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EFEITOS DA INTERRUPÇÃO DA TERAPIA DE REPOSIÇÃO ENZIMÁTICA (TRE) EM MODELO MURINO DE MUCOPOLISSACARIDOSE TIPO I

Porto Alegre

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Trabalho de conclusão de curso de graduação apresentado ao Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do título de Bacharela em Biomedicina.

Orientador: Prof.ª Dr.ª Ursula da Silveira Matte

Co-orientador: Prof. Dr. Guilherme Baldo

Porto Alegre

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“... toda a nossa ciência, comparada à realidade, é primitiva e inocente; e, portanto, é o que temos de mais valioso.”

(Albert Einstein)

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SUMÁRIO

LISTA DE ABREVEATURAS

DLD – Doenças lisossômicas de depósito

GAGs – Glicosaminoglicanos

IDUA – α-L-iduronidase

MPS – Mucopolissacaridoses

MPS I – Mucopolissacaridose do tipo I

OMIM – *Online Mendelian Inheritance in Men*

TCTH – Transplante de Células Tronco Hematopoiéticas

TRE – Terapia de Reposição Enzimática

RESUMO

A terapia de reposição enzimática para MPS I (Mucopolissacaridose tipo I) foi aprovada a mais de uma década, e vem sendo amplamente aplicada. Embora seja eficiente em corrigir alguns aspectos da doença, os pacientes precisam obter a enzima pelo Sistema Público de Saúde, um processo que envolve demandas judiciais, e eventualmente acarreta na interrupção do tratamento. Portanto, nesse trabalho buscamos avaliar os efeitos da interrupção do tratamento de TRE (Terapia de Reposição Enzimática) e sua posterior reintrodução em camundongos com MPS I. Para isso, analisamos quatro grupos de animais. No primeiro grupo, os camundongos MPS I foram tratados com TRE (Laronidase®, 1,2 mg / kg a cada 2 semanas) desde o nascimento, sem interrupção. Um segundo grupo de camundongos teve o mesmo tratamento, porém com interrupção dos 2 aos 4 meses. Estes camundongos foram comparados a camundongos normais e MPS I não tratados. Analisamos os níveis de GAGs (glicosaminoglicanos) urinários e teciduais, a função cardíaca, o comportamento, a distensão da parede da aorta, a presença de neuroinflamação e a formação de anticorpos. Todos os animais foram eutanasiados aos 6 meses. Nossos resultados mostraram que os níveis de GAGs apresentaram redução considerável em todos os órgãos com o tratamento iniciado logo após o nascimento. Porém, também vimos que em certos órgãos como o coração, o córtex cerebral e a aorta, a interrupção do tratamento levou à perda parcial dos efeitos benéficos do mesmo. Aos 6 meses, os animais com o tratamento interrompido e reintroduzido apresentaram testes comportamentais, parâmetros de funções cardíacas e espessura da parede da aorta piores, quando comparados aos animais sem interrupção. A interrupção do tratamento não conduziu à formação de anticorpos após a sua reintrodução, sugerindo que a TRE neonatal induz uma tolerância imunológica que foi mantida. Esses dados sugerem que a interrupção do tratamento pode ter efeitos deletérios sobre os órgãos que, durante o curso da doença, sofrem mudanças estruturais, tais como as aortas. Além disso, os níveis de GAGs urinários podem não refletir com precisão o estado da doença, uma vez que não se apresentaram alterados mesmo depois de algumas semanas de interrupção, independentemente da existência de efeitos deletérios nos outros órgãos.

INTRODUÇÃO

1. Doenças lisossômicas de depósito

Por ser uma organela presente em todos os tipos celulares, com exceção dos eritrócitos, o lisossomo tem papéis importantes na estrutura e função celular, e o seu mau funcionamento tem efeitos generalizados e multissistêmicos (Vellodi, 2005). As Doenças Lisossômicas de Depósito (DLDs) formam um grupo de aproximadamente 50 doenças, causadas por defeitos na hidrólise ácida lisossomal de macromoléculas endógenas (Greiner et al., 2005). Estas doenças ocorrem devido a mutações que afetam enzimas lisossômicas ou proteínas responsáveis pela biogênese e maturação dos lisossomos (Meikle et al., 2004). Por isso, este grupo é caracterizado pelo acúmulo de substratos não degradados dentro e fora desta organela.

Os inúmeros eventos bioquímicos e estruturais secundários ao acúmulo de substrato afetam as células, causando manifestações como hepatoesplenomegalia e cardiomiopatias (Vellodi 2005). Além disso, essas doenças apresentam um curso progressivo, o que pode levar à morte de pacientes no início da vida, como visto nas mucopolissacaridoses (Neufeld & Muenzer, 2011).

Praticamente todas as DLDs são de caráter autossômico recessivo, com exceção de três que são ligadas ao X: a MPS II, a doença de Fabry e a doença de Danon (Sugie et al., 2003). Já o amplo espectro de fenótipos clínicos encontrados revela que a gravidade é intimamente relacionada com a atividade enzimática residual (Conzelmann e Sandhoff, 1983).

2. Mucopolissacaridoses

As mucopolissacaridoses (MPS) são um conjunto de 11 doenças caracterizadas por mutações em genes que codificam enzimas da via de degradação dos glicosaminoglicanos (GAGs) ou, como chamados antigamente, mucopolissacarídeos. A ausência ou deficiência de uma enzima provoca um “bloqueio” no mecanismo catabólico, levando a um acúmulo progressivo de metabólitos intermediários dentro da organela (Neufeld e Muenzer, 2001). Clinicamente, estas doenças se caracterizam por alterações multissistêmicas, incluindo organomegalia, alterações ósseas (disostose múltipla), diminuição do crescimento, infecções recorrente, rigidez articular, problemas de audição e visão, e nas formas mais graves, retardo mental (Clarke e Heppner, 2011).

3. Mucopolissacaridose do tipo I

3.1 Aspectos gerais da MPS I:

A mucopolissacaridose do tipo I é causada por mutações no gene da alfa-L-iduronidase (IDUA), localizado no cromossomo 4 (4p16.3) em humanos (Scott et al., 1990). A IDUA é uma glicosidase e tem como função a hidrólise de heparan e dermatan sulfato (Figura 1) (Neufeld e Muenzer, 2001). Estes GAGs estão presentes em praticamente todos os tecidos do corpo, o que corrobora com as manifestações clínicas observadas em tecidos onde são amplamente distribuídos. A MPS I é considerada o protótipo de doença de depósito lisossômico, progressiva e multissistêmica. Devido ao amplo espectro de fenótipos, os pacientes são classificados em 3 síndromes de acordo com suas manifestações clinicas: a síndrome de Hurler (OMIM # 607014) é a mais grave, com morte dos pacientes na primeira década de vida e envolvimento do sistema nervoso central. A síndrome de Hurler-Scheie (OMIM # 607015) é a forma intermediária e a Síndrome de Scheie (OMIM # 607016) é a forma atenuada.

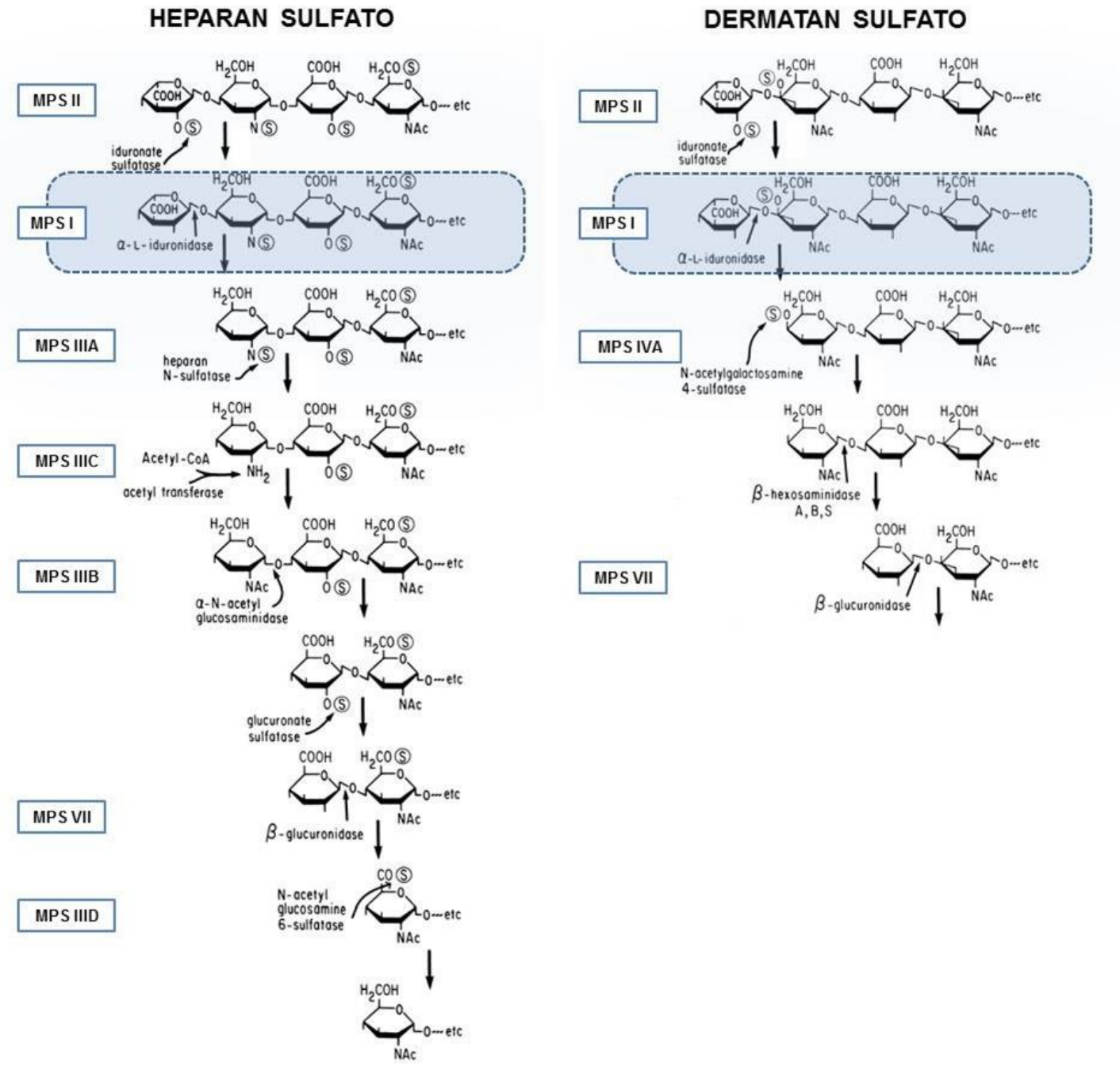

Figura 1 - Via de degradação dos glicosaminoglicanos heparan e dermatan sulfato. Nos quadrados estão indicadas as mucopolissacaridoses e as respectivas enzimas envolvidas. Em destaque temos a MPS I. Adaptado de Neufeld e Muenzer, 2001.

A MPS I tem uma incidência estimada de 1 em cada 100.000 nascidos vivos (Neufeld and Muenzer, 2001). Seu diagnóstico primeiramente é feito através da história clínica do paciente e análise de GAGs urinários, mas deve ser confirmado por ensaio enzimático utilizando amostras de sangue seco (*dried blood spot – DBS*), plasma, leucócitos ou fibroblastos e ainda pela análise molecular do gene IDUA (Lehman et al. 2011).

A sintomatologia da doença é variada e os pacientes podem apresentar hérnias umbilical ou inguinal no momento do nascimento, faces grosseiras, disostose múltipla, hepatoesplenomegalia, opacificação da córnea, perda auditiva, disfunções intelectuais e dificuldades de aprendizagem, doença valvar cardíaca, entre outros (Clarke e Heppner, 2011). Na síndrome de Hurler os sintomas surgem logo após o nascimento e progridem rapidamente, de modo que a maioria dos pacientes morre ainda na primeira década de vida. Contrapondo, na síndrome de Scheie, o início dos sintomas é mais tardio, sendo estes normalmente mais leves e com uma progressão mais lenta, de modo que a maioria dos pacientes mostra inteligência normal e sobrevive até a idade adulta. Já na síndrome de Hurler-Scheie, cujo fenótipo é intermediário, existe pouco ou nenhum comprometimento cognitivo, porém sintomas somáticos reduzem a expectativa de vida na segunda ou terceira década (Tabela 1) (Muenzer, Wraith & Clarke, 2009).

Tabela 1 - Prevalência e idade de início dos sinais e sintomas em pacientes com MPS I por fenótipo. Só são demonstrados sintomas que apareceram em pelo menos 25% dos pacientes com fenótipo. Os dados sobre idade são médias em anos. Adaptado de Beck et al., 2014.

	Fenótipo de MPS I					
	Hurler		**Scheie**		**Hurler-Scheie**	
Sinais e sintomas	%	Idade (anos)	%	Idade (anos)	%	Idade (anos)
Hérnia	58,9	0,8	53,5	4,6	59,9	3,2
Características faciais	86,4	0,9	48,0	8,7	72,2	3,4
Cifose/ gibosidade	70,0	1,0	-	-	33,5	4,6
Disostose múltipla	43,6	1,1	35,4	8,0	37,4	4,2
Opacidade da córnea	70,9	1,1	70,1	10,5	68,3	4,4
Hepatomegalia	70,0	1,1	48,0	9,4	66,5	4,4
Esplenomegalia	50,9	1,2	27,6	11,0	47,1	4,6
Língua alargada	41,3	1,2	-	-	38,3	4,0
Distúrbios do sono	51,6	1,2	26,8	8,7	48,9	4,0
Dano cognitivo	46,4	1,2	-	-	31,3	3,8
Anormalidades em válvulas cardíacas	48,9	1,3	67,7	11,7	59,0	5,7
Tonsilas alargadas	28,6	1,5	-	-	33,0	4,1
Contraturas articulares	37,9	1,6	69,3	7,6	57,3	4,2
Síndrome do túnel do carpo	-	-	51,2	12,5	27,8	7,4
Displasia de quadril	-	-	25,2	8,4	25,6	6,2

3.2 Tratamentos para MPS I

Não existe cura para as MPS em geral, entretanto, existem tratamentos que podem melhorar alguns sintomas da doença. Os principais tratamentos para as MPS são: o transplante de células-tronco hematopoiéticas (TCTH), a Terapia de Reposição Enzimática (TRE), a terapia de inibição da síntese do substrato e a terapia gênica (Guarany et al, 2012).

Os dois tipos de tratamentos mais utilizados para os pacientes com MPS I são o TCTH e TRE. Ambos os tratamentos foram baseados no conhecimento de que enzimas lisossômicas podem ser secretadas e captadas por células vizinhas via receptores de manose-6-fosfato. Estes substituíram o tratamento com apenas terapias de suporte, consistido por fisioterapia, cirurgias e medicações.

O TCTH tem sido uma opção de tratamento há muitos anos e é o tratamento de escolha em crianças com menos de 2 anos de idade que possuam a forma mais grave da doença, antes do aparecimento dos sintomas neurológicos. Porém, muitas vezes isso não é possível devido ao diagnóstico tardio e ao tempo necessário para encontrar doadores compatíveis (Vieira et al, 2008). Devido a isso, a TRE é a forma de tratamento mais utilizada no Brasil.

A TRE foi aprovada pela ANVISA (Agência Nacional de Vigilância Sanitária) no Brasil em 2005. Ela consiste na administração por via intravenosa da versão recombinante da enzima IDUA (*Aldurazyme®, Genzyme Corporation e BioMarin Pharmaceutical, USA*), podendo ser semanal (dose 0.6 mg/kg de peso corporal) ou quinzenal (1.2 mg/kg de peso corporal) (Giugliani et al., 2009). Vários estudos demonstram os efeitos benéficos deste tratamento, por exemplo, na redução no volume do fígado e baço (Sifuentes et. al, 2007), porém existem resultados conflitantes em relação à sua eficácia em outros órgãos, como na função do miocárdio (Sifuentes et al., 2007). Dentre os problemas encontrados neste tratamento podemos citar a formação de anticorpos contra a enzima (Brooks, 2003), e a interrupção do mesmo. Porém, estudos recentes mostraram que quando a TRE é iniciada no período neonatal há melhoras em aspectos importantes como formação de anticorpos, espessura de válvulas cardíacas, dilatação cardíaca e da aorta e mesmo na doença neurológica

(Baldo et al. 2013). No entanto, dados demonstram que, nas doses administradas, a enzima recombinante não atravessa a barreira hemato-encefálica (Munoz-Rojas et al., 2008). Por isso, a TRE acaba sendo iniciada assim que o diagnóstico é estabelecido para melhorar os sintomas que não são neurológicos.

3.3. Interrupção da Terapia de Reposição Enzimática

O alto custo da TRE faz com que o tratamento de poucos pacientes gere um gasto muito alto ao sistema público de saúde. Segundo dados do Departamento de Informática do SUS e processos judiciais do arquivo do Ministério da Saúde, os gastos com a TRE para MPS I no período de 2006 a 2010 foram de R$ 5.890.646,00 (Diniz et al., 2012). Isso faz com que os pacientes brasileiros frequentemente entrem na justiça para garantir o acesso à enzima. Além disso, a necessidade de infusões repetidas afeta a qualidade de vida dos pacientes e de suas famílias, uma vez que estas têm de se deslocar de suas casas e cidades, o que também pode acarretar em descontinuação do tratamento. Ambos os fatores podem resultar em períodos de interrupção do tratamento, tanto devido a fatores burocráticos quanto pela dificuldade de manutenção dass infusões semanais.

Deste modo, estudos que mostrem os efeitos de interrupções na TRE são necessários, visto que, no contexto brasileiro, a interrupção do tratamento é um cenário comum e os seus efeitos deletérios são pouco conhecidos, podendo levar a uma diminuição da eficácia do tratamento. Atualmente, existe apenas um relato de caso que evidencia os efeitos deletérios da interrupção da TRE em um paciente com MPS I (Anbu, Mercer, and Wraith 2006). Em outros tipos de DLDs, porém, isso já foi relatado e parece ocorrer uma piora dos sinais e sintomas clínicos que podem não se revertida após a re-introdução do tratamento (Drelichman et al, 2007).

OBJETIVOS

Objetivo geral

Avaliar os efeitos da interrupção da Terapia de Reposição Enzimática (TRE) em camundongos com MPS I.

Objetivos específicos

- Avaliar se os níveis de GAGs são alterados com a interrupção da TRE em amostras de órgãos e urina através de método bioquímico;

- Analisar se os depósitos de GAGs são alterados com a interrupção da TRE em amostras de órgãos por meio de análise histológica;

- Analisar os efeitos da interrupção da TRE sobre parâmetros ecocardiográficos e comportamentais;

- Avaliar a presença de neuroinflamação através de Imunohistoquímica para GFAP;

- Verificar a presença de anticorpos contra a enzima recombinante em animais com interrupção do tratamento.

TRABALHO EXPERIMENTAL EM FORMA DE ARTIGO CIENTÍFICO

O presente trabalho será apresentado nas normas da revista *Molecular Genetics and Metabolism*.

Deleterious effects of discontinuing enzyme replacement therapy in MPS I mice

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Abstract

Mucopolysaccharidosis type I (MPS I) is caused by the deficiency of α-L-iduronidase (IDUA), leading to accumulation of glycosaminoglycans (GAGs) in tissues and damage to multiple organs. Although enzyme replacement therapy (ERT) is efficient, obtaining the enzyme is expensive and lifelong weekly infusions are necessary, which may result in discontinuing treatment periods. So, we evaluated the effects of discontinuing ERT in MPS I mice. We used knockout mice for the *Idua* gene treated with ERT (laronidase®, 1.2 mg / kg / 15 days) from birth, with or without discontinuing. All animals were euthanized at 6 months. We evaluated the urinary and tissue GAG levels, cardiac function, aortic wall distension, neuroinflammation, behavior and antibody formation. GAG levels were in the upper normal limit even after two months of discontinuing ERT. However, pulmonary vascular resistance, heart contractility and aortic wall thickness were not normalized despite treatment reintroduction. In cerebral cortex, GFAP positive cells presented intermediate results in both treatment groups. Interestingly, no antibody formation was detected after reintroduction of treatment, evidencing that an immunological tolerance was maintained. In conclusion, discontinuing ERT should be avoided in order to maintain the benefits achieved by ERT. Some structures, such as the aorta and the heart, seem to have a special deleterious effect with discontinuation.

Keywords: Enzyme replacement therapy; Mucopolysaccharidosis type I; treatment interruption.

1. Introduction

Mucopolysaccharidosis type I (MPS I) is progressive life-threatening lysosomal storage disorder caused by deficiency of the enzyme α-L-iduronidase (IDUA, EC 3.2.1.76), which mediates the degradation of the glycosaminoglycans (GAGs) dermatan sulphate and heparan sulphate. Patients present multiple alterations, including learning difficulties, facial and skeletal abnormalities, hepatosplenomegaly, joint stiffness, heart enlargement, aorta distention and valvular heart disease [1].

Initially treated only with supportive measures, several studies in the last decades have shown that hematopoietic stem cell transplantation (HSCT) can be efficient for MPS I if performed before the onset of brain manifestations to prevent cognitive decline (which occurs usually at 2 years of age). However, mortality and morbidity rates related to the process and the delayed diagnosis are to date problems that limit the use of HSCT for MPS I patients [2, 3].

Enzyme replacement therapy (ERT) with recombinant α-L-iduronidase (Laronidase) was approved for MPS I in 2003 and is currently the most used treatment for this disease. It has shown to significantly reduce urinary GAGs levels, as well as liver volume, heart enlargement and other visceral abnormalities [4]. However it is believed that the enzyme when applied intravenously is not able to cross the blood-brain-barrier and reach the brain [5]. Other difficult-to-treat organs are the poor vascularized aorta, heart valves, joints and bones [4, 6, 7].

The ERT is a very expensive medication (approximately U$ 300,000/patient/year) [8]. It is considered an orphan drug, and, at least in Brazil, very often patients suffer from treatment interruptions. Major reasons for that are the need of weekly injections that implies in patients often moving to another city to reach treatment centers, or due to lawsuits to gain access to the enzyme. A previous study described a case of a MPS I patient that had to discontinue ERT because she was pregnant. Treatment interruption caused a significant and rapid deterioration of the patient, and after treatment reintroduction, important parameters such as forced vital capacity and 6-min walk test

only showed partial improvement [9]. Even though, the effects of treatment interruption were never systematically evaluated.

We have previously shown that neonatal treatment with ERT in MPS I mice lead to reduced urinary GAG levels, benefits in visceral organs, improved heart function, and reduced aorta dilatation, as well as a reduction in antibody formation against the enzyme. Furthermore, some enzyme seemed to be able to cross the blood-brain-barrier and reach the brain, even improving behavior skills [10]. Our goal in the current work is to compare the results from mice continuously treated from birth with ERT previously reported (Baldo et al, 2013) with mice who were subjected to treatment discontinuing and reintroduction of ERT.

2. Material and Methods

2.1. Experimental design

The present study was approved by our institutional ethics review board and mice on a C57BL/6 background (kindly donated by Dr Elizabeth Neufeld, UCLA) were used. MPS I and normal mice were genotyped by PCR as previously described [11]. The MPS I mice developed a phenotype compared to Hurler syndrome [12].

The animals were separated into four groups (n= 3-16/ group): in the first group MPS I mice (*Idua* $^{-/-}$) received ERT intravenous (Laronidase® Genzyme, 1.2 mg / kg every 2 weeks) from birth (Neo-ERT) to 6 months without interruption. Most of the results from these mice were already reported [10] and are being used for comparison. The second group received the same treatment from birth to 6 months, but with interruption of treatment at 2 months and reintroduction at 4 months (Stop-ERT). We compared those groups to untreated MPS I mice (MPS I) and to wild-type mice (Normal).

During treatment interruption, urine samples were collected. Two weeks after the last injection, mice were subjected to behavioral testing (open field test) and

echocardiography. At 6 months of age, euthanasia was performed through the inhalation anesthetic isoflurane and cervical dislocation, and blood samples and organs were collected. The samples were stored at -80°C for biochemical analysis and embedded in paraffin for histological studies.

2.2. GAG measurement

Samples of heart, lung, liver, and kidney with approximately 20 mg were macerated in 600 uL of phosphate buffer 0.5 M pH 6.5 with 0.24 g/L L-cysteine and 0.4% EDTA 0.5 M using automatic homogenizer for 20 seconds. GAGs were separated after chloroform extraction and centrifugation at 10,000 G (9,000 rpm) for 15 minutes followed by separation of the supernatant. Aorta samples were macerated in 150 uL of acetate buffer 0.1 M pH 5.5, due to its small size. GAGs were quantified by Dimethyl Blue (DMB) technique, where 25 uL of supernatant is mixed with freshly prepared DMB solution (DMB 0.3 mol/L with 2 mol/L Tris) and absorbance was read at 530 nm. The results are expressed in ug GAG/mg protein. Protein content was measured using the Lowry assay.

Urine samples were centrifuged and 25 uL were used to measure levels of GAGs through Dimethyl Blue technique. The results were calculated as ug GAG/mg creatinine. Creatinine was measured using the Picric acid method [13].

2.3. Histological analysis

Aorta, heart, lung, liver, kidney, testis, and cortex tissue samples were properly fixed in 10% formalin for 48 hours, embedded in paraffin, and histological sections of 4mm were made. The cross sections were stained according to routine techniques with Hematoxylin-Eosin and Alcian Blue and analyzed. The aorta wall thickness was measured in at least 5 different points using the QCapture software (Q Imaging, British Columbia, Canada) and the average was used as a measure of aorta wall distention. Immunohistochemistry for glial fibrillary acidic protein (GFAP) was performed using a specific antibody (Abcam, Polyclonal Rabbit anti-GFAP) diluted 1/5000, and a

peroxidase-conjugated streptavidin molecules (Dako LSAB® 2 System-HRP). Slides were analyzed counting positive cells in 5 fields. All analyzes were performed in 400X magnification.

2.4. Behavioral tests

2.4.1. Open field test

The locomotor and exploratory activities were analyzed through the open field test. This test consists of a square area (52x52 cm^2) surrounded by walls 60 cm clear walls. The floor of this apparatus is divided into 16 squares by parallel and perpendicular lines, of which 4 are centrals and 12 are peripherals. Mice were placed in the right corner square, and locomotion and exploratory behavior was observed for 5 minutes for all groups. Locomotion is the number of times the animal was crossing one of the lines on the floor with 4 legs, and exploratory behavior is the number of times the animal was on the lower limbs. The open field apparatus was cleaned between each mouse using 70 % Ethanol.

2.4.1. Repeated open field

To analyze the habituation memory, mice were put in the open field apparatus for 5 minutes and number of crossings and rearings is measured. The test was repeated two more times, 30 and 60 minutes after the first trial, to evaluate habituation to the new environment. The results from the third trial are compared to the first.

2.5. Echocardiographic analysis

At six months, the mice were subjected to analysis of cardiac function by transthoracic two-dimensional echocardiography. For that, the mice were anesthetized with isoflurane and placed in the lateral decubitus (45 ° angle) to obtain the images. The

images were captured by a trained professional with experience in small animals echocardiography.

Parameters such as left ventricular ejection fraction (LVEF), left ventricular shortening fraction (LVSF), fractional area change (FAC), acceleration / ejection time in the pulmonary valve (AT / ET), systolic diameter of the left ventricle (DS) and diastolic diameter of the left ventricle (DD) were evaluated.

To calculate these parameters, we use the following equations: LVEF = (end diastolic volume − end-systolic volume)/ end-diastolic volume × 100. The end volumes were calculated using *Sympson's rule.* LVFS = (diastolic diameter − systolic diameter) / diastolic diameter × 100. FAC = (diastolic area—systolic area)/ diastolic area. As the AT/ET ratio is used as an index of pulmonary vascular resistance, their measures were obtained using Doppler echocardiography [14].

2.6. Antibody formation.

Immediately after collection of blood samples by retro-orbital puncture, the samples were centrifuged to obtain serum. Then, we evaluated the formation of antibodies against the recombinant enzyme present in the serum of animals at time of euthanasia. For this assay, 96-well ELISA plates were coated with 4 µl/mL of Laronidase® (Aldurazyme, Genzyme Corporation) overnight in PBS and blocked with 3% BSA. The diluted serum (1:50) was added to the plate and incubated for 2 hours. A secondary antibody conjugated to peroxidase (anti-mouse IgG) was diluted 1:1000, incubated for 3 hours and developed with TMB for 6 minutes. The reaction was stopped with 1M H_2SO_4 and then absorbance was read at 450 nm.

2.7. Ethics and statistics

All experiments in this study were approved by institutional ethics committee (Committee of Ethics in Research from Hospital de Clínicas de Porto Alegre – permit number 08-658). All procedures with animals were monitored by a veterinary and

designed to minimize animal suffering. Statistical analysis was performed using IBM SPSS Statistics version 20. According distribution of each variable, the data were compared using ANOVA and Tukey or Kruskall-Wallis and Mann-Whitney. Continuous data are expressed as mean (± standard deviation). P values < 0.05 were considered statistically significant.

3. Results

3.1. Urinary GAG

Previously, our group showed that urinary GAG levels in MPS I mice increase since 1 month of age and remains constant up to 8 months [12]. It was also shown that neonatal treatment reverses the urinary GAG levels to normal levels [10]. Here, we observed that GAG levels 2 weeks after treatment interruption were still within normal range. Surprisingly, GAG levels remained low and within normal levels during the whole 2 months of treatment discontinuation differing from untreated MPS I mice with 2 months (P<0.01) (Fig. 1).

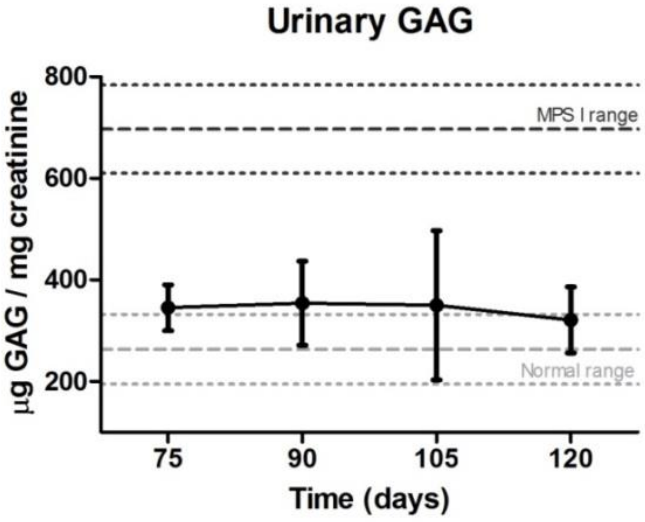

Fig 1. Urinary GAG levels at discontinuing treatment period. Urine was collected from 2 (day 75) to 4 months (day 120) fortnightly. P<0.001 compared to MPS I range, ANOVA and Tukey.

3.2. GAG storage in visceral organs

In all tissues analyzed, GAG levels of MPS I mice were mighty increased. In liver and kidney MPS I GAG levels are about 15 ($P<0.001$) and 8 ($P<0.01$) fold the normal group, respectively. In lung and heart, this difference is about 5 and 3 fold, respectively ($P<0.001$). Neo-ERT reduced GAG levels in all organs analyzed, being different from the untreated MPS I mice ($P<0.001$ for lung, kidney and heart and $P<0.05$ in liver). GAG levels in Stop-ERT after treatment reintroduction were similar to Neo-ERT in both liver and kidney. In the lungs (1.6-fold normal, $p= 0.02$ vs Normal) and heart (1.4-fold normal, $p=0.047$ vs Neo-ERT) GAG levels were slightly increased (Fig. 2A).

Histological analyses demonstrated several blue vacuoles in the MPS I mice tissues. These were absent in normal mice and also in both treatment groups, in agreement with biochemical measurement (Fig. 2B).

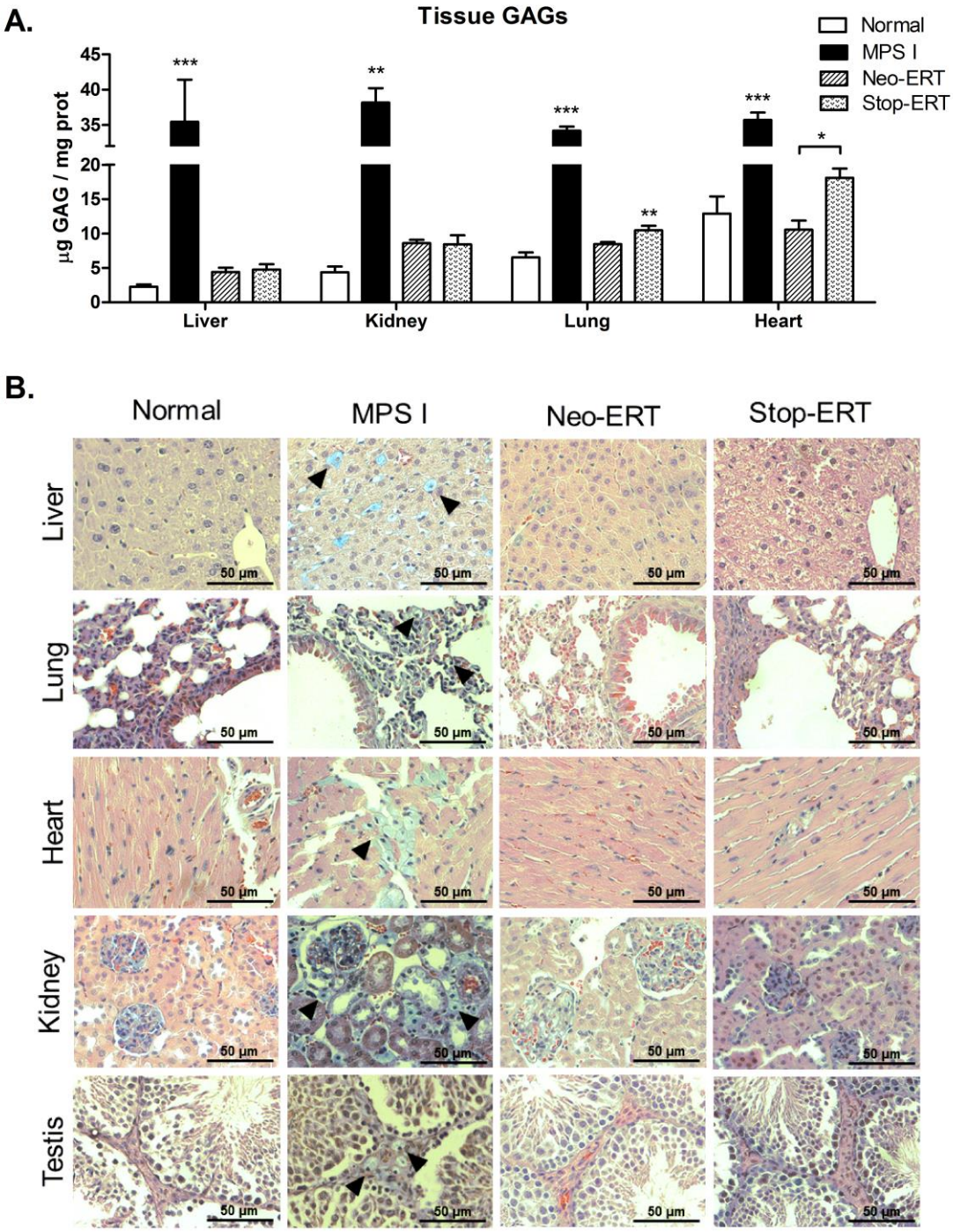

Fig 2. Tissues GAG levels. A) Tissue GAG from Normal (n = 3-5), MPS I (n = 3/4), Neo-ERT (4-6) and Stop-ERT (n=5) mice collected two weeks after last Laronidase injection. Both treatments were different of MPS I mice in these organs (P<0.001). *P>0.05, **P<0.01 and ***P<0.001 ANOVA and Tukey. B) Representative histological sections stained with H-E/alcian blue (which show GAG storage in blue, indicated by black arrows) in visceral organs.

3.3. Heart function

Among the parameters of cardiac function analyzed, we found that Neo-ERT caused a significant improvement in LVSF and AT/ET ratio when compared to untreated MPS I mice (P<0.01 for LVSF; P<0.05 for AT/ET) [10]. With discontinuing treatment (Stop-ERT), LVSF was not different from control groups, but the AT/ET ratio was not improved compared to Normal group (P<0.05). In parameters such as LVEF, FAC, SD and DD the values found were similar to normal (P<0.01) suggesting that left ventricle function and heart dilatation are not altered after treatment interruption or recover upon ERT reintroduction (Table 1).

Table 1. Myocardial parameters analyzed by echocardiography at 6 months old *Idua*$^{-/-}$ treated or untreated, compared to normal mice.

Parameters	Normal	MPS	Neo-ERT	Stop-ERT
LVSF (%)	37.52 ± 8.74	24.51 ± 4.78*	41.64 ± 7.71$^{\#\#}$	35.84 ± 7.32
LVEF (%)	60.23 ± 8.66	49.57 ± 12.19*	58.55 ± 6.97	59.61 ± 15.91
FAC (%)	51.53 ± 10.41	42.52 ± 11.06	53.32 ± 7.10	48.05 ± 6.52
AT/ET ratio	0.26 ± 0.04	0.18 ± 0.04***	0.24 ± 0.04$^{\#}$	0.19 ± 0.03*
SD (cm)	0.25 ± 0.05	0.35 ± 0.08**	0.22 ± 0.04$^{\#\#\#}$	0.23 ± 0.05$^{\#}$
DD (cm)	0.39 ± 0.03	0.46 ± 0.08*	0.37 ± 0.03$^{\#\#}$	0.36 ± 0.04$^{\#\#}$

Legend: LVEF - left ventricular ejection fraction; LVSF - left ventricular shortening fraction; FAC – fractional area change; DD – left ventricle diastolic diameter; SD – left ventricle systolic diameter. *P<0.05, **P<0.02 and ***p=0.001 compared to normal. #P<0.02, ##P<0.01 and ###P<0.001 compared to MPS I. (n=4-16 animals/ group).

3.4. Aortic analysis

When we analyzed GAG levels in aorta, we found that MPS I mice have about 9 fold the normal mice values (P<0.001). In both treated groups, Neo-ERT and Stop-ERT, GAG levels in aorta are higher than in the Normal group (2-3 fold, P<0.05) (Fig. 3A). However, when we measure aortic wall thickness, we observe that the MPS I and Stop-ERT groups present statistically different values when compared to normal mice (P<0.001 and P<0.003, respectively) (Fig. 3B). This finding is corroborated by a larger number of vacuoles in the aorta of MPS I (Fig. 3D) and Stop-ERT (Fig. 3E) mice.

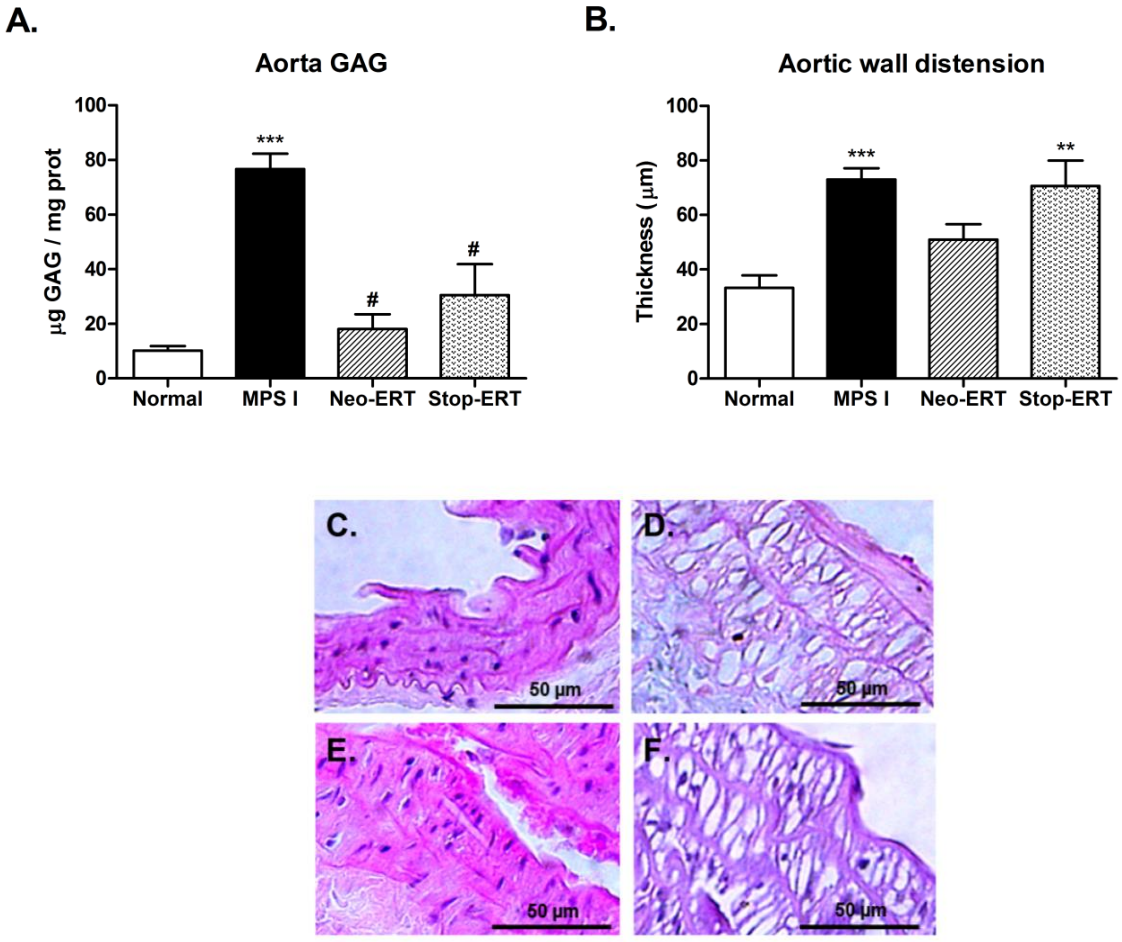

Fig 3. Aortic analysis. A) GAG measure in aorta. B) Quantification of aortic wall distension in the 4 groups (n=3-7 animals/group). **P<0.01 and P<0.001 compared to normal, ANOVA and Tukey. #P<0.05 compared to MPS I, ANOVA and Tukey. C-D) Representative sections from 6 month mouse aorta normal (B), MPS I (C), Neo-ERT (D) and Stop-ERT (E) groups stained for H-E/Alcian Blue (400× magnification).

3.5. Behavioral analysis and brain GAG levels

In the open field analysis, MPS animals showed decreased locomotor activity (crossings) and exploratory behavior (rearings) when compared to normal and Neo-ERT mice (P<0.003 and P<0.001, respectively). Animals in the Stop-ERT group showed intermediate values in both parameters (Fig. 4A).

In repeated open field, the MPS I mice showed statistical difference from normal mice only in number of crossings (P<0.01). Both treated groups showed a wide range of values, without statistically significant difference from normal (Fig. 4B).

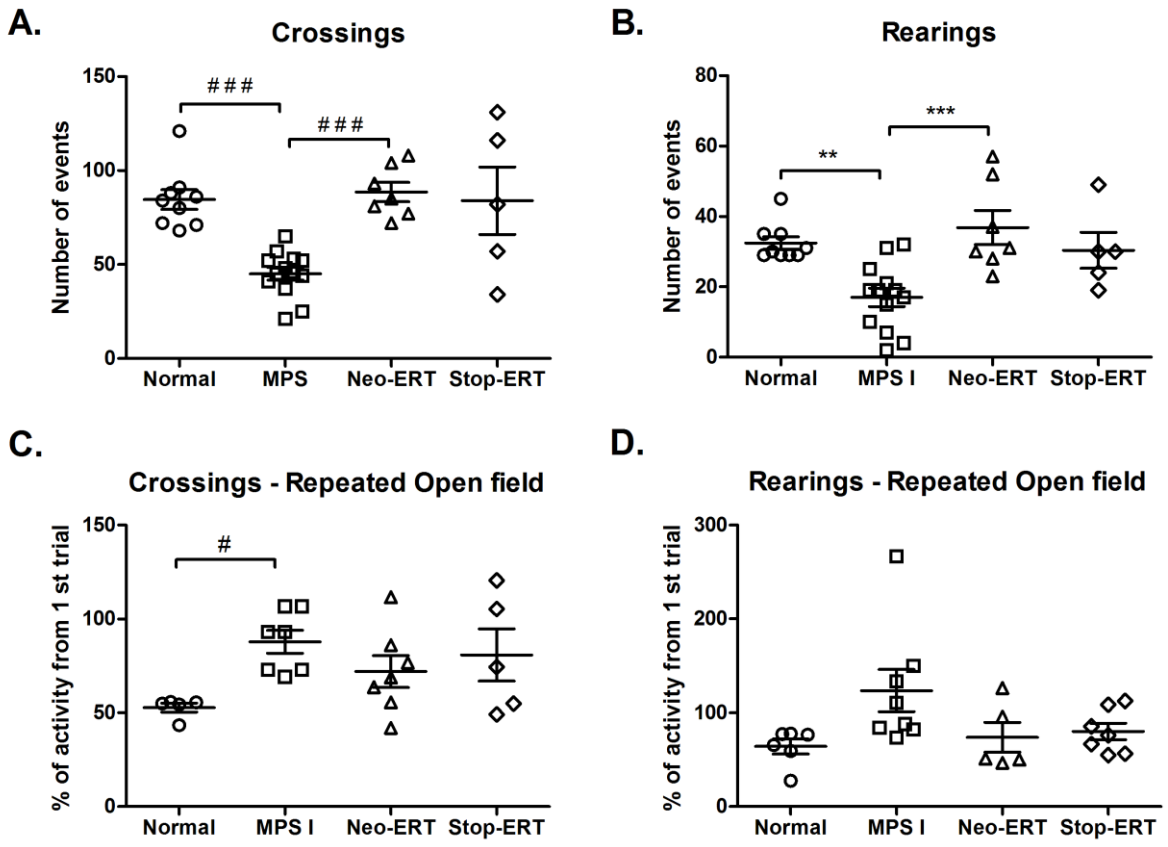

Fig 4. Brain involvement. A -B) Open field test was used as a measure locomotor (crossings) and exploratory (rearings) activities (n=5-13 animals/group). C-D) The repeated open field test is a measure of habituation (non-aversive memory). The activity in the 3rd trial was compared to the 1st trial, as results are expressed as percentage of activity from 1st trial (n=5-7 animals/group). **P<0.01 and ***P=0.001 ANOVA and Tukey. #P<0.05 and ###P<0.003 Kruskal-Wallis and Mann-Whitney.

3.6. Neuroinflammation

In MPS I mice is common the occurrence of cognitive deficit. So we decided to investigate the existence of neuroinflammation in the cerebral cortex by counting GFAP positive cells, a marker of neurologic damage. MPS I mice showed a large number of GFAP positive cells when compared to normal (P <0.001). However, both treated groups, Neo-ERT e Stop-ERT, showed values between MPS I and normal groups (P<0.01) (Fig. 5).

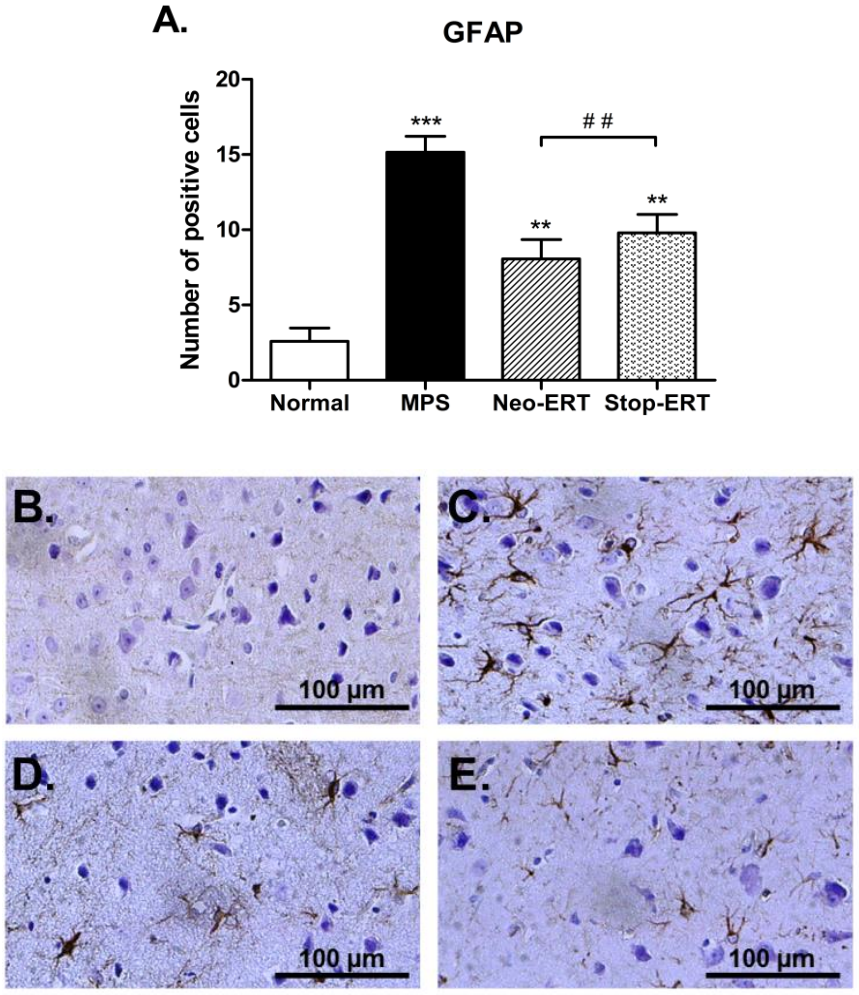

Fig 5. Evaluation of the presence of neuroinflammation in the cerebral cortex. A) Number of positive cells counted in 5 fields (n= 5 animals/group). B-E) Representative sections from 6 month mouse cerebral cortex normal (B), MPS I (C), Neo-ERT (D) and Stop-ERT (E) groups stained for glial fibrillary acidic protein (GFAP).). **P<0.05 and ***P<0.001 compared to normal, ANOVA and Tukey. ##P<0.01 compared to MPS I, ANOVA and Tukey.

3.7. Antibody formation

The next step was to check for the formation of recombinant anti-IDUA antibodies when ERT after ERT reintroduction. Using ELISA, we observed that Neo-ERT prevents antibody formation with only one of seven animals presenting detectable antibodies against the enzyme [10]. This immune tolerance is maintained even if treatment was interrupted. In control groups, MPS I and Normal, antibodies were not detected (Fig. 6).

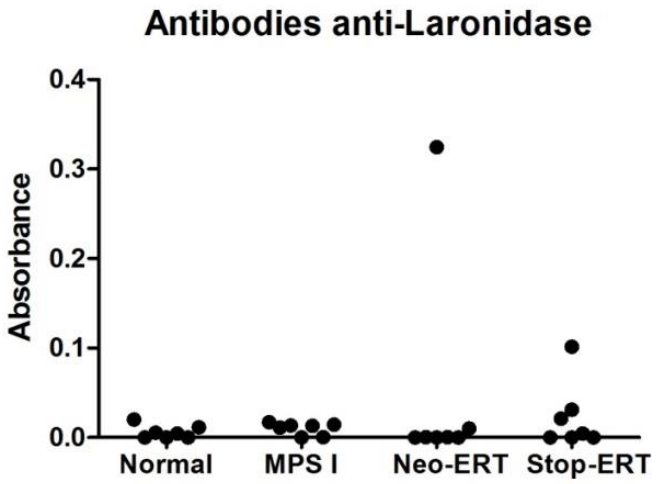

Fig 6. Antibody formation. Serum IgG-anti-Laronidase antibodies in mice at 6 months. Each dot represents results from individual mice. No statistical difference was detected between groups.

4. Discussion

In MPS I the damage triggered by GAG accumulation appears in various organs, and considerably decreases the quality and life expectancy of patients. In 2003, enzyme replacement therapy (ERT) was approved by FDA and brought with it many benefits to patients, especially for those who were not eligible for hematopoietic stem cell transplantation [15]. However, due to different reasons it is not unusual for these patients to have periods of discontinuing ERT.

It is known that ERT initiated immediately after birth, reverts various parameters of MPS I such as GAG levels in the various organs affected by the disease [10]. Here, we

have shown that the discontinuing of ERT in animals followed by its reintroduction didn't compromise the beneficial effects of treatment in the liver, kidney, and aorta whose GAG levels decreased considerably. However, in the heart and lungs, GAG levels were not completely normal. Urinary GAG levels, on the other hand, remained in the upper normal values throughout treatment discontinuation. This observation suggests that urinary GAG must be used with caution as a biomarker of disease status, as it has already been pointed out [16].

The heart is an organ largely affected by the accumulation of GAGs, which makes the presence of heart diseases very common. Heart diseases are seen in approximately 60% of patients and contribute significantly to morbidity [17]. Among the abnormalities are valvular disease, narrowing and/or occlusion coronary artery [18], and distension or reduction of the thickness of large vessels [19, 20]. It has been shown that several parameters of cardiac function improve in neonatal ERT [10]. Discontinuing ERT is particularly damaging in pulmonary vascular resistance, which reflects in pulmonary hypertension. Other parameters such as left ventricle function and heart dimensions seem to be unaffected or reverse upon ERT reintroduction.

Abnormalities in the aorta are also common [17]. Among them, we can mention the reduction of elasticity of the aorta [21] which is related to the GAG effects in assembly of tropo-elastin, resulting in decreased amount or abnormal structure of elastin [20]. Several studies in MPS I animals demonstrated that there is involvement of matrix metalloproteinase and cathepsins in elastin degradation, which is associated with dilatation of the aorta [6, 22]. In contrast to visceral organs, abnormalities in the aorta once established are irreversible [6]. Treatment initiated at birth reduces the level of GAGs, and these levels were kept after the interruption. However, even with lower GAG levels, the aortic wall thickness is larger than normal in animals undergoing treatment discontinuation. This may occur because during the neonatal treatment, the status of disease is being controlled; and during the period of discontinuing treatment, this status evolves and stabilizes, not being reversed even after the reintroduction of the enzyme.

The severe phenotype of MPS I has an important neurological involvement [23]. Despite the great improvement observed in various tissues by the use of ERT, this does

not apply to the brain. The conventional dose used allows only a very small enzyme quantities exceeding the blood brain barrier (BBB), and data about corrections promoted by ERT in central nervous system are contradictory [24]. Studies in MPS I patients demonstrated that improvement can be associated with age at the beginning of treatment [25]. Also, experiments in MPS I mice treated with neonatal or high-dose ERT showed improvements in GAG levels in the cerebral cortex, in locomotor and exploratory behavior and habituation to new environments [10, 26]. In the behavioral tests, animals with discontinuing treatment had intermediate values, i.e., showing a small worsening compared to Neo-ERT.

The presence of neuroinflammation in animals with discontinuation of treatment was also evaluated, since this has been reported in studies with animals treated with neonatal or late ERT [10, 27]. Glial activation in the cortex showed no difference between Neo-ERT and Stop-ERT, and both still had a number of GFAP positive cells intermediate to that of normal animals. These results are in accordance to the data found in behavioral tests of these animals.

Antibody formation against the enzyme has been demonstrated in animals and patients, and is related to the decrease in available enzyme [28]. It has also been reported that when starting ERT at birth, the number of antibodies produced against the recombinant enzyme is smaller than if started at 2 months of age [10]. The question was if there was antibody formation against the enzyme with the discontinuing neonatal ERT and subsequent reintroduction. Our results showed that only one in seven animals with treatment develops antibodies against the enzyme. This may be explained by induction of immunological tolerance in neonates, previously shown in treated dogs [29], which is maintained into adulthood. This reinforces that negative results are from discontinuing treatment and not for the capture of the enzyme by antibodies and sequential decrease in ERT effectiveness.

In conclusion, our findings show that the discontinuing of neonatal ERT maintain low GAG levels in urine and viscera. However, in areas where the enzyme has limited access, as the brain, aorta and the heart, deleterious effects caused by the GAG build-up during interruption are not reverted even after restoration of treatment. Therefore

discontinuing ERT should be avoided to not reverse the beneficial effects achieved by patients.

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CONSIDERAÇÕES FINAIS

Estudos com a TRE neonatal demonstraram melhora em diversos parâmetros encontrados na MPS I, tais como: redução dos níveis de GAGs teciduais e urinários, melhora da função cardíaca e em testes comportamentais e a baixa produção de anticorpos contra a enzima recombinante.

Aqui, foi demonstrado que camundongos MPS I mesmo com a interrupção da TRE e sua reintrodução apresentam níveis de GAGs reduzidos em órgãos amplamente afetados pela doença, como fígado e rim. Entretanto, apesar do coração ter seus níveis de GAGs reduzidos, eles permanecem acima do normal mesmo após a reintrodução do tratamento. Dessa forma, problemas causados pelo acúmulo e seus efeitos secundários são observados. Na análise da função cardíaca, por exemplo, foi visto uma piora na hipertensão pulmonar quando ocorre a interrupção da TRE. De mesmo modo, a espessura da parede da aorta não retornou ao normal após a reintrodução do tratamento. No cérebro, ainda existia a presença de neuroinflamação. Porém, não houve a formação de anticorpos contra a enzima recombinante após a reintrodução da TRE, o que nos faz acreditar que uma tolerância imunológica foi mantida.

Dessa forma demonstramos que existem efeitos concretos da interrupção do tratamento sobre órgãos vitais. Portanto a interrupção da TRE deve ser evitada para não reverter os efeitos benéficos alcançados pelos pacientes.

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ANEXOS

1. Orientações da revista *Molecular Genetics and Metabolism*

GUIDE FOR AUTHORS

INTRODUCTION

Molecular Genetics and Metabolism is a contribution to the understanding of the metabolic basis of disease. The journal publishes articles describing investigations that use the tools of biochemistry and molecular biology for studies of normal and diseased states.

Research Areas include:

•Inherited Metabolic Diseases

- Biochemical studies of primary enzyme defects

- Molecular genetic analyses of mutations

- Pathogenesis of these disorders, including not only primary but also secondary metabolic alterations

• Systems Biology

- Functional integration of biochemical network modules

- Moonlighting functions of proteins

• Intercellular and Intracellular Metabolic Relationships

- Biochemical interactions between cells

- Functional roles of and interactions between subcellular compartments and distinct regions within

these cellular spaces, termed microcompartments

- Metabolic relations between individual enzymes and pathways

• Cellular Catalysts

- Protein and nonprotein catalyst in normal and deranged cellular metabolism

- Relationships between the structure and function of catalytic molecules

- Interaction of these catalysts with other cellular components

• Disease Pathogenesis

- Underlying mechanisms of inherited and acquired diseases

- Relationships between genotype and phenotype at the biochemical and molecular levels

• Treatment

- Drug, protein and dietary interventions

- Transplantation and gene therapy

- Multicenter clinical trials

- Pharmacogenetics / Pharmacogenomics

Language (usage and editing services)

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's WebShop (http://webshop.elsevier.com/languageediting/) or visit our customer support site (http://support.elsevier.com) for more information.

References

There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted

article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct.

Formatting requirements

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Divide the article into clearly defined sections.

Figures and tables embedded in text

Please ensure the figures and the tables included in the single file are placed next to the relevant text in the manuscript, rather than at the bottom or the top of the file.

Article structure

Subdivision - numbered sections

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 not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

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.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• Title. Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• Author names and affiliations. Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lowercase superscript letter immediately after the author's name and in front of the appropriate address.

Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• Corresponding author. Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be

defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Nomenclature and units

Whenever enzymes are the subject of reporting, the Enzyme Commission (EC) number should be used for accurate identification and retrieval purposes. The Internet address for the Enzyme Commission is http://www.bis.med.jhmi.edu/bio/search/FILT/enzyme.html.

Math formulae

Please submit math equations as editable text and not as images. Present simple formulae in line with normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.

• Preferred fonts: Arial (or Helvetica), Times New Roman (or Times), Symbol, Courier.

• Number the illustrations according to their sequence in the text.

• Use a logical naming convention for your artwork files.

• Indicate per figure if it is a single, 1.5 or 2-column fitting image.

• For Word submissions only, you may still provide figures and their captions, and tables within a single file at the revision stage.

Figure captions

Ensure that each illustration has a caption. A caption should comprise a brief title (not on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal

communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

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Example: '..... as demonstrated [3,6]. Barnaby and Jones [8] obtained a different result ....'

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