UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
PROGRAMA DE PÓS GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

Edição gênica em mucopolissacaridose tipos I e II utilizando o sistema CRISPR-Cas9: uma abordagem não-invasiva para o tratamento do comprometimento neurológico e sistêmico

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Orientador: Prof. Guilherme Baldo

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Luisa Natalia Pimentel Vera

Tese submetida ao Programa de Pós Graduação em Genética e Biologia Molecular da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do grau de Doutor em Genética e Biologia Molecular.

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MPS I – Mucopolissacaridose tipo I

MPS II – Mucopolissacaridose tipo II

MPS III – Mucopolissacaridose tipo III

MPS III – Mucopolissacaridose tipo III

MPS VI – Mucopolissacaridose tipo VI

MPS IX – Mucopolissacaridose tipo IX

OMIM – do inglês *Online Mendelian Inheritance in Man*

CS – Condroitin Sulfato

KS – Keratan Sulfato

DS – Dermatan Sulfato

HS – Heparan Sulfato

GAG – Glicosaminoglicanos

IDUA – alpha-L-iduronidase

IDS- Idutonato-2-sulfatase

M6F – Manose-6-fosfato

M6FR – Receptor de manose-6-fosfato

TCTH – Transplante de Células Tronco Hematopoiéticas

TRE – Terapia de Reposição Enzimática

SNC-Sistema nervoso central

DNA – Ácido Desoxirribonucleico

RNA – Ácido ribonucléico

TALEN – do inglês *transcription activator-like effector nuclease*

ZFN – do inglês *zinc finger nucleasse*

CRISPR – do inglês *clustered regularly interspaced palindromic repeats*

CRISPR-Cas9 – do inglês *Clustered regularly interspaced short palindromic repeat associated Cas9*

UENH – união de extremidades não homólogas

UEH – união de extremidades não homólogas

gRNA – RNA guia

PAM – do inglês *protospacer adjacent motifs*

PEG– polietilenoglicol

RESUMO

As Mucopolissacaridose tipo I e tipo II são doenças de depósito lisossomal causadas por variantes patogênicas em genes que codificam para as enzimas lisossomais IDUA e IDS, respectivamente. A ausência ou mau funcionamento dessas enzimas leva a um comprometimento da via catabólica dos glicosaminoglicanos Heparan e Dermatan sulfato, que resulta em um acúmulo progressivo desses substratos em lisossomas de múltiplos tecidos. As manifestações fenotípicas dos pacientes podem variar da forma atenuada à grave, sendo malformação esquelética, rigidez articular, doença valvar pulmonar e cardíaca e hepatoesplenomegalia as mais comuns. O comprometimento do sistema nervoso central é a característica mais preocupante entre os fenótipos graves em ambas as doenças. A falta de tratamentos eficazes e seguros que retardem ou previnam os sintomas neurológicos e somáticos leva à busca de abordagens inovadoras que superem as limitações das terapias existentes. Assim, o objetivo geral desta tese foi propor uma abordagem de edição de genoma usando vetor não-viral visando ao tratamento dos sintomas neurológicos em MPS I e II. Neste trabalho preparamos, caracterizamos e testamos in vivo um vetor lipossomal capaz de transportar e entregar o sistema CRISPR-Cas9 ao cérebro para correção gênica em camundongos MPS I e II pela via nasal. Nosso complexo foi capaz de atingir e editar células no bulbo olfatório, o coração e pulmões após trinta administrações nasais, e as células editadas foram capazes de produzir cerca de 1% dos níveis normais de enzimas, o que levou a uma diminuição do acúmulo de substrato em alguns tecidos, urina e soro em camundongos MPS I e II. Isso também significou uma melhora nas características motoras e de memória em camundongos MPS I e II. Por fim, comprovamos que nosso sistema não foi capaz de melhorar sua eficácia com o uso trinta administrações adicionais, uma vez que não foram observadas melhoras metabólicas e fenotípicas maiores após sessenta doses em comparação com trinta doses. Assim, esses achados sugerem que uma otimização de todo o sistema proposto é necessária para atingir níveis mais elevados de eficácia e eficiência do sistema. No entanto, esta abordagem não viral e não invasiva pode ser promissora para o tratamento de sintomas neurológicos e somáticos após abordar suas limitações reais.

ABSTRACT

Mucopolysaccharidosis type I and Type II are lysosomal storage diseases caused by pathogenic variants on genes encoding for lysosomal enzymes IDUA and IDS, respectively. The absence or malfunction of these enzymes leads to impaired degradation of glycosaminoglycans Heparan and Dermatan sulfate, which causes progressive accumulation of these substrates in lysosomes of multiples tissues. The patient's phenotypical manifestations can vary from the mild to the severe form, being skeletal malformation, stiffened joints, pulmonary and heart valve disease, and hepato-splenomegaly the most commons. Central nervous system impairment is the most concerning feature among severe phenotypes in both diseases. The lack of effective and safe treatments that delay or prevent neurological and somatic symptoms lead to seeking out innovative approaches that may overcome the limitations of the existing therapies. Thus, the overall objective of this thesis was to propose a non-viral genome editing approach targeting neurological symptoms in MPS I and II. In this work we prepared, characterized, and tested in vivo a liposomal vector capable of carrying and delivering the CRISPR-Cas9 system to the brain for gene correction in MPS I and II mice using the nasal route. Our complex was capable of reaching and editing cells in the olfactory bulb, the heart, and lungs after thirty nasal administrations, and edited cells were able to produce about 1 % of the normal enzyme levels which led to a decrease in of glicosaminoglycan levels in some tissues, urine and serum in MPS I and II mice. This also produced an improvement in motor and memory behaviors. Finally, we proved that our system was unable to improve its efficacy when sixty administrations were used, since no metabolic and phenotypic changes were seen after sixty doses compared to thirty doses. Thus, these findings suggest that a major optimization of the whole system proposed is necessary to achieve higher levels of efficacy and efficiency. Nevertheless, this non-viral and non-invasive approach may hold promise for the treatment of neurological and somatic symptoms after addressing its actual limitations.

1. INTRODUÇÃO

1.1. Mucopolissacaridoses tipo I e II

1.1.1. *Genética e bioquímica*

As Mucopolissacaridoses do tipo I e II são erros inatos do metabolismo pertencentes ao grupo de as doenças lisossômicas (**Tabela 1**). Como o seu nome menciona, nestas doenças a organela celular que se encontra afetada é o lisossomo (Poswar et al. 2019). Ambas são o resultado de um desequilíbrio metabólico multissistêmico causado por variantes patogênicas em genes que codificam para duas enzimas lisossômicas cruciais no ciclo de reciclagem celular (D′avanzo et al. 2020; Kubaski et al. 2020).

A Mucopolissacaridose do tipo I (MPS I), também chamada na sua forma grave de Síndrome do Hurler (OMIM #607014) é causada por variantes patogênicas em homozigose ou heterozigose composta no gene *IDUA* (4p16.3) que codifica para a produção da enzima alfa-L-iduronidase (IDUA). Dependendo da variante, a enzima pode ser expressa de uma forma aberrante (truncada ou com acometimento da sua estrutura terciária) ou no caso extremo, não ser expressa. Devido às diferentes alterações genéticas, ocorrem níveis de gravidade variados, dentro de um espectro fenotípico contínuo (Parini et al. 2017). Sua herança é autossômica recessiva e, portanto, afeta homes e mulheres em igual proporção, sendo que indivíduos heterozigotos são favorecidos pelo efeito compensatório do alelo normal, e não desenvolvem a doença (Campos et al. 2014). No Brasil, estudos recentes estimam uma taxa de incidência de aproximadamente 0,24 por 100.000 recém-nascidos vivos (Federhen et al. 2020).

A Mucopolissacaridose do tipo II ou Síndrome de Hunter (OMIM #309900) é também causada por variantes patogênicas, mas no gene *IDS* (Xq28) que codifica para a produção da enzima iduronato-2-sulfatase (IDS). Do mesmo modo que a MPS I e outras doenças lisossômicas, a MPS II apresenta diferentes fenótipos relacionados na maioria dos casos a um genótipo particular. Por ser esta uma doença ligada ao X, a grande maioria dos casos afetam homens, com uma taxa de incidência de aproximadamente 0,38 por 100.000 recém-nascidos vivos no Brasil (D′avanzo et al. 2020). Mulheres afetadas são

raras, mas se acredita-se que isso aconteça pela inativação não aleatória do cromossomo X (Meikle et al. 2005).

Tabela 1. Representantes das doenças lisossômicas, divididas em categorias.

Categoria principal	Principais representantes	Principais sinais e sintomas *
Mucopolissacaridoses	MPS I a MPS IX	Face grosseira, hepatoesplenomegalia, opacificação da córnea, anormalidades esqueléticas, limitação articular e baixa estatura; retardo mental progressivo
Mucolipidoses	Tipo I a IV	Face grosseira, hepatoesplenomegalia, disostose múltipla, contraturas dos dedos, escoliose, baixa estatura; retardo mental progressivo (casos graves).
Esfingolipidoses	Gangliosidoses; Niemann-Pick (tipos A, B, e C); doença de Gaucher (tipos I, II, e III); doença de Fabry; Leucodistrofia metacromática; doença de Krabbe; doença de Farber.	Neurodegeneração, mancha "vermelho cereja" na retina, hepatoesplenomegalia, comprometimento pulmonar, paralisia do olhar, ataxia, alterações ósseas, parestesias, insuficiência renal.
Oligossacaridoses	α-manosidose; β-manosidose; fucosidose; aspartilglucosaminúria; Síndrome de Schindler; ISSD; Síndrome de Salla; Galactosialidose	Face grosseira, disostose múltipla; Mancha "vermelho cereja" na retina, hepatoesplenomegalia, retardo mental, ataxia, perda auditiva, angioqueratoma.
Lipofuscinoses ceróides neuronais	Tipos 1 a 14	Neurodegeneração, problemas de visão, convulsões, ataxia

Abreviaturas: MPS-mucopolissacaridose; ISSD: Doença infantil de armazenamento de ácido siálico.
* Pode não estar presente em todas as doenças da mesma categoria. Adaptado de Poswar (2019).

Ambas enzimas mencionadas, a IDUA e a IDS, pertencem ao grupo das hidrolases lisossômicas, envolvidas diretamente no catabolismo de cadeias complexas de açúcares chamados glicosaminoglicanos (GAGs), compostas maioritariamente por sulfato de keratan, condroitin, dermatan ou heparan (Alroy and Lyons 2014). Os GAGs são encontrados na estrutura de moléculas proteicas maiores, os proteoglicanos, os quais fazem parte dos componentes principais da matriz extracelular (Ghatak et al. 2015) (**Figura 1**). Estes componentes, assim como muitos outros componentes celulares como proteínas, lipídios e ácidos nucleicos, são transportados por meio de vesículas e endossomos (rota endossômica)

até o lisossomo para inicializar o processo de reciclagem ou degradação dependendo dos requerimentos metabólicos da célula (Cullen and Steinberg 2018; Trivedi et al. 2020) .

Durante o tráfego dos proteoglicanos, os primeiros processos de clivagem iniciam no endossomo tardio, onde participam diferentes endo-enzimas que reduzem a estrutura (Meikle et al. 2005). Após, no lisossomo, os GAGs culminam o processo de degradação mediado pelas exo-enzimas (hidrolases lisossômicas). Neste processo, glicosidases, sulfatases e acetiltransferases hidrolisam resíduos não redutores nas cadeias de oligossacarídeos dos diferentes GAGs de uma maneira organizada e sequencial, até a sua redução a monossacarídeos e sulfato (Meikle et al. 2005). Estas hidrolases lisossômicas, como a grande maioria das enzimas lisossômicas solúveis, sofrem modificações pós-traducionais durante sua síntese, que permite que sejam levadas até o lisossomo. As N-glicosilações com resíduos de manose 6-fosfato são as principais encarregadas deste processo, pois este resíduo medeia a translocação das enzimas na membrana lisossômica, que tem na sua composição receptores para manose-6 fosfato (Braulke and Bonifacino 2009).

