 inherited lysosomal storage disorders caused by NPC protein impairment that leads to unesterified cholesterol accumulation in late endosomal/lysosomal compartments. The clinical manifestations of NP-C includes 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. In this work, we evaluated cholestane-3β,5α,6β-triol and chitotriosidase levels, Filipin staining and molecular analysis for NPC mutation in 76 individuals with NP-C suspicion. Also, we analyzed cholestane-3β,5α,6β-triol levels in 7 patients with previous NP-C diagnosis under treatment with miglustat, in order to verify its value as a tool for therapy monitoring. For cholestane-3β,5α,6β-triol analysis using molecular assay as golden standard we found 88 % of sensibility, 96.08 % of specificity, a positive and negative predictive value calculated in 91.67 % and 94.23 %, respectively. 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 in 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. These 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.

Keywords

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 .

Molecular and biochemical biomarkers for diagnosis and therapy monitorization of Niemann-Pick type 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 [1]. In 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 [2]. 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 [2]. Regarding its rarity, NP-C incidence is estimated in 1/89,000, but this data may be significant underascertainment of a late-onset NPC1 phenotype [3].

Despite there is no cure for NP-C, the management of symptoms is an important goal in therapy for these patients [4]. 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 [5]. 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 [5,6]. Cyclodextrins are also showing some promising results in several studies, but the mechanisms are not yet completely established [7,8].

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 [9]. 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 sensibility and specificity of this assay [9]. 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 [10]. Cholesterol can suffer oxidation in different ways, and it could be mediated by enzymes or in non-enzymatic reactions (Figure 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 [11]. 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 [11]. 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 [12,13]. 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.
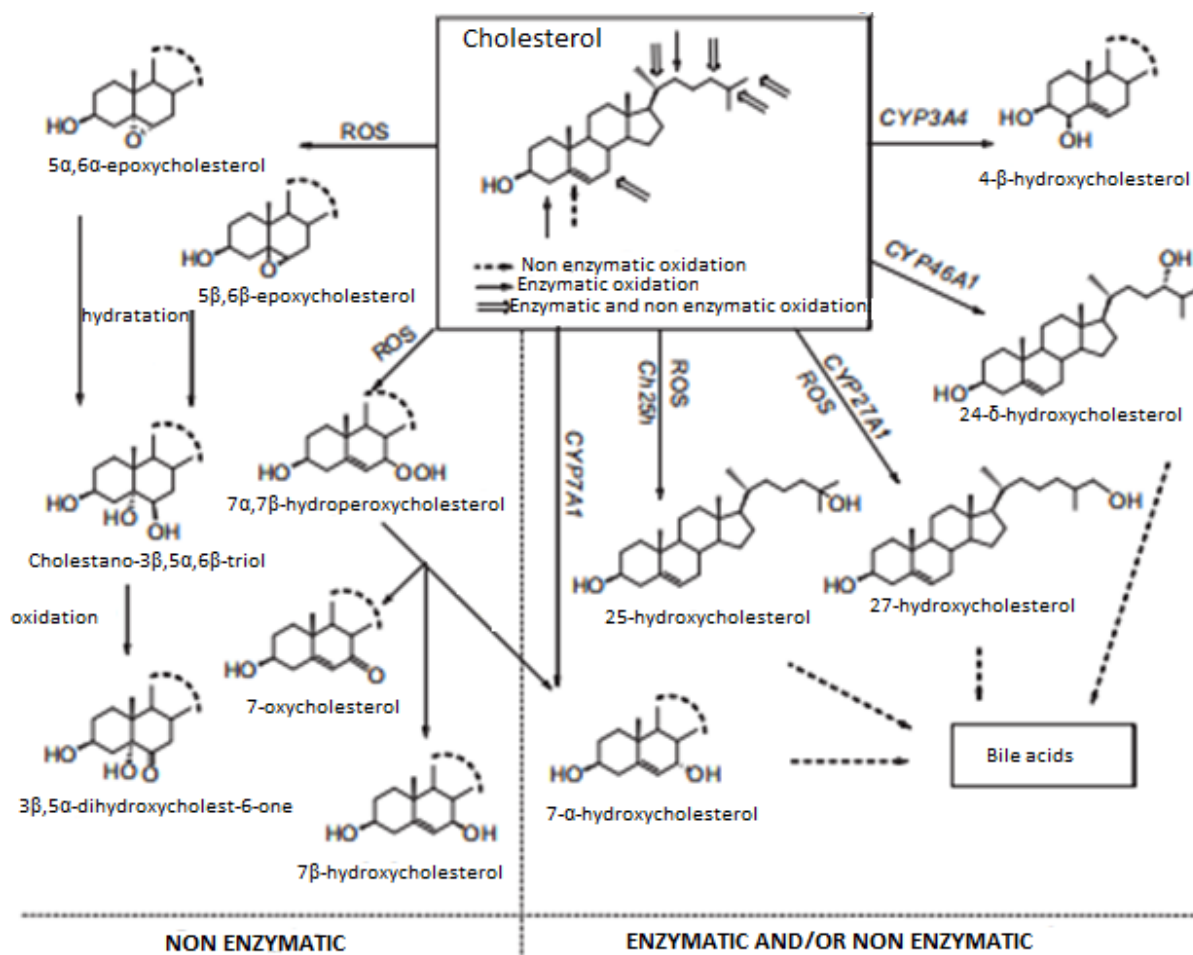

Fig. 1: Different pathways of cholesterol oxidation

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 ºC. The clinical features presented by these patients include 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 it 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 esters, according to Jiang et al [11], 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 [14].

2.3. Chitotriosidase assay

Plasma enzyme determination was performed according to Hollak et al [15], 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), obtained a total volume of 105 µL. This mixture was incubated for 15' at 37 ºC. 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/mL.

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 [16]; '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 3130xl 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 [17]. Molecular analysis showed that 2 patients from group A did not have any mutation in NPC gene, excluding the existence of the disease. In 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 has 88 % of sensibility, 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. Filipin staining was not performed in all individuals since 3β,5α,6β-triol analysis was previously established as a good screening assay for NP-C [14].

The therapy with miglustat and its effect in 3β,5α,6β-triol levels was also evaluated. It can be observed in table 3 and figure 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 monitorization in NP-C.

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)	[1]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 (n=24)	164.6± 10.67	929.3± 346.5	10	0	2	22	2
Group B (n=52)	34.7± 3.803	174.5± 49.73	1	7	24	3	49

[1]: Chitotriosidase

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.I923V and p.A1035 V	1
Heterozygous for mutations p.N195Kfsp*2 and p.F1221SfsX*20	1
Heterozygous for mutations c.114-122del19 and p.F1221SfsX20	2
Homozygous for mutation p.V694M	2
Homozygous for mutation p.F1221SfsX20	2
Heterozygous for mutations p.R1186H and p.F1221SfsX20	1
Heterozygous for sequence variation p.G992R and heterozygous for mutation p.G1140V.	1
Heterozygous for mutations p.A1035V and p.E1166K	1
Heterozygous for mutations p.S151Ffs*70 and p.F1221SfsX20	1
Heterozygous for variations p.G910S and p.G992W	1
Homozygous for mutation p.Q710Rfs*27	1
Homozygous for mutation p.P1007V	1
Homozygous for mutation p.P1007A.	1
Homozygous for mutation p.R518M	1
Heterozygous for variation p.G992R and heterozygous for mutations p.A1035V.	1
Heterozygous for mutations p.P1007A V and p.E1166K	1

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

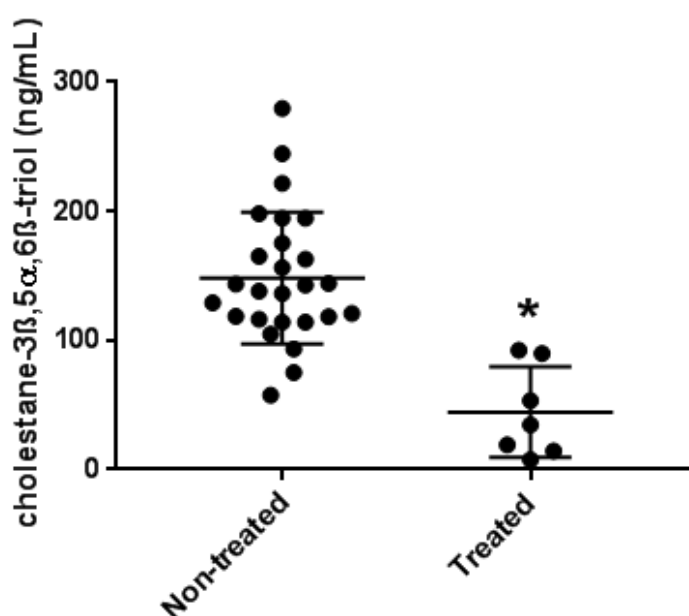|	3β,5α,6β-triol concentration (ng/mL; mean±SD)	Age (years; mean±SD)	Treatment duration (years; mean±SD)
Treated (n=7)	44.47 ± 13.3	23.44 ± 11.95	4.29 ± 1.80
Non-treated (n=26)	148.5 ± 10.0	12.5 ± 13.63	-

Fig 2: Cholestane-3β,5α,6β-triol concentration in treated and non-treated patients with NP-C. Data is represented as mean±SD. Student's *t* test was performed, and *p<0.0001 when compared with non-treated individuals.

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 [17]. 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 and used as biomarkers for diagnosis and therapy monitorization of NP-C [11]. 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 [18]. For Filipin analysis, it was found 1 false positive, 7 false negative and 24 inconclusive cases, showing the limitations of this assay. The sensibility (88 %) and specificity (96.08 %) of 3β,5α,6β-triol analysis verified by this work is consistent with previous studies [11,14,19], and reaffirm the high potential of this metabolite for screening and its importance in NP-C diagnosis, especially when Filipin cannot be performed or it 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.

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 along with the appropriate use of symptomatic and disease-specific therapies can dramatically improve life's quality for all those affected. Currently therapy for NP-C patients consists in a symptomatic treatment altogether with the use of miglustat to reduce neurological 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 [20]. Besides, miglustat improves the traffic lipids in lymphocytes type B of NP-C patients [21], and also decrease lipid peroxidation and increase antioxidant status in NP-C1 patients [10]. In this context, our study found a significant 3β,5α,6β-triol decrease levels in patients treated with miglustat when compared to non-treated patients, probably caused by reduced cholesterol availability for oxidation [22] and also providing a less oxidative environment at a cellular level of these patients. Currently monitorization 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) [20]. In this way, our data shows that miglustat provides an improvement in biochemical status in treated individuals reducing 3β,5α,6β-triol levels and this metabolite can be successfully used as a therapy monitorization for NP-C.

Porter et al found an increase of 3β,5α,6β-triol levels in mice brain tissue and in NP-C patients cerebrospinal fluid (CSF) [13], showing a possible role of this metabolite in neurological manifestations of these individuals. As we found a decrease in 3β,5α,6β-triol plasma levels in patients treated with miglustat, and once this therapy can slow down, or even decrease neurodegeneration, we can hypothesize that 3β,5α,6β-triol reduction could be associated with this improvement. However, further studies are necessary to explain how this mechanism works in NP-C disease.

5. Conclusion

Taken together, our presented data shows that the cholestane-3β,5α,6β-triol analysis by LC-MS/MS can be used for Niemann-Pick type C disease diagnosis and screening with good sensibility and specificity. This biomarker emerges as a potential candidate to substitute the current test for NP-C: the Filipin staining. In the other hand, the 3β,5α,6β-triol analysis also can be used for therapy monitoring, since a significant decrease in this metabolite levels was observed in patients treated with miglustat when compared to non-treated patients. Although the mechanism of this effect is not completely clear, it may be related to improve in lipid traffic and decrease in oxidative stress caused by this treatment.

6. Aknowledgements

7. Declaration of interest

The authors declare that there is no conflict of interests.

8. Funding informations

9. References

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ANEXO I - NORMAS PARA PUBLICAÇÃO NA REVISTA INTERNATIONAL JOURNAL OF DEVELOPMENTAL NEUROSCIENCE

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Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2,), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do

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Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

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Results should be clear and concise.

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Conclusions

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Appendices

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