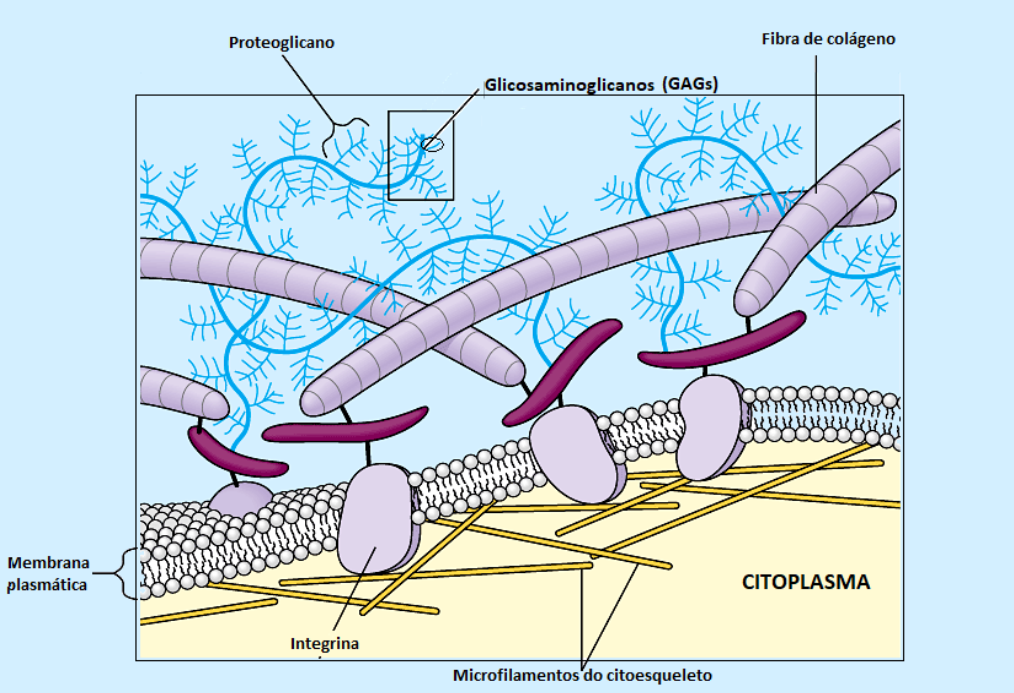

Figura 1. Componentes da matriz extracelular (MEC), com especial ênfase para os glicosaminoglicanos (GAG). Adaptado de Pearson, education Inc. Copyright©.

O Sulfato de dermatan e o sulfato de heparan são os principais substratos das enzimas IDUA e IDS (Meikle et al. 2005). IDUA pertence ao grupo das glicosidases e hidrolisa as ligações N-glicosídicas do ácido idurônico não sulfatado dos substratos, enquanto a IDS pertence ao grupo das sulfatases e realiza a hidrólise dos grupos sulfato do C2 das unidades alfa L-idurônicas destes mesmo substratos (Meikle et al. 2005). Ao final, o sulfato de heparan e dermatan são degradados por uma série de exo-enzimas além das duas mencionadas anteriormente, que agem sequencialmente na extremidade não redutora (**Figura 2**). Com isto tudo, em ausência de alguma das duas enzimas, não é possível continuar o processo natural de degradação dos sulfato de dermatan e heparan o qual leva a o seu acúmulo no lisossomo, e eventualmente no espaço extracelular de diferentes tecidos, desenvolvendo doença multissistêmica (Ponder 2013).

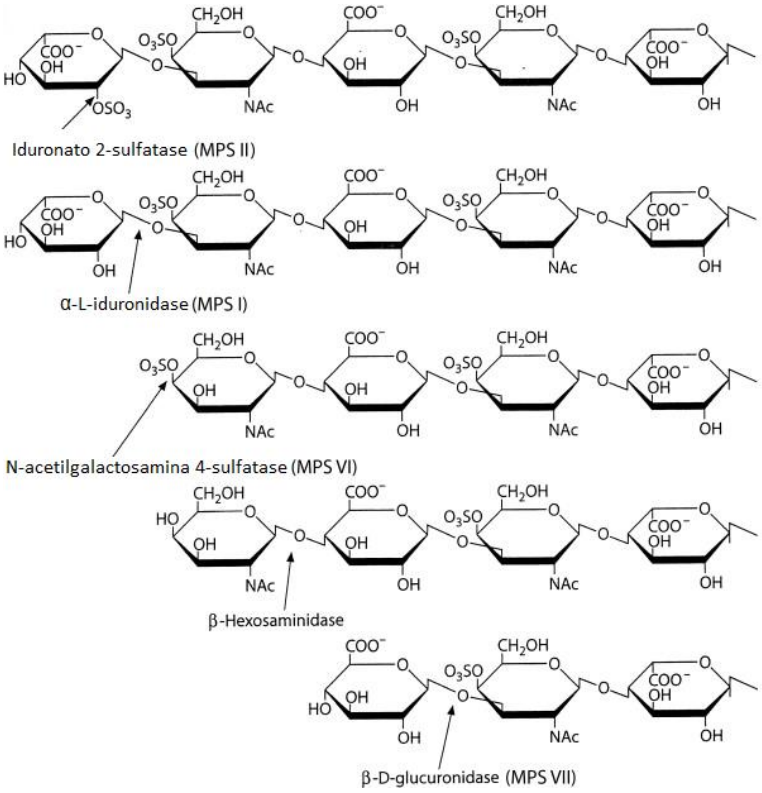

Figura 2. Processo de degradação do Sulfato de dermatan mediado por hidrolases lisossômicas. A deficiência em alguma destas enzimas causa diferentes tipos de Mucopolissacaridoses, apontadas nos parênteses. Adaptado de (Wraith 2013).

1.1.2. Fisiopatologia

As mucopolissacaridoses (MPSs), como a MPS I e MPS II se caracterizam pelo acúmulo principalmente de sulfato de heparan e dermatan em lissosomos e tecidos onde estes GAGs se encontram em maior proporção. Por esta razão, estas doenças levam a um comprometimento multissistêmico envolvendo tecidos viscerais, conectivos, esquelético e neuronal. Também por isso, suas manifestações clinicas são similares **(tabela 2)** (Alroy and Lyons 2014)**.**

As manifestações somáticas das MPS I e II incluem problemas no desenvolvimento esquelético e articular. Além disso, há o desenvolvimento de doença cardíaca, com espessamento de valvas, hipertensão pulmonar e dilatação do miocárdio. Infecções de vias áereas superiores e outras alterações na função pulmonar também são frequentes. Eles também apresentam hérnias e hepatoesplenomegalia, relacionadas aos processos inflamatórios causados pelo acúmulo de substratos, além de opacidade da córnea (principalmente em pacientes MPS I) e perda auditiva (D′avanzo et al. 2020; Hampe et al. 2020)**.**

Os sintomas neurológicos também são característicos destas doenças, com déficit cognitivo. No entanto, tanto as manifestações somáticas e neurológicas se apresentam em um amplo espectro de gravidade. Na MPS I as formas clínicas são subdivididas de maneira histórica em três classificações: Síndrome de Hurler (grave), síndrome de Hurler-Scheie (intermediário) e síndrome de Scheie (atenuada). Pacientes Scheie apresentam sintomas clínicos mais discretos e geralmente têm desenvolvimento psicomotor e desenvolvimento neurológico normal em seus primeiros anos de vida, mas as dificuldades de aprendizagem podem se tornar aparentes à medida que envelhecem (Melbouci et al. 2018). Os pacientes atenuados menos afetados apresentam poucas complicações e subsequente expectativa de vida quase normal. Por outro lado, complicações esqueléticas, perda auditiva e deficiência intelectual são comuns na MPS I grave e a morte ocorre na primeira década de vida, normalmente devido à insuficiência cardiorrespiratória (Melbouci et al. 2018).

Na MPS II também existem descritos dois fenótipos: atenuado e grave. Similar aos pacientes MPS I, pacientes com fenótipo atenuado são normalmente diagnosticados entre 4 e 8 anos de idade devido à sintomatologia difusa ou em alguns casos ausente. Embora sejam descritos frequentemente como não tendo sintomas neurológicos, os pacientes podem desenvolver dificuldades cognitivas em longo prazo, com um déficit de atenção significativo (Bigger et al. 2018). Todos os pacientes com fenótipos graves têm envolvimento do sistema nervoso central, problemas graves de aprendizagem, problemas psicomotores e um estado neurológico deteriorado. A morte para esses pacientes normalmente ocorre durante a segunda década de vida por doença cardíaca ou respiratória (Melbouci et al. 2018).

Tabela 2. Deficiência enzimática, genética e características clínicas das mucopolissacaridoses.

Doença	**Deficiência Enzimática**	**Substrato armazenado**	**Local**	Gene (OMIM)	**Principais Características Clínicas**
MPS I (Hurler, Scheie, Hurler/Scheie)	Iduronidase (EC 3.2.1.76)	DS, HS	4p16.3	*IDUA* (252800)	HSM, CNS, SD, DYS, OPH, CAR
MPS II (Hunter)	Iduronato-2-sulfatase (EC 3.1.6.13)	DS, HS	Xq27–28	*IDS* (300823)	HSM, CNS, SD, DYS, OPH, CAR, SK
IIIA (Sanfilippo)	Heparan-n-sulfatase (EC 3.10.1.1)	HS	17q25.3	*HSS* (605270)	CNS, SD (+/−), DYS (+/−)
IIIB (Sanfilippo)	*N*-acetil-glucosaminidase (EC 3.2.1.50)	HS	17q21.1	*NAGLU* (609701)	CNS, SD (+/−), DYS (+/−)
IIIC (Sanfilippo)	Acetil CoA glucosamina n-acetil transferase (EC 2.3.1.3)	HS	8p11.1	*HGSNAT* (610453)	CNS, SD (+/−) DYS (+/−)
IIID (Sanfilippo)	*N*-acetil-glucosamina-6-sulfatase (EC 3.1.6.14)	HS	12q14	*GNS* (607664)	CNS, SD(+/−), DYS (+/−)
IVA (Morquio)	Galactose-6-sulfatase (EC 3.1.6.4)	KS	16q24	*GALNS* (612222)	SD, CAR, OPH (+/−)
IVB (Morquio)	β-Galactosidase (EC 3.2.1.23)	KS	3p21-pter	*GLB1* (611458)	SD, CAR
MPS VI (Maroteaux–Lamy)	Galactosamina-4-sulfatase (EC 3.1.6.12)	DS	5q13–q14	*ARSB* (611542)	HSM, SD, DYS, OPH, CAR
MPS VII (Sly)	β-Glicuronidase (EC 3.2.1.31)	HS, DS	7q21.1–q22	*GUSB* (611499)	HF, HSM, CNS, SD, DYS, OPH, CAR
MPS IX	Hialuronidase (EC 3.2.1.35)	HA	3p21.3	*HYAL1* (607071)	U/K

Abreviaturas: CAR, doença cardíaca; CNS, retardo mental; DS, sulfato de dermatan; HS, sulfato de heparan; KS, sulfato de queratano; HA, ácido hialurônico; SA, ácido siálico; DYS, aparência dismórfica; HF, hidropisia fetal; HSM, hepatoesplenomegalia; OPH, sinais dos olhos - opacificação da córnea; SD, disostose múltipla; SK, sinais dermatológicos; (+/−), sinal nem sempre presente ou leve. Adaptado de (Wraith 2013).

Embora estas doenças sejam principalmente de depósito, uma vez que os substratos não degradados se acumulam nos lisossomos, os sintomas e sinais manifestados não são exclusivamente consequência deste acúmulo intracelular primário. Os GAGs, além de serem constituintes da matriz extracelular e das membranas, também desempenham um papel fundamental na sinalização celular e modulam vários processos bioquímicos que são fundamentais para a biologia celular (Fecarotta et al. 2020). O Sulfato de heparan, em locais

fora do lisossomo, como a superfície celular ou na matriz extracelular, desempenha um papel essencial na sinalização celular, distribuição de fatores de crescimento e citocinas, além de influenciar a motilidade e adesão celular. Níveis excessivos de heparan e sulfatação anormal deste no sistema nervoso central de pacientes com MPS I e II podem alterar a sinalização dependente de HS e desencadear inflamação; isso pode alterar os processos intracelulares e a neurotransmissão, que por sua vez afetam o funcionamento neurológico (Bigger et al. 2018). Em articulações e tecido sinovial o sulfato de dermatan acumulado é um mediador direto da ativação de uma cascata complexa (principalmente macrófagos e citocinas pro-inflamatórias) que leva à apoptose dos condrócitos, hiperplasia sinovial e destruição progressiva da articulação na MPS I e II (Clarke 2011). No tecido cardíaco, a patogênese se apresenta com hipertrofia do miocárdio, anormalidades na elastina dos vasos cardíacos e aórticos. Estes eventos parecem ser causados pelo aumento da expressão de proteínas de degradação da elastina, como metaloproteinases e catepsinas, secundários ao acúmulo do sulfato de dermatan (Clarke 2011; Hampe et al. 2020).

Além disso, o lisossomo desempenha um papel central em muitas funções celulares, e o desequilíbrio na homeostase lisossômica que ocorre pelo acúmulo dos substratos desencadeia uma série de eventos secundários que são atores importantes na patogênese das MPS. Estes eventos incluem armazenamento de substratos secundários não relacionados à enzima defeituosa, composição anormal de membranas, fusão aberrante e tráfego intracelular de vesículas, comprometimento da autofagia, disfunção mitocondrial e estresse oxidativo, desregulação das vias de sinalização e ativação da inflamação, anormalidades da homeostase e sinalização do cálcio, que leva a apoptose (**Figura 3**) (Fecarotta et al. 2020).

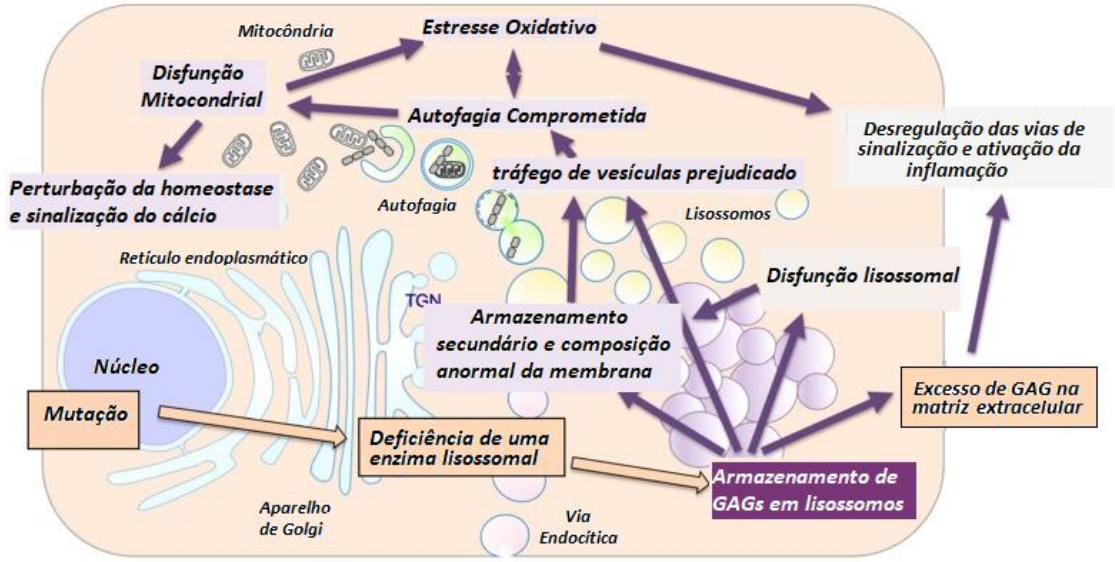

Figura 3. Cascata de eventos patogênicos das mucopolissacaridoses. Eventos patogênicos múltiplos: armazenamento primário de glicosaminoglicanos (GAGs) e vias secundárias interrompidas. Adaptado de Fecarotta 2020.

1.1.3. Diagnóstico e terapias

As manifestações clínicas das MPS são muito semelhantes em alguns casos, e muitas vezes, também semelhantes a outros distúrbios do desenvolvimento e neurológicos. Portanto, para o diagnóstico da MPS I e II se requer uma combinação de técnicas clínicas, avaliações físicas, bioquímicas e moleculares (Alroy and Lyons 2014) .

O diagnóstico das MPS pode ser determinado por: 1) análise de oligossacarídeos em urina e sangue. Devido à deficiência enzimática, os GAGs se acumulam em vários tecidos, são parcialmente eliminados na urina e se encontram em altas concertações na circulação (Zhou et al. 2020). Determinar o tipo de GAG pode dar pistas sobre a qual a enzima deficiente. A quantificação/ tipificação dos GAGs nos fluidos pode ser feita por meio de eletroforese em gel de poliacrilamida ou por espectrometria de massas em tandem, sendo este último o mais utilizado nos últimos anos para a triagem neonatal (Kubaski et al. 2017b). 2) Ensaio enzimático das hidrolases lisossômicas em leucócitos, fibroblastos ou plasma, para avaliar o nível de atividade da enzima envolvida, sendo este considerado o padrão-ouro e 3)

a determinação da variante patogênica por técnicas moleculares (Alroy and Lyons 2014; Zhou et al. 2020). O diagnóstico precoce representa uma oportunidade para tratamentos precoces, para prevenir a progressão da doença.

O transplante de células-tronco hematopoiéticas (TCTH) foi a primeira terapia utilizada para o tratamento das doenças lisossômicas. As CTH são as únicas células do organismo com a capacidade de se autorenovar (se dividir) e diferenciar em células do sistema sanguíneo (Drize and Petinati 2015). Esta terapia consiste no transplante de medula óssea de uma pessoa com genótipo normal para o paciente com a finalidade de substituir uma grande parte das células sanguíneas do paciente por células-tronco de um doador saudável, cujas células são capazes de sintetizar e secretar a enzima deficiente. O transplante alogênico se encontra aprovado pela FDA (Food and Drug Administration) para o tratamento de pacientes com MPS I (síndrome de Hurler), pois quando bem-sucedido, o TCTH tem resultados satisfatórios em nível sistêmico com ação, inclusive, no sistema nervoso central (Beck 2018). Embora esteja aprovado para uso em pacientes MPS I, estes devem atingir alguns critérios de seleção baseado na chance de prevenir as manifestações neurológicas, pois, mesmo com resultados muito favoráveis, a terapia não consegue reverter a patologia já estabelecida (Vera and Baldo 2020). Nestes casos, o diagnóstico molecular precoce contribui na correlação fenótipo genótipo e possibilita estabelecer o prognóstico (Clarke et al. 2019). Esta terapia tem sido testada em pacientes com MPS II, apresentando alguns resultados promissores, mas ainda não se encontra aprovada (Poswar et al. 2019). No caso da MPS II ainda é difícil prever o prognóstico somático e neurológico em pacientes com base na relação genótipo-fenótipo, e as decisões de transplante podem ser difíceis (Chiesa et al. 2016).

O segundo tratamento disponível para estas doenças é a terapia de reposição enzimática (TRE). A TRE fornece ao paciente por via sistêmica a enzima funcional. Isso aumenta a atividade enzimática por um período de tempo e leva à melhora ou prevenção da progressão de vários sintomas clínicos (Li 2018). A Laronidase (Aldurazyme®; Genzyme Europe B.V., Gooimeer 10, NL-1411 DD Naarden, The Netherlands) para a MPS I e a Idursulfase (Elaprase®; Shire Human Genetic Therapies, Inc., Cambridge, MA, USA) para MPS II foram aprovadas pelo FDA desde 2003 e 2006 respectivamente. Estas enzimas são produzidas em células de mamífero, garantindo que ela possua N-glicosilações com resíduos

de manose-6 fosfato, que permitem seu transporte intracelular (Concolino et al. 2018). A TRE é uma terapia que requer infusões intravenosas semanais ou quinzenais. Ela tem apresentado eficácia revertendo sintomas como a redução do GAG urinário a valores aproximadamente normais, e melhora do tamanho do fígado e do baço (Concolino et al. 2018). No entanto, algumas alterações cardiovasculares e a motilidade articular apresentam apenas melhora limitada, e as anormalidades cerebrais não são corrigidas devido à incapacidade da enzima de atingir o sistema nervoso central (Vera and Baldo 2020). Assim como para o TCTH, existem riscos associados ao tratamento, que incluem reação imunogênica à enzima, além de resultados muitas vezes insatisfatórios (Vera and Baldo 2020). A TRE ainda possui o agravante de ter um custo muito elevado, onerando o sistema público de saúde.

1.2. Terapia Gênica

A terapia gênica tornou-se uma opção para o tratamento de diferentes enfermidades. É baseada na entrega de um material genético exógeno (transgene) em células, que mais tarde será transcrito e traduzido em uma proteína funcional. Para doenças monogênicas, como as doenças lisossômicas de depósito, esse material genético seria a "versão normal" do gene mutado, que leva à produção da enzima/proteína não funcional, a qual, na maioria dos casos, é a causa da doença. A terapia gênica clássica tem utilizado principalmente vírus modificados para entregar este gene ao paciente (Vera and Baldo 2020). São utilizados majoritariamente vírus integrativos de RNA como os retrovírus ou lentivírus que, após transduzir as células, se integram em uma região aleatória do genoma, e consequentemente irão transcrever o transgene; e vírus de DNA não integrativos, como os adenovírus ou vírus adenoassociados, que permanecem em as células de forma episssomal (sem se integrarem), podendo, no caso dos AAV, expressar o transgene por longos períodos de tempo (Ørstavik and Apold 2001). Os vetores não-virais são uma importante alternativa ao uso de vetores virais, conforme será relatado a seguir.

1.2.1. Vetores de entrega

A terapia gênica se tornou possível por meio de avanços da genética e da bioengenharia que possibilitaram a manipulação de vetores para entrega de material extracromossômico às células alvo, pois grande parte da eficácia deste tipo de terapia depende das características e qualidades destes (Yi et al. 2021). Os vetores estão classificados em duas grandes categorias, virais e não virais **(Figura 4)**, sendo a primeira a mais comum no desenvolvimento de terapias para as MPS (Baldo et al. 2014).

O vetor viral tem sido amplamente utilizado desde o início da terapia gênica, devido à excelente capacidade de adentrar em células e inserir seu material genético, assim como alcançar expressão de longo prazo de genes desejados para efeitos terapêuticos sustentados (Yi et al. 2021). Os retrovírus e lentivírus são vírus de RNA. Sua replicação é baseada na cópia do RNA em DNA (transcrição reversa) e integração no genoma de uma célula hospedeira. Antes da descoberta dos sistemas CRISPR-Cas9, os retrovírus eram a principal maneira utilizada para modificar o genoma de um paciente (Lukashev and Zamyatnin 2016). Esses vetores têm uma capacidade de empacotamento de quase 10 kb, o que permite transportar genes grandes. Enquanto os retrovírus transduzem apenas células em divisão, lentivírus podem transduzir células quiescentes (Lukashev and Zamyatnin 2016). Ambos apresentam efeitos colaterais como genotoxicidade, causada pela sua integração no genoma. Mas os lentivírus não apresentam uma integração preferencial próximo ao início das unidades de transcrição e assim a indução de oncogênese é significativamente menos frequentemente do que vetores retrovirais (Lukashev and Zamyatnin 2016).

Na categoria não integrativa, o tipo de vetor viral mais amplamente utilizado em enfermidades monogênicas são os vírus adenoassociados (AAVs). Esses são vetores baseados em DNA fita simples, que não se integram ao genoma do hospedeiro, mas ainda fornecem expressão de longa duração. Seu genoma tem cerca de 4,5 kb, então sua capacidade de empacotamento é menor. Esses vetores tem sido amplamente estudados devido à possibilidade de manipular sua estrutura do capsídeo para tornar o vetor sitio-especifico, possibilitando a transdução de diferentes tipos celulares (Vera and Baldo 2020).

Os lentivirus e AAVs são os vetores mais comuns empregados em ensaios clínicos. Os lentivirus são vetores de integração com expressão geralmente mais alta com uma multiplicidade de infecção inferior (MOI), enquanto os AAVs normalmente resultam em uma

propagação in vivo superior devido ao tamanho relativamente pequeno das partículas virais (Yi et al. 2021). No entanto existe uma grande preocupação em relação ao fato de que parte da população possui anticorpos contra esses vírus, o que geralmente leva a uma ineficácia da terapia.

Embora a terapia gênica com vetores virais tenha mostrado a capacidade de atingir alto nível de entrega do transgene em vários modelos de doenças in vivo e ensaios clínicos, a incerteza de desencadear respostas imunogênicas, riscos de ativação de oncogênese e a dificuldade de empacotar grandes ácidos nucléicos são questões que permanecem sem solução (Foldvari et al. 2016). Os vetores não virais podem fornecer várias vantagens em relação à segurança pois podem ser administrados repetidamente sem induzir uma resposta imune detectável além de poder fornecer uma expressão gênica localizada e capacidade de transportar grandes genes terapêuticos (Foldvari et al. 2016).

A entrega do plasmidio nu é a estratégia não viral menos recomendável, uma vez que um ácido nucléico desprotegido (RNA ou DNA) não é estável por muito tempo em um ambiente biológico. Além disso, o ácido nucleico não é capaz de entrar sem auxílio no citoplasma (onde o RNA pode realizar sua função) ou no núcleo, onde o DNA é transcrito e o genoma celular pode ser modificado (Lukashev and Zamyatnin 2016). O uso de nanomateriais como as partículas de ouro, e nanoparticulas lipídicas, tem sido estudado e proposto como uma alternativa a vetores virais, que também protegem o material gênico e facilitam a entrega às células (Keles et al. 2016; Liu et al. 2017).

As nanopartículas de ouro são um novo método de entrega para o sistema CRISPR-Cas9, formando um complexo com a proteína Cas9 e sgRNA. Este sistema de entrega atinge mais de 90% de eficiência de entrega e 30% de eficiência de edição de genes em uma ampla variedade de tipos de células. A entrega mediada por nanopartículas é obtida por meio de um processo de fusão de membrana dependente do colesterol que é distinto da endocitose celular, o que pode enfatizar a notável eficiência de entrega desse sistema. Este método fornece uma nova plataforma para edição in vitro (Liu et al. 2017). As nanopartículas lipídicas como os lipossomos, estão entre os principais veículos de entrega de genes. São compostos por um grupo de cabeça polar catiônica, um domínio hidrofóbico e um ligante. O grupo principal catiônico atrai os grupos fosfato carregados negativamente da molécula de DNA para assim

formar um complexo chamado lipoplex. A porção hidrofóbica é composta na maior parte do tempo por esteroides, e seu comprimento e tipo afetam sua eficiência de transfecção. O grupo de ligação conecta o grupo de cabeça polar com a porção hidrofóbica e determina a estabilidade química, biodegradabilidade e também eficiência de transfecção (Keles et al. 2016). Os lipossomos catiônicos e as moléculas de DNA / RNA formam um complexo protegendo o material genético da degradação enzimática. Este tipo de vetor interage com a célula por meio de interações eletrostáticas com a membrana celular carregada negativamente promovida por a sua natureza lipídica. A internalização destes complexos na célula é realizada por diferentes vias endocíticas. A escolha da via para o processo de internalização depende principalmente do diâmetro do complexo e da eficiência de transfecção, que varia entre as diferentes rotas de entrega (Keles et al. 2016). Além de sua composição básica, os lipossomas são comumente protegidos com um revestimento de polietilenoglicol (PEG) o qual pode fornecer um tempo de circulação prolongado, com meia-vida de 1–10 h, evitando o sistema reticuloendotelial e permitindo melhor estabilidade e maior entrega de genes direcionados, além de promover melhor a biocompatibilidade (Pan et al. 2003).

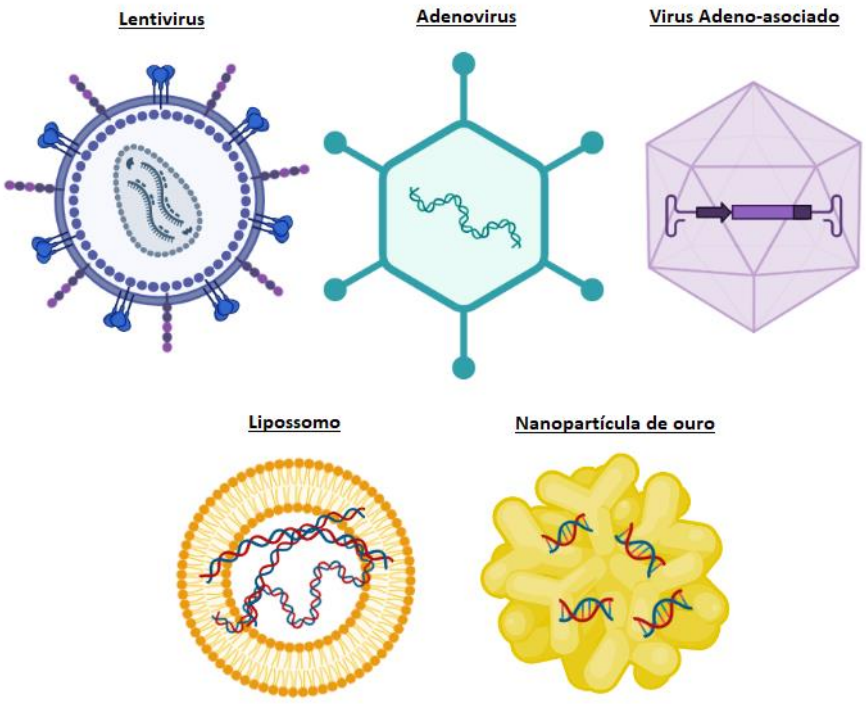

Figura 4. Principais vetores de entrega em terapia gênica e edição genômica.

1.2.2. Vias de administração

Um dos maiores desafios para a terapia gênica é a entrega do transgene terapêutico nos tecidos diretamente afetados, uma vez que a aplicação terapêutica dos vetores de entrega é limitada, em grande parte, devido a várias barreiras fisiológicas extracelulares e intracelulares (Wang et al. 2012). Contudo, junto com o desenvolvimento de novos vetores de entrega, diferentes vias de administração têm sido estudadas visando superar estes obstáculos e garantir a eficiência e eficácia da terapia.

Além da terapia genica ex vivo (**Figura 5**), onde as células do paciente são modificadas em laboratório para posterior infusão (Anguela and High 2019) (Naldini 2011) (Wang and Gao 2014) uma das principais formas de terapia gênica testada para as MPS é a terapia in vivo.

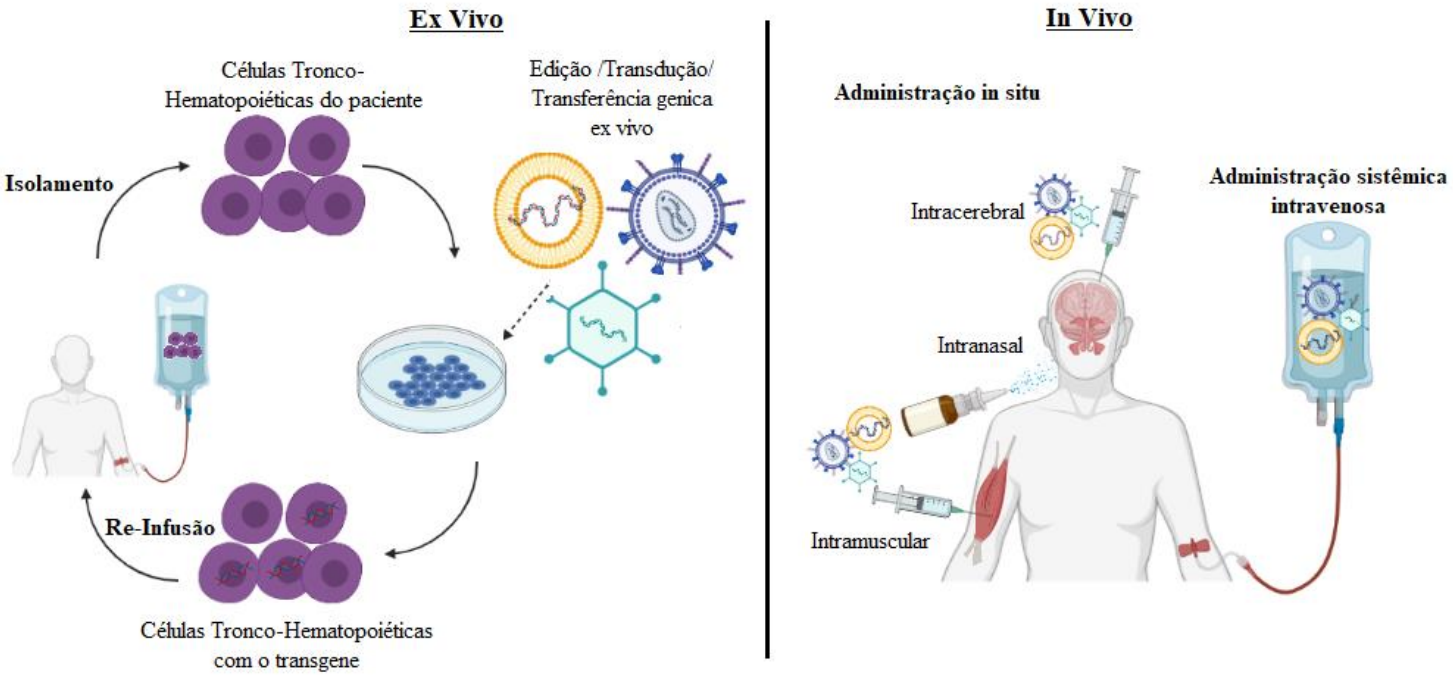

Figura 5. Tipos de terapia gênica na prática clínica. Ex vivo e In vivo

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Na terapia in vivo, a transferência de genes é feita diretamente no paciente, mediante administração do vetor terapêutico ao órgão alvo (administração in situ) ou por meio do sistema vascular aos vasos sanguíneos que alimentam os órgãos envolvidos (administração sistêmica intravenosa) (**Figura 5**) (Rajawat et al. 2019). Esta estratégia proporciona algumas vantagens sobre a estratégia ex vivo, incluindo facilidade de administração, sem necessidade de coleta, manipulação e cultura de células tronco fora do corpo e, uma segurança maior pela ausência de condicionamento mieloablativo para o transplante (Rajawat et al. 2019).

A administração sistêmica intravenosa é a via de entrega mais comum, pois é possível atingir um número de tecidos maior por meio do sistema circulatório. Entre os vetores disponíveis, os virais têm normalmente as maiores eficácias de entrega de genes, e são os mais utilizados neste tipo administração (Reynolds and Danilo 2002). No entanto, a presença de anticorpos neutralizantes na população, além de impossibilidade da grande maioria dos vetores virais de transpassar a barreira hematoencefálica (Bourdenx et al. 2014), fazem com que a administração intravenosa não seja a opção mais viável. Com relação aos vetores não virais, a principal limitante desta via de administração é a degradação endossomal na circulação, o que diminui a eficácia do sistema (Reynolds and Danilo 2002). Com isto tudo, a administração in situ tem sido utilizada para superar estas limitações. Administrações intramusculares, intratumorais ou intranasais são estratégias promissórias para o tratamento de doenças onde um órgão só é o alvo principal, pois limita a propagação do vetor para aquela região, além de diminuir as respostas inmunes geradas pela administração sistêmica.

A administração intranasal de produtos biológicos de grande peso molecular, como proteínas ou genes, é uma estratégia potencialmente útil para tratar uma variedade de doenças ou distúrbios do sistema nervoso central. É um método não invasivo de entrega que pode contornar a barreira hematoencefálica para permitir o acesso direto de substâncias terapêuticas ao cérebro (Lochhead and Thorne 2012). Além de sua aplicação no sistema nervoso, também é considerada uma das formas mais promissoras de pesquisa em terapia gênica pulmonar, e oferece uma forma alternativa para o tratamento de pacientes que sofrem de doenças pulmonares como fibrose cística ou câncer (Podolska et al. 2012).

A cavidade nasal se encontra dividida em três regiões principais, que participam ativamente como diferentes rotas de absorção e internalização (**Figura 6**)**.** A menos

importante entre as três regiões, devido à sua pequena área superficial, é a região vestibular, a qual está localizada imediatamente nas aberturas das narinas (aproximadamente 0,6 cm2) (Hong et al. 2019). A região respiratória ocupa a maior parte da cavidade nasal e possui grande área superficial. Caso passem ilesos pela depuração mucociliar na região vestibular, os produtos biológicos administrados por via intranasal deslocam-se para as regiões posteriores da cavidade nasal e entram em contato com o epitélio respiratório da região respiratória (Hong et al. 2019). Essa região é o principal local de absorção e entrega do produto biológico para a circulação sistêmica, pois é área de maior superfície (~ 130 cm2), além de ter uma alta densidade de vasos sanguíneos. No entanto, alguns de estes produtos podem ser transportados através das inervações dos nervos trigêmeos situados na região, sendo internalizados nos neurônios trigêmeos periféricos por endocitose, e posteriormente transportados ao tronco encefálico e outras estruturas conectadas (Bonferoni et al. 2019).

A última região é a região olfatória, localizada na parte superior profunda da cavidade nasal sobre o osso cribriforme que separa a cavidade nasal do cérebro. Este possue perfurações que fornecem acesso para as terminações nervosas e, portanto, constituem a parte única do SNC que se conecta diretamente ao ambiente externo (Bonferoni et al. 2019). O mecanismo de absorção de moléculas por esta última região é dividido em três rotas: a intracelular, que compreende as células neurais olfatórias; a extracelular, que compreende os espaços entre as células nos canais próximos ao nervo olfatório; e o transporte transcelular, através das células epiteliais basais. O transporte direto do nariz para o cérebro pode ocorrer por meio de uma única via ou uma combinação de diferentes vias, de acordo com a natureza e as características da molécula administrada (Hong et al. 2019)

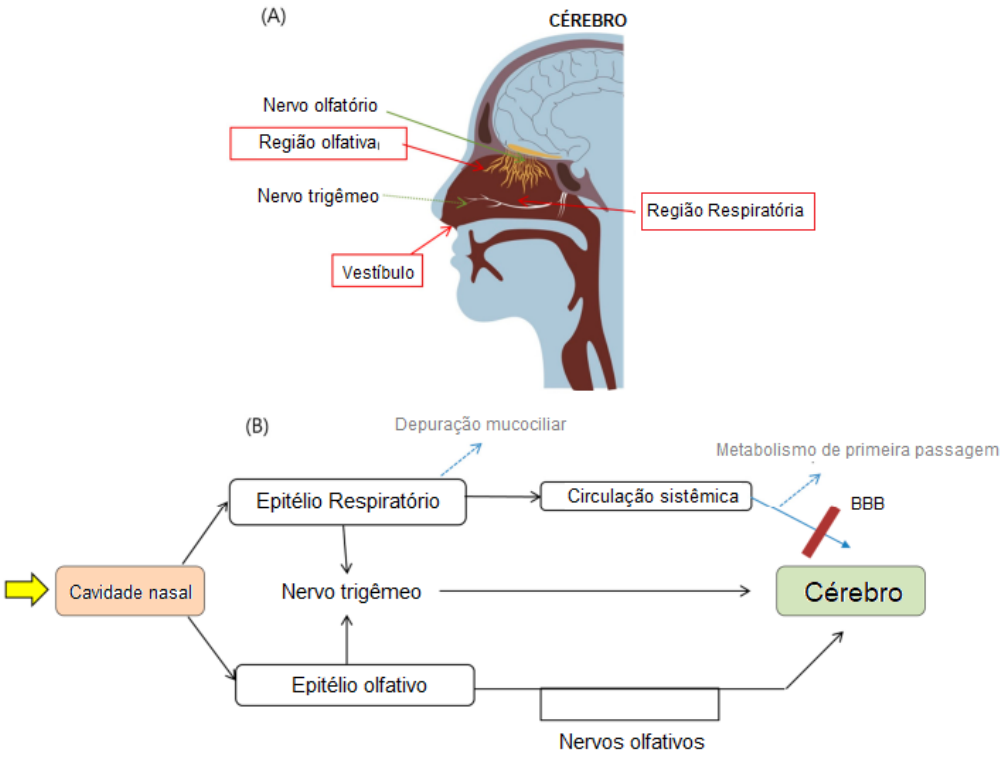

Figura 6. Via nasal.**A)** Estruturas envolvidas no possível transporte de produtos biofarmacêuticos pela via nasal; **B)** As potenciais vias de transporte que levam à captação pelo cérebro após administração intranasal. Modificado de Hong et al 2019.

1.3. Edição genômica

O uso das ferramentas de edição genômica surge como método alternativo e inovador à terapia gênica "convencional", pois fornecem a possibilidade de escolher e editar o local do genoma em que o transgene irá ser inserido. As plataformas mais relevantes para fins terapêuticos são baseadas em nucleases programáveis e incluem nucleases de dedo de zinco (ZFNs), as Nucleases com efetores do tipo ativador transcricional (TALENs) e, mais recente, o sistema CRISPR-Cas9 (Repetições Palindrômicas Curtas Agrupadas e Regularmente Interespaçadas) (Poletto et al. 2020).

O sistema CRISPR-Cas9 na natureza é encontrado em bactérias, servindo como um sistema imunológico adaptativo, pois seus componentes detectam e consequentemente degradam DNAs invasores recorrentes (principalmente DNA de bacteriófagos) (Ma et al. 2014). Este sistema tem sido adaptado para sua aplicação em diversas áreas da biotecnologia e no desenvolvimento de ferramentas terapêuticas. A Cas9 e o sgRNA (do inglês "single-

guide RNA") são os componentes essenciais para a edição do genoma pelo sistema CRISPR-Cas9 (**Figura 7**). O sgRNA ou RNA guia consiste em uma sequência de 20 nucleotídeos, complementar a uma região especifica no genoma (local alvo). Este sgRNA é responsável pelo direcionamento e acoplamento da endonuclease Cas9 no alvo. Entretanto, a endonuclease Cas9 é a responsável pela clivagem sitio-especifico do DNA, gerando quebra de fita dupla. Dentro do local alvo, a região PAM (com sequência NGG) é necessária para garantir seu reconhecimento. Assim, uma sequência de DNA que contenha aqueles 20 nucleotídeos seguidos da região -NGG pode ser reconhecida pela nuclease (Ma et al. 2014). Finalmente, o potencial terapêutico do sistema é fornecido pelos mecanismos de reparo de DNA intrínsecos do organismo. As quebras de dupla fita podem ser reparadas por duas vias diferentes, sendo uma delas a via de reparo por união de extremidades não-homólogas (NHEJ), que pode produzir inserções / deleções (Indels) no local da quebra e leva à introdução de mutações no local alvo. A segunda via é a de recombinação homóloga (RH), que procura a presença de uma sequência de DNA homóloga (sequência doadora) e, ao encontrá-la, leva à recombinação da sequência clivada pela doadora, inserindo uma nova sequência de DNA no genoma celular (**Figura 7**) (Gupta et al. 2019). A Cas9 pode ser facilmente programada para atingir novos locais, alterando sua sequência de RNA guia. Assim, facilita a edição de diferentes genes (Wang et al. 2016). No entanto, uma grande preocupação no processo de desenvolvimento da tecnologia tem sido a frequência relativamente alta de atividade de nuclease em sítios fora do alvo, ou "off-targets" em células de mamíferos, podendo levar à mutagênese em regiões diferentes de seus alvos inicialmente propostos (Gupta et al. 2019).

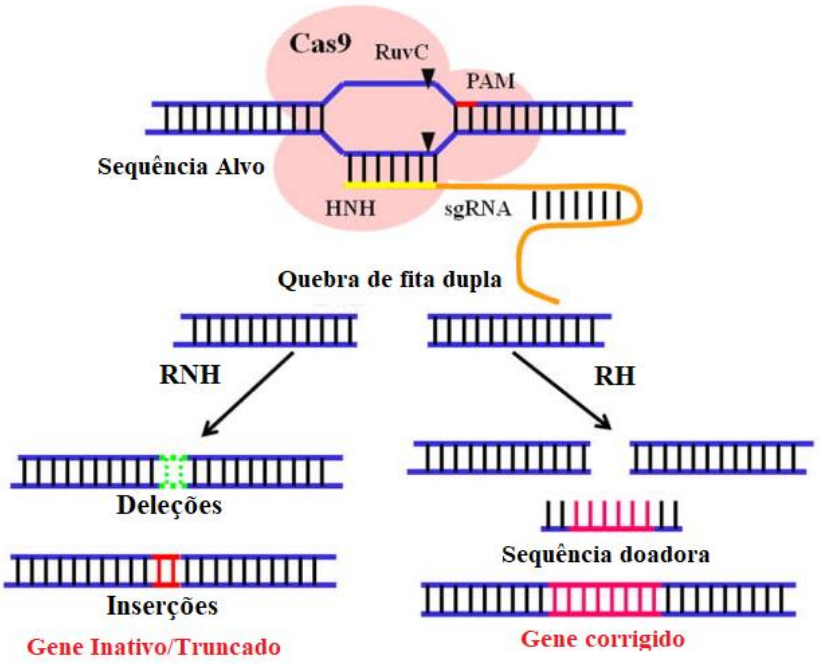

Figura 7. Ilustração esquemática dos dois diferentes mecanismos de reparo de quebras de fita dupla mediadas por CRISPR / Cas9. Adaptado de (Li et al. 2018).

Nos últimos 10 anos a edição genômica tem se apresentado como uma plataforma versátil e, em alguns casos, mais eficiente que a terapia gênica convencional, uma vez que promete uma terapia única e precisa, a qual, dependendo da estratégia de entrega, tem potencial para tratar órgãos como cérebro e osso (Poletto et al. 2020).

2. JUSTIFICATIVA

Apesar dos avanços na terapia gênica visando o desenvolvimento de tratamentos para as doenças lisossômicas, até a data existem vários obstáculos a serem resolvidos, os quais envolvem a baixa biodistribuição dos vetores utilizados, reduzindo sua entrega a tecidos alvos importantes como o cérebro, e a ativação de respostas imunes que levam à diminuição da eficácia do tratamento, entre outros. Avaliar estratégias alternativas que permitam um tratamento seguro e efetivo a longo prazo para o paciente continua sendo o alvo das pesquisas na área das doenças lisossômicas. Com a aparição de as diversas ferramentas de edição genômica em os últimos anos, e o aumento de terapias aprovadas baseadas nesse enfoque para tratamento de outras diversas doenças, o uso dos ZFN tem chegado a estudos clínicos em pacientes MPS I e II. Embora os resultados pré-clínicos tenham sido satisfatórios, a translação ao paciente não tem exibido resultados muito alentadores após da entrega sistêmica da ferramenta por meio de vetores virais. Assim, continua sendo imperativo estudar estratégias de entrega de sistemas de edição gênica que tentem superar as limitações existentes.

3. OBJETIVOS

3.1. Objetivo geral

Esta tese tem por objetivo geral a avaliação dos efeitos da administração intranasal da ferramenta de edição genômica CRISPR-Cas9 complexada a um vetor não viral tipo lipossomal como possível terapia não invasiva visando o tratamento dos sintomas sistêmicos e neurológico das MPS I e II.

3.2. Objetivos específicos

- Produzir e caracterizar vetores lipossomais contendo o sistema CRISPR-Cas9 junto das sequências para recombinação homóloga, para uso em animais com MPS I e II;

- Estudar a biodistribuição dos vetores após administração nasal em modelo murino;

- Avaliar o efeito de administrações repetidas do vetor sobre a atividade enzimática tecidual e sérica da enzima deficiente na doença, acúmulo tecidual de GAGs e melhora fenotípica por testes comportamentais no modelo de MPS I;

- Comparar o efeito de administrações nasais cumulativas (30 ou 60 administrações) sobre os mesmos parâmetros do objetivo anterior no modelo animal de MPS II;

- Avaliar se as administrações repetidas levam a alguma resposta inflamatória persistente nos animais, através da dosagem de interleucinas inflamatórias séricas.

4. RESULTADOS

4.1 Artigo I: The potential of gene therapy for Mucopolysaccharidosis type I

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REVIEW

The potential of gene therapy for mucopolysaccharidosis type I

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ABSTRACT

Introduction: Mucopolysaccharidosis type I (MPS I) is a lysosomal storage disorder caused by mutations in the IDUA gene, characterized by deficient IDUA enzyme production and storage of glycosaminoglycans in tissues. Currently, therapeutic strategies approved have shown an improvement in quality of life of patients, but the majority of severe symptoms including cognitive and skeletal alterations persist. Gene therapy aimed to correct the genetic defect holds promise. Indeed, preclinical results show that it may be possible to develop a gene therapy strategy that may overcome the present limitations. In this review, authors review studies involving gene therapy for MPS I in the last years and highlight the most promising approaches.

Areas covered: Authors review main studies involving gene therapy and genome editing for MPS I in the last 2–3 decades, from the initial in vitro studies up to the first clinical trials, and prospect what the future may hold for this technology in this disease.

Expert opinion: Among all strategies studied, viral gene therapy and genome editing are being applied in clinical trials. Some of the results are inconclusive while scaling the process from animal models to human. The key for better outcomes relies on giving patients a proper therapy.

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KEYWORDS
MPS I; gene therapy; genome editing

1. Background

Mucopolysaccharidosis type I (MPS I-H) is an autosomal recessive disease caused by mutations in the *IDUA* gene localized at 4q16.3. This gene encodes the lysosomal hydrolase alpha-L-iduronidase (IDUA, EC 3.2.1.76), which is responsible for the hydrolysis of alpha-L iduronic acid residues of glycosaminoglycans (GAGs) Dermatan sulfate and Heparan sulfate [1,2]. This process is a fundamental step for the breakdown of these molecules in the lysosome and mutations in *IDUA* result in a deficient production or function of the enzyme. It causes progressive accumulation of GAGs within lysosomes, impairing its function [3]. As a result, loss of lysosomal function also involves secondary impairment of other pathways as autophagy and activation of inflammation. These alterations trigger a series of severe symptoms in patients, resulting in ocular, skeletal, visceral, and neurological manifestations, causing death at early ages [1,4,5].

Patients with MPS I can present different phenotypes. The severe form (called Hurler syndrome) has an early manifestation, starting between the first and third year of life, with a profound and progressive systemic and neurologic impairment. The intermediary phenotype, described as Hurler-Scheie may not display an aggressive cognitive decline at early stage, but learning disabilities can be present. There is also a more attenuated (or less severe) form, where the patients present a residual enzyme activity, called Scheie syndrome. These patients are cognitive normal, but still develop several other abnormalities seen in the most severe forms [6,7].

An essential characteristic of IDUA, shared by most lysosomal enzymes, is the presence of specific post-translational modifications that allow the traffic to the lysosome. The Mannose 6 phosphate (M6P) residues that are added during protein synthesis to interact with their specific receptors and allow the uptake of the enzyme [8,9]. This interaction can occur not only with the lysosomal membrane but also with other neighboring cells. Part of the enzyme that is being synthesized can be secreted to the extracellular media and can be recognized by extracellular membrane receptors [10,11]. This mechanism makes the disease suitable for treatment using different approaches, once 'cross-correction' can occur due to the presence of extracellular enzyme. The enzyme may be given intravenously or produced by other cells and be captured by multiple tissues.

Currently, there are two different treatments for MPS I patients available. The first one is enzyme replacement therapy (ERT), approved for human use in 2003 (Aldurazyme®, Sanofi/Genzyme). This enzyme is a human recombinant variant of IDUA produced in CHO cells, that displays human-like post-translational modifications that allow cross–correction after intravenous administration [7,12]. Patients that received treatment showed variate responses. It is an exogenous enzyme, therefore, antibody production has been detected in almost every patient. Also, side effects such as anaphylactic reactions have been described. The treatment has demonstrated positive outcomes as the significant reduction in secreted urinary GAGs, spleen and liver volume. Nevertheless, cardiac alterations, pulmonary function and

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Article highlights

- ERT and HCSC transplantation strategies for the treatment of MPS I patient show several limitations.
- Gene therapy and genome editing approaches have shown in different ways its therapeutic potential for MPS I.
- For most of the integrative viral and non-viral strategies, concerning related to their random integration still persists.
- AAVs vectors are the most used vectors used in vivo due to its neural tissue-specific tropism.
- Lentiviral vectors are promising for ex vivo approaches;
- Genome editing approaches such as ZNFs nuclease and CRISPR-Cas9 system are becoming the late research interest in the field.

This box summarizes key points contained in the article.

joint motility show only limited improvement, and brain abnormalities are not corrected due to the inability of the enzyme to reach the central nervous system [12,13].

Hematopoietic stem cell transplantation (HSCT) has become the standard procedure for treatment of MPS I patients in developed countries. The criteria of choosing patients is based on the chance to prevent the neurological manifestations [14,15]. The administration of normal allogenic hematopoietic populations to restore the blood system allows correction of multiple peripheral organs and also delivery of the enzyme to the brain via trafficking of myeloid/macrophage populations across the blood–brain barrier [16]. Even so, there exist a risk of death during/after the procedure because of the possibility of severe immunogenic reaction and also unexpected unsatisfactory outcomes due to the heterogeneous patient population [3].

Gene therapy has become a prominent alternative for the treatment of monogenic diseases. It is based on the delivery of an exogenous genetic material into cells that later will be transcribed and translated into a functional protein [17]. For monogenic diseases, this genetic material would be the normal version of the mutated gene that leads to the production of an unfunctional protein that is, in most of the cases, the cause of the disease [18]. The normal version of the gene is delivered to the cells by either nonviral or viral vectors. Nonviral vectors are either physical methods or lipids used to protect the DNA, while viral vectors are common viruses that were adapted to become nonpathogenic.

Among the most used viral vectors for this purpose, and specifically used in the search for MPS I therapeutic options, we found the integrative RNA-based viruses (Retrovirus and lentivirus). Those viruses have in their genetic material viral proteins that allow nuclear import and integration in the genome, a transgene packaged capacity about 8 kb, and while retroviruses are capable of transducing only dividing cells, lentiviruses can also do it in non-dividing cells. Both may trigger immune response in host [19,20].

In the non-integrative category, there are the adeno-associated viruses vectors (AAVs). These are DNA-based vectors that do not integrate with the host genome but still provide long-lasting expression. Its genome is about 4.5 kb, so its package capacity is smaller [21]. These vectors have been extensively studied because of the possibility to manipulate its capsid structure to facilitate tissue-specific target [22].

In this review, we summarize studies involving gene therapy for MPS I in the last years (Tables 1 and 2) and try to highlight promising technologies that may guide future steps in the field.

2. Gene therapy for the treatment of MPS I

2.1. Viral gene therapy

Early in the 90s researchers began to study viral vectors as a possible gene therapy approach for MPS I. At that moment, retroviral vectors were the most common vectors used in the emerging field of gene therapy. Previously, there were studies showing the potential of these vectors in the treatment of fibroblasts from Gaucher and Sly diseases, other lysosomal storage diseases [23–25]. In MPS I fibroblasts the approach also showed positives results [26]. Researchers were focused on the design of the retroviral vector, so different alternatives such as choosing the right promoter for high, long and stable transgene expression were attempted. They demonstrated cases of extreme high expression (250-fold normal). In these situations, the capacity of cells to modify the protein product to its mature form was limited due to saturation of its enzymatic machinery. Therefore, high gene expression, despite desirable, may affect cells metabolism [26].

The first attempts for transducing MPS I patient hematopoietic stem cells and perform *ex vivo* gene therapy started in 1996, using retroviral vectors. CD34+ cells isolated from MPS I patients were successfully transduced by different retroviral vectors carrying the IDUA gene [27,28]. An average of 50% of transduction efficient in bone marrow cells was obtained, with high level of IDUA expression for several months *in vitro*. Moreover, cross correction studies confirmed the ability of the enzyme produced by hematopoietic cells to correct MPS I fibroblasts [29]. Later, studies also were made to investigate mesenchymal stem cells (MSC) as a possible target for gene therapy [30]. Treatment led to overexpression of IDUA and normalization of GAGs mesenchymal stem cells.

The MPS I mouse model was developed late in 90s [31] and along with feline and canine models [32,33] allowed gene therapy for MPS I to move on from patients' cells to *in vivo* and *ex vivo* approaches. The first autologous hematopoietic stem cell transplants with modified cells were performed in the MPS I canine model in 1999 [34]. The transduced cells showed high expression of IDUA *in vitro*, from 10 to 200-fold normal levels. After transfusion in dogs, the IDUA cDNA was also detected in blood and marrow leucocytes up to 3 years after the procedure, showing a good engraftment. Despite this first good result, the enzyme was not detected in dogs due to the immune response activated after enzyme production [34].

To avoid activation of the immune response, researchers tried different strategies. An *in utero* transplantation of retroviral transduced hematopoietic stem cells directed into fetal pups was attempted [35]. Despite no immune response, surprisingly enzyme levels and transcript were not detected, and dogs ended up developing the disease. The researchers finally

Table 1. Some gene delivery strategies for gene therapy in MPS I.

Vector Characteristic		Vector	Tissue target	Model tested	Relevant outcomes	References
Viral vectors	Integrative	RT-IDUA	HSC, visceral tissue, Brain tissue, Skeletal tissue	Fibroblast Canine Murine	Short-term expression of the enzyme after HSC transplant. *In utero* strategies show long-term expression, ameliorating biochemical, physical and behavioral impairment.	[30–35,38]
		LT-IDUA-apoE	Brain/visceral tissues	Murine	Enzyme produced by other organs can reach brain tissue	[49]
		LT-IDUA	HSC	Murine Clinical trial	Long-term expression of IDUA in different tissues after HSCT engraftment NR	[50] NCT03488394
	Non-integrative	AAV/1/2/5/-IDUA	Brain/visceral tissues	Murine, canine, non-human primate	*In situ*, intranasal and systemic administration prevent neurological impairment and correct the biochemical alterations in most tissues.	[53–59]
		AAV/9-IDUA	Brain	Clinical trial	NR	NCT03580083
		Sleeping-beauty Transposon	MSC	Murine	Short-term expression of the enzyme in tissue	[64–66]
Non-viral vectors	Integrative		Liver			
		PhiC31 integrase	Liver	Murine	Short-term enzyme detected in blood	[68]

R: Retroviral; LT: Lentiviral, AAV: Adeno-associated vector, HSC: Hematopoietic stem cells, MSC: Mesenchymal stem cells. NR: Not reported.

established that the reason of the unsatisfactory results was due to the possibility of gene silencing after retroviral integration, which was also proved by other authors [35]. Silencing of the transgene happens by endogenous epigenetic mechanisms of the organism [36,37].

It was not until 2007 that the attempts to develop gene therapy using retroviruses gave their first positive results. Via intravenous injection, MPS I dogs were treated with a gamma retrovirus carrying IDUA gene and a human alfa-1-antitrypsin promoter at 2–3 days [38]. After almost 2 years all dogs had a significant improvement in skeletal, cardiac, ocular and visceral disease, and there was no detectable immune response. The authors hypothesized that probably, the success of the results was due to the mutations that dogs originally had in its *idua* gene. The mutation causes production of the protein in small amounts but without signal sequences that avoids its secretion or transport to the lysosomes. That was a very important outcome since that enzyme still has epitopes that could be recognized by the immune system and therefore, create an adaptive tolerance [38]. Regarding the brain tissue, even when all treated dogs showed a significant reduction on lysosomal storage in neurons, one important feature to point out is that cognitive impairment was not easy to recognize since the animals presented normal behavior [38].

As mentioned, the MPS I mice model was developed around that time [31], and because it reproduces the phenotypic features of the disease, researchers started to focus experiments on the mouse model. There are several studies that report neonatal or adult retroviral administration with a sustained expression of the enzyme and improvement of the systemic impairment [39–42]. They also show brain tissue cross-correction that ameliorated biochemical [43,44], behavioral and cognitive manifestations [45].

Lentiviral vectors are also another group of vectors used in several protocols for MPS I gene therapy, whose main difference from retroviral vectors is the capability of infected not only dividing but non-dividing cells equally efficiently [19], a desirable feature in this therapy.

Almost a decade after retroviral studies began, the first attempts to use lentiviral vectors carrying the IDUA gene in MPS I fibroblasts and MPS I mice started to show up with positive results. In fibroblasts, a 1.5 fold-normal expression of the enzyme was obtained, with cross correction of untreated fibroblasts [46]. Scaling up to mice revealed low expression of the enzyme, near to 1% from normal levels, but this was enough to normalize GAGs levels in visceral organs [47]. The benefits of neonatal treatment were also observed at this time [48].

An interesting proposal was shown by researchers trying to reach central nervous system with lentiviral vectors. They designed a vector with a fusion protein between IDUA and a peptide that allows the enzyme to cross the blood–brain barrier. That enzyme led to biochemical correction and improvement of cognitive deficits in treated mice [49]. That approach served as proof-of-concept for development of an ERT that uses the same approach to reach the central nervous system, currently in clinical trials.

Lentiviral vectors are particularly efficient in transducing hematopoietic stem cells [50,51]. An initial study compared MPS I mice treated either with transduced HSCT or normal HSCT. Overexpression of the transgene in transduced cells allows to reduce GAGs levels in all tissues and also

Table 2. Genome editing strategies in MPS I.

	Vector Characteristic	Vector	Tissue target	Model tested	Relevant outcomes	References
Genome editing systems	Integrative-Viral	ZFNs-AAV8	Liver	Hepatocytes Murine	Albumin locus as a 'safe harbor'. Stable and long term expression of the enzyme/reduction in visceral GAGs.	[82,85]
				Clinical Trial	No severe side effects. No increase in enzyme activity.	NCT02702115
			HSC	Human HSC	CCR5 locus as a 'safe harbor'. After engraftment, enzyme was detected in all tissues.	[87]
		sgRNA/Cas9-RNP		Murine	Prevention of cognitive and skeletal impairment. No off-targets detected.	
		Plasmidial CRISPR-Cas9			Partial correction of cells	[91]
	Non-integrative	System-liposomes		Fibroblast		
			Liver	Murine	Improvement of cardiac function, enzyme activity and GAG levels	[92]

ameliorated the cognitive impairment, being better than those mice treated with hematopoietic stem cells from a health donor [50]. After, the safety and tolerability of lentivirus transduction in HSCT was proved in vitro and in vivo with mice and human cells [51]. High level and long-term expression of IDUA was detected, and the ability of the HSCT to differentiate into multiple cell types after transplant in mice was demonstrated including the low rate of toxicity and tumorigenic potential [51].

Later, adeno-associated vectors (AAVs) have arisen as a promising alternative. The AAVs present desirable characteristics: as non-integrative vectors, they reduce the possibility of insertional mutagenesis. Their non-immunogenic nature allow long-term gene expression in almost all types of cells and the possibility to engineering its capsid to target specific tissues also exists [22,52].

Both *in-situ* and systemic administration of AAVs has been studied. With the necessity to develop a system that reaches the brain, intrathecal and intraventricular injection of diverse AAV serotypes in mice, feline, canine, and non-human primate models were evaluated. In mice, it was observed a complete biochemical correction of GAGs and other biomarkers in brain tissue after injections of AAV-2, AAV-5 and AAV-9 [53–55]. The prevention of neurological dysfunction when neonatal intrathecal injection was performed was also observed [55]. In feline and canine models, almost the same biochemical results were obtained using AAV-9, but an immune response was also activated and decreased the enzyme production over time [56,57]. Aimed to study the tropism of several AAVs to brain tissue, researchers used non-human primates as the model more similar to humans, and assessed the distribution of these vectors after direct central nervous system injection of AAV-1 or AAV-2 or AAV-5 [58], showing that either AAV serotype could achieve a global distribution through brain tissue and surroundings.

A latter approach in this field was the intranasal administration of AAV-9, which has also shown satisfactory outcomes. In the study, an intranasal administration of an IDUA-encoding AAV-9 was performed in MPS I mice with at 3 months [59]. This resulted in IDUA activity levels up to 50-fold normal mice in the olfactory bulb and reduction of tissue glycosaminoglycan accumulation in all brain tissue. The enzyme diffused from the olfactory bulb and the nasal epithelium to other areas. Mice also presented a normal cognitive development. Besides, the study proved that a pre-treatment with ERT before gene therapy prevent immune response. At the moment, AAV gene therapy is moving to clinical trials, and seems to be a very promising alternative to MPS I.

2.2. Non-viral gene therapy

Other strategies were attempted in parallel throughout the years, as alternatives to viral vectors. Non-viral vectors avoid immunogenicity and also have lower cost of production. Sleeping beauty transposon (SBT) is a non-viral vector that combines some advantages of viruses (capacity of integration) without the need of a viral particle [60]. It is composed of a transposomic region, which is replaced by the transgene to be expressed and a transposase, that generates double-stranded breaks in the DNA and allow the insertion of the transgene [61]. Among its advantages is the fact of (i) not relying on an endogenous DNA repair machinery to integrate into the genome, (ii) the system is not restricted to a cell cycle phase, so it is possible to reach and transpose a wide range of cell types including those with high therapeutic potential and (iii) it allows stable and efficient transgene production once transposed [62,63].

The capability of this system to treat MPS I mice was first studied in 2007 by Aronovich *et al*. They proposed the use of this system as a tool for gene therapy of MPS I and VII (Sly syndrome) combined with immunomodulation. In their study, they showed that after a hydrodynamic injection of transposon/transposase plasmid carrying *IDUA* gene, enzyme activity in plasma reached up to 100-fold wild-type (WT) levels but was maintained for only 4 weeks post-injection in mice that did not receive immunomodulation. It remained high for over 3 months in some of the inmunomodulated ones, and that enzyme levels were enough to correct the pathology in liver and some other organs [64,65]. This strategy was also tested in mesenchymal stem cells from MPS I mice but the outcomes were not as satisfactory [66], as high antibody titer were detected in blood [66]. As an important point to highlight, results did not show any neurological improvement data, possibly because the system is unable to reach the brain tissue.

So far, SBT has been successfully used in the clinical trials with T-CAR cells for lymphomas, leukemia, and also in Alzheimer's disease [63,67]. Clinical trial for SBT treatment for MPS I developed by Immusoft Corporation engineering human B lymphocytes is still waiting for approval [63]. On

the other hand, this approach still has a probability to cause undesirable insertional oncogenesis.

Another studied strategy was the use of a serine recombinase from the phage *Streptomyces lividans*., PhiC31 integrase, which is somewhat similar to transposons. It integrates a donor gene tagged with attB-enhancers sequence into pseudo-attP sites present in the mammalian genome [68]. Adding this recombinase to a vector carrying the IDUA transgene given by hydrodynamic injection into MPS I mice resulted in a high level of enzyme activity (over a 1000 U/ml) in blood. Unfortunately, this activity drops fast [68].

Non integrative non-viral approaches were also studied. A minicircle (MC) DNA vector carrying *IDUA* gene was used to treat MPS I mice. The vector design was focused in achieving long-term expression of the protein and avoiding epigenetic gene silencing [69]. Treatment resulted in biochemical correction of lung, liver, spleen, kidney and also cerebellum, but enzyme only maintained expression for a longer time with immune modulation [69]. Although these options had some positive outcomes, since then, limited research in the field has been performed.

3. Gene editing approaches

Due to the discovery and development of genome editing tools based on nucleases, researchers are trying to use them as a treatment approach for monogenic diseases. Three different platforms were developed (Figure 1), and all of them share two characteristics: first, the ability to create double-strand breaks on DNA, that will be repaired using endogenous DNA-repair machinery; and the second one is the specificity of the nuclease, recognizing the target sequence in a desired gene that will be cleaved [70,71].

The first two systems described were the Zinc Finger Nuclease (ZFNs) and the transcription activator-like effector nucleases (TALEN). They are artificially designed restriction enzymes with a DNA recognition domain. The ZFNs system comprise a pair of nucleases of 30 amino acids linked to zinc finger proteins, that recognize target sequence specifically by codons (nine nucleotides each) [72]. TALEN, on the other hand, do not recognize triplets of nucleotides but a 17-nucleotide region for each nuclease domain which works similarly to ZFNs [73].

More recently, the CRISPR-Cas9 complex was described and rapidly became the most used platform for genome editing worldwide. It comprises an endonuclease, mostly Cas9, which forms a complex with a 20-nucleotide sequence called guide-RNA [74]. This complex recognizes specific regions in the genome, which then creates a double-strand break that will further be repaired by either Non-Homologous End Joining (NHEJ) or Homologous Recombination (HR). This last mechanism allows gene therapy to happen since it is possible to add a new DNA sequence in the cleaved site [70].

These tools were widely used for the creation of animal and cell models that allowed the study of physiopathological processes and the screening of different drugs for selected conditions [75,76]. Currently, the use of these approaches allows multiple applications in monogenic diseases [77]. In particular, the develop of a safe and efficient platform that allows genome editing in human somatic and pluripotent cells has been pursued [77].

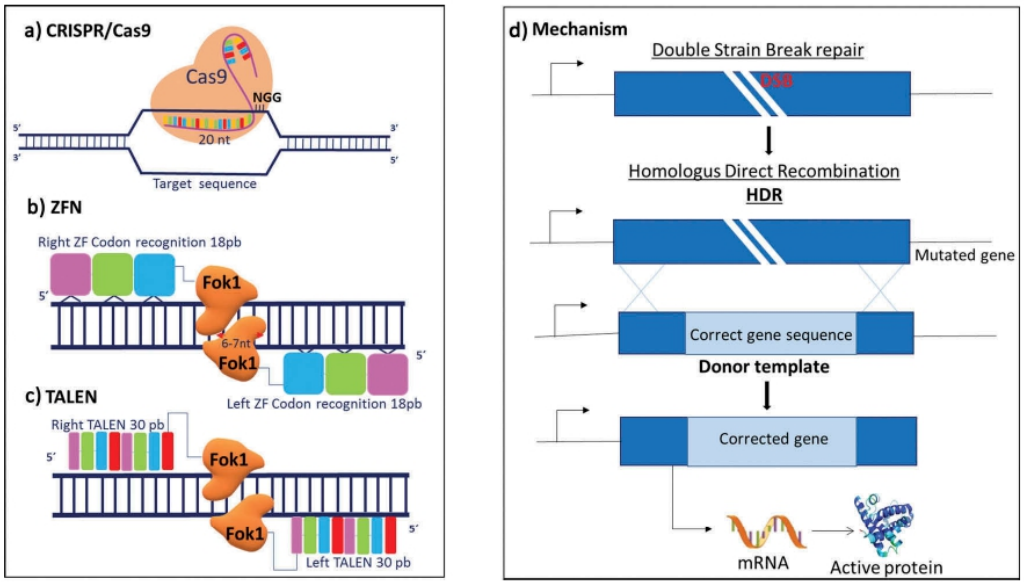

Figure 1. Gene editing approaches. (a) CRISPR-Cas: this system uses CAs9 as the endonuclease, who recognizes a 20-nt guide-RNA sequence close to a PAM (NGG) sequence in the genome. (b) Zinc-Finger Nucleases (ZFN) uses Fok1 to cleave DNA, guided by a synthetic protein that recognizes a 18nt genome sequence. (c) Transcription activator-like effector nucleases (TALEN) also uses Fok1 to cleave DNA, guided by a protein that attaches to a 30-nt genome sequence. (d) Mechanism for gene correction. A Double-strand break (DSB) occurs after DNA cleavage by an endonuclease. A corrected gene sequence is then added and homologous recombination occurs, changing the mutated sequence for the corrected one, producing a functional protein.

3.1. Viral delivery

Delivery of DNA to cells is still a point to be considered when working with gene editing tools. Viral vectors are still preferred, but the same limitations mentioned previously are still valid here [78–80]. However, once the new transgene integrates, there is no need for having a constant production of the transgene by the vector [81].

Researchers proposed a strategy based on the utilization of an AAV-8 vector to carry and deliver donor template DNA for HR, together with Zinc Finger nucleases, to integrate the *IDUA* gene in the albumin locus in liver cells [82,83]. The albumin locus works as a 'safe harbor' site to target the recombinant integration of the gene. A genomic safe harbor is described as any 'non-essential' gene, that is not involved in pathological conditions if deleted [84]. Loci such as ROSA26 in human and mice, AAVS1 and CCR5 have been reported as 'safe harbors' [82,84,85].

Using the albumin locus, IDUA insertion and high expression was achieved in hepatocytes, 10 to 16-fold wild-type mice, 4 months after administration [83]. They also reported high IDUA activity in serum, the secretion of the enzyme from the liver and its capacity to reach secondary tissues like spleen, heart, lungs, and muscles from 11% to 26% of the normal enzyme activity. The brain tissue achieved a smaller proportion, from 0.5% to 1% [83]. Consequently, a significant GAG reduction in visceral organs was observed. This strategy is now in phase I/II clinical trials.

Thanks to gene editing the possibility to transplant autologous edited hematopoietic stem cells to MPS I patients holds high promise [86]. Gomez-Ospina *et al.*, in 2018 presented the first pre-clinical study that shows the potential of human genome-edited HSCT in the improvement of biochemical, cognitive, and bone impairment of MPS I mice after transplant [87]. Their strategy was using an AAV vector to deliver a donor template for HR combined with a sgRNA/Cas9 ribonucleoprotein targeting *CCR5* safe harbor gene into CD34+ cells [87]. The use of Ribonucleoprotein Cas9 complex (RNP) has become a suitable option to help overcome the concern about immunogenicity and possible off-target activity of CRISPR/Cas9 technology [88]. With this complex, a more limited amount of editing machinery is delivered to the cell, which is safer.

A stable insertion of IDUA gene in *CCR5* locus and a long-lasting expression of the enzyme from modified HSTC during 30 days was achieved, 660-fold normal values [87]. It was also proved that the procedure did not affect the cell ability to differentiate into macrophages, that migrated to the affected tissues. No significant presence of off-targets after sequencing and no tumorigenicity was detected in mice [87]. Overall, the protocol developed by Ospina-Gomez and collaborators seems to have managed to solve the biodistribution problem that existing therapies have, reaching tissues such as brain [54,55,89].

3.2. Non-viral delivery

Among the strategies to deliver a transgene to a cell, nanoparticles are now raising the attention of researchers. These nanotechnological approaches provide some advantages related to its biocompatible nature and also its low manufacture cost [90]. The use of nanoemulsions complexed with CRISPR/cas9 and a short donor oligonucleotide for the *in-vitro* edition and correction of a point mutation in an MPS I patient fibroblast was reported [91]. The researchers performed the correction of p.Trp402* variant in MPS I patient skin cell with constant enzyme activity during 30 days. The enzyme produced was also enough to recovery the lysosomal mass and morphology caused by GAGs accumulation. The low cytotoxicity of the vector in cells was also shown [91,92]. This strategy was then tested in neonatal MPS I mice, using as safe harbor the ROSA26 locus [92].

A hydrodynamic injection of cationic liposomes complexed with CRISPR/Cas9 and donor plasmid carrying the IDUA gene was given to newborn MPS I mice. The administration of naked plasmids and liposome complexed plasmids was compared. The liposomal complexes went mainly to the heart, lung, and liver. Due to the high levels of enzyme in heart, the researchers observed an improvement in cardiac function in MPS I mice after 6 months. With all, the possibility to deliver genetic material without the use of viral vector is an alternative, nevertheless, a better strategy to reach problematic tissues as brain needs to be studied.

4. Human trials

Despite several pre-clinical attempts, to date, there are only three approved clinical trials using gene therapy and genome editing platforms for MPS I treatment. Using genome editing platform with AAV vectors, Sangamo therapeutics in 2016 started the first clinical trial in 3 MPS I patients (NCT02702115). SB-318, a ZNF genome editing technology delivered by AAV-6, aims the integration of IDUA gene at the albumin locus in liver cells after intravenous injection. Sangamo has shown the preliminary results early in 2019. In terms of adverse event, the patients did not show severe side effects. The higher dose of the vector was well tolerated (5e13 copies/kg), but plasma IDUA values were still unchanged from pre-treatment levels. Two of the three patients showed a decrease in GAGs excretion meanwhile GAGs values for the other remain above the normal range [93]. Thus, these results for this promising treatment have to be taken as a challenge for the industry and researchers, because there is still a large lack of knowledge regarding immunological and physiological mechanisms that are affecting the translation between the results obtained from pre-clinical compares to clinical studies [94].

In 2018, hospital San Raffael and Theleton foundation in Italy started a phase I/II study to evaluate Safety and Efficacy of the transplant of autologous hematopoietic stem and Progenitor cells CD34+ collected from peripheral blood and modified with lentiviral vector encoding for IDUA gene in pediatric patients (NCT03488394). The trial is planning to follow up patients initially for 1 year after treatment and the first outcomes are expected in 2020.

Also in 2018, another company, REGENXBIO, started to recruit patients to test safety, biodistribution, and possible improvements in neuropathological symptoms. The therapy, RGX-111 (NCT03580083), is a recombinant AAV9 that carries a human IDUA gene. The intent is to deliver the vector to the nervous system since the capsid of that virus serotype has a tropism for that tissue. Two different experimental doses of viral vector will be tested and the preliminary results are expected for 2020–2022.

5. Expert opinion

In the last 2–3 decades, researchers have worked rigorously and tirelessly to develop new, safe, and effective therapies for the treatment of MPS I as well as the other lysosomal diseases. Gene therapy is a potential alternative that could offer better results compared to current treatments. Despite continuous research, a few strategies manage to reach clinical trials. Gene therapy and genome editing strategies both have shown its potential to be a suitable therapy for MPS I patients if started at early stage of the disease. But the translation from lab results to a clinical scenario has been challenging.

Normally, in studies involving animal models, the variables are easier to control: the starting point of the treatment, the lack of mutation variability, the immune response to the transgene and the enzyme, and specially, the vector dose. Scaling up from mice to bigger animals, such as dogs and macaques, and then to humans has been particularly difficult for gene therapy. Viral vectors are efficient, but high titers of some vectors such as AAVs are hard to obtain, and increase the cost of vector production dramatically. The immune response to the vector in higher doses is also something to be considered. At this moment, in vivo approaches using AAVs as well as ex vivo transduction of hematopoietic stem cells followed by their transplant seem to be the two gene therapy platforms with better results for MPS I.

Gene editing studies are being conducted, but the efficiency of gene editing in vivo also needs to be improved. Ex vivo gene editing with the CRISPR-Cas9 system holds great promise, and this platform could be potentially reaching clinical trials in the next years.

Recently, the first clinical trials were finally started. The gene editing technology used, despite not showing any severe adverse effect, also failed to increase enzyme activity as shown in preliminary data. High titers of the AAV vector were used in two patients, and increasing the dose to even high titers implies in increasing the cost of production if such vectors, as well as verification of safety of such a high i.v vector dose. The other trials are also occurring but without any preliminary data.

Based on this scenario, it is likely that multiple approaches will be tested in MPS I clinical trials in the next years. The design of the trials will probably benefit patients with different characteristics (for example, presence or absence of neurological symptoms or presence of previous antibodies against a particular vector) and the physician could then decide which approach should be performed to a better outcome for a particular patient.

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References

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Rao KS, Adhikari S, Singh S, et al. Hurler syndrome. J Nepal Paediatric Soc. 2016. DOI:10.3126/jnps.v36i3.16349
2. Scott HS, Bunge S, Gal A, et al. Molecular genetics of mucopolysaccharidosis type I: diagnostic, clinical, and biological implications. Hum Mutat. 1995;6(4):288–302.
3. Parini R, Deodato F, Di Rocco M, et al. Open issues in mucopolysaccharidosis type I-Hurler. Orphanet J Rare Dis. 2017;12. DOI:10.1186/s13023-017-0662-9
4. Settembre C, Fraldi A, Rubinsztein DC, et al. Lysosomal storage diseases as disorders of autophagy. Autophagy. 2008;4:113–114.
5. Parenti G, Andria G, Ballabio A. Lysosomal storage diseases: from pathophysiology to therapy. Annu Rev Med. 2015;66:471–486.
• **This is a good review on pathophysiology of lysosomal disorders.**
6. Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Stephens K, Amemiya A, editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2020.
7. Jameson E, Jones S, Remmington T. Enzyme replacement therapy with laronidase (Aldurazyme®) for treating mucopolysaccharidosis type I. Cochrane Database Syst Rev. 2019. DOI:10.1002/14651858.CD009354.pub5
8. Braulke T, Bonifacino JS. Sorting of lysosomal proteins. Biochim Biophys Acta Mol Cell Res. 2009;1793:605–614.
9. Neufeld EF. In: Mehta A, Beck M, Sunder-Plassmann G, editors. Fabry Disease: Perspectives from 5 Years of FOS. Oxford: Oxford PharmaGenesis; 2006. Chapter 10.
10. Fratantoni JC, Hall CW, Neufeld EF. Hurler and Hunter syndromes: mutual correction of the defect in cultured fibroblasts. Science. 1968;162:570–572.
11. Biffi A. Gene therapy for lysosomal storage disorders: a good start. Hum Mol Genet. 2016;25:R65–R75.
12. Concolino D, Deodato F, Parini R. Enzyme replacement therapy: efficacy and limitations. Ital J Pediatr. 2018;44. DOI:10.1186/s13052-018-0562-1
13. Muenzer J. Early initiation of enzyme replacement therapy for the mucopolysaccharidoses. Mol Genet Metab. 2014;111:63–72.
14. Muenzer J, Wraith JE, Clarke LA, et al. Management and treatment guidelines. Pediatrics. 2009;123:19–29.
15. Wolf, Daniel Adam. (2010). Molecular therapy for mucopolysaccharidosis Type I. Retrieved from the University of Minnesota Digital Conservancy, http://hdl.handle.net/11299/144098.
16. Holley RJ, Wood SR, Bigger BW. Delivering hematopoietic stem cell gene therapy treatments for neurological lysosomal diseases. ACS Chem Neurosci. 2019;10:18–20.
17. Macpherson JL, Rasko JEJ. Clinical potential of gene therapy: towards meeting the demand. Intern Med J. 2014;44:224–233.
18. Ørstavik KH, Apold J. Gene therapy for monogenic inherited diseases. Tidsskr Nor Laegeforen. 2001 Jan 30;121(3):349-350.
19. Milone MC, O'Doherty U. Clinical use of lentiviral vectors. Leukemia. 2018;32:1529–1541.
20. Anson DS. The use of retroviral vectors for gene therapy-what are the risks? A review of retroviral pathogenesis and its relevance to retroviral vector-mediated gene delivery. Genet Vaccines Ther. 2004;2:9.

21. Jager L, Ehrhardt A. Emerging adenoviral vectors for stable correction of genetic disorders. Curr Gene Ther. 2007. DOI:10.2174/156652307781369074
22. Büning H, Srivastava A. Capsid modifications for targeting and improving the efficacy of AAV vectors. Mol Ther - Methods Clin Dev. 2019;12:248–265.
23. Choudary PV, Barranger JA, Tsuji S, et al. Retrovirus-mediated transfer of the human glucocerebrosidase gene to Gaucher fibroblasts. Mol Biol Med. 1986 Jun;3(3):293-299.
24. Sorge J, Kuhl W, West C, et al. Complete correction of the enzymatic defect of type I Gaucher disease fibroblasts by retroviral-mediated gene transfer. Proc Natl Acad Sci. 1987;84:906–909.
25. Wolfe JH, Schuchman EH, Stramm LE, et al. Restoration of normal lysosomal function in mucopolysaccharidosis type VII cells by retroviral vector-mediated gene transfer. Proc Natl Acad Sci. 1990;87:2877–2881.
26. Anson DS, Bielicki J, Hopwood JJ. Correction of mucopolysaccharidosis type I fibroblasts by retroviral-mediated transfer of the human alpha-L-iduronidase gene. Hum Gene Ther. 1992;3(4):371–379.
27. Fairbairn LJ, Lashford LS, Spooncer E, et al. Long-term in vitro correction of alpha-L-iduronidase deficiency (Hurler syndrome) in human bone marrow. Proc Natl Acad Sci U S A. 1996 Mar 5;93(5):2025-2030.
28. Fairbairn LJ, Lashford LS, Spooncer E, et al. Towards gene therapy of Hurler syndrome. Cas Lek Cesk. 1997 Jan 8;136(1):27-31.
29. Huang MM, Wong A, Yu X, et al. Retrovirus-mediated transfer of the human α-L-iduronidase cDNA into human hematopoietic progenitor cells leads to correction in trans of Hurler fibroblasts. Gene Ther. 1997;4:1150–1159.
30. Baxter MA, Wynn RF, Deakin JA, et al. Retrovirally mediated correction of bone marrow-derived mesenchymal stem cells from patients with mucopolysaccharidosis type I. Blood. 2002;99:1857–1859.
31. Clarke LA, Russell CS, Pownall S, et al. Murine mucopolysaccharidosis type I: targeted disruption of the murine α-L-iduronidase gene. Hum Mol Genet. 1997;6:503–511.
32. Haskins ME, Jezyk PF, Desnick RJ, et al. Morphologic and biochemical studies of canine mucopolysaccharidosis I. Am J Pathol. 1984 Mar;114(3):487-495.
33. Haskins ME, Aguirre GD, Jezyk PF, et al. The pathology of the feline model of mucopolysaccharidosis I. Am J Pathol. 1983;112(1):27–36.
34. Lutzko C, Kruth S, Abrams-Ogg AC, et al. Genetically corrected autologous stem cells engraft, but host immune responses limit their utility in canine alpha-L-iduronidase deficiency. Blood. 1999 Mar 15;93(6):1895-1905.
35. Lutzko C, Omori F, Abrams-Ogg ACG, et al. Gene therapy for canine alpha-l-iduronidase deficiency: in utero adoptive transfer of genetically corrected hematopoietic progenitors results in engraftment but not amelioration of disease. Hum Gene Ther. 2002. DOI:10.1089/10430349950017851
36. Oleg E. Tolmachov, Tatiana Subkhankulova and Tanya Tolmachova (February 27th 2013). Silencing of Transgene Expression: A Gene Therapy Perspective, Gene Therapy - Tools and Potential Applications, Francisco Martin Molina, IntechOpen, DOI:10.5772/53379. Available from: https://www.intechopen.com/books/gene-therapy-tools-and-potential-applications/silencing-of-transgene-expression-a-gene-therapy-perspective
37. Meertens L, Kohn D, Kruth S, et al. In utero injection of α-L-iduronidase-carrying retrovirus in canine mucopolysaccharidosis type I: infection of multiple tissues and neonatal gene expression. Hum Gene Ther. 2002;13:1809–1820.
38. Traas AM, Wang P, Ma X, et al. Correction of clinical manifestations of canine mucopolysaccharidosis I with neonatal retroviral vector gene therapy. Mol Ther. 2007;15:1423–1431.
39. Liu Y, Xu L, Hennig AK, et al. Liver-directed neonatal gene therapy prevents cardiac, bone, ear, and eye disease in mucopolysaccharidosis I mice. Mol Ther. 2005;11:35–47.
40. Ma X, Liu Y, Tittiger M, et al. Improvements in mucopolysaccharidosis I mice after adult retroviral vector-mediated gene therapy with immunomodulation. Mol Ther. 2007;15:889–902.
41. Herati RS, Ma X, Tittiger M, et al. Improved retroviral vector design results in sustained expression after adult gene therapy in mucopolysaccharidosis I mice. J Gene Med. 2008. DOI:10.1002/jgm.1229
42. Metcalf JA, Ma X, Linders B, et al. A self-inactivating γ-retroviral vector reduces manifestations of mucopolysaccharidosis i in mice. Mol Ther. 2010;18:334–342.
43. Chung S, Ma X, Liu Y, et al. Effect of neonatal administration of a retroviral vector expressing α-l-iduronidase upon lysosomal storage in brain and other organs in mucopolysaccharidosis I mice. Mol Genet Metab. 2007;90:181–192.
44. Wang D, Zhang W, Kalfa TA, et al. Reprogramming erythroid cells for lysosomal enzyme production leads to visceral and CNS cross-correction in mice with Hurler syndrome. Proc Natl Acad Sci. 2009;106:19958–19963.
45. Baldo G, Wozniak DF, Ohlemiller KK, et al. Retroviral-vector-mediated gene therapy to mucopolysaccharidosis i mice improves sensorimotor impairments and other behavioral deficits. J Inherit Metab Dis. 2013;36:499–512.
46. Di Natale P, Di Domenico C, Villani GRD, et al. In vitro gene therapy of mucopolysaccharidosis type I by lentiviral vectors. Eur J Biochem. 2002;269:2764–2771.
47. Domenico CD, Villani GRD, D Di N, et al. Gene therapy for a mucopolysaccharidosis type I Murine model with lentiviral-IDUA vector. Hum Gene Ther. 2005;16:81–90.
48. Kobayashi H, Carbonaro D, Pepper K, et al. Neonatal gene therapy of MPS I mice by intravenous injection of a lentiviral vector. Mol Ther. 2005;11:776–789.
49. El-Amouri SS, Dai M, Han JF, et al. Normalization and improvement of CNS deficits in mice with hurler syndrome after long-term peripheral delivery of BBB-targeted iduronidase. Mol Ther. 2014;22:2028–2037.
50. Visigalli I, Delai S, Politi LS, et al. Gene therapy augments the efficacy of hematopoietic cell transplantation and fully corrects mucopolysaccharidosis type I phenotype in the mouse model. Blood. 2010;116:5130–5139.
    - **In this study the authors show that gene therapy in hematopoietic stem cells improves results compared to traditional bone marrow transplant.**
51. Visigalli I, Delai S, Ferro F, et al. Preclinical testing of the safety and tolerability of lentiviral vector–mediated above-normal alpha-L-iduronidase expression in murine and human hematopoietic cells using toxicology and biodistribution good laboratory practice studies. Hum Gene Ther. 2016;27:813–829.
52. Breyer B, Jiang W, Cheng H, et al. Adenoviral vector-mediated gene transfer for human gene therapy. Curr Gene Ther. 2006. DOI:10.2174/1566523013348689
53. Watson G, Bastacky J, Belichenko P, et al. Intrathecal administration of AAV vectors for the treatment of lysosomal storage in the brains of MPS I mice. Gene Ther. 2006;13(11):917–925.
54. Janson CG, Romanova LG, Leone P, et al. Comparison of endovascular and intraventricular gene therapy with adeno-associated virus-α-L-iduronidase for hurler disease. Neurosurgery. 2014;74:99–111.
55. Wolf DA, Lenander AW, Nan Z, et al. Direct gene transfer to the CNS prevents emergence of neurologic disease in a murine model of mucopolysaccharidosis type I. Neurobiol Dis. 2011;43:123–133.
56. Hinderer C, Bell P, Gurda BL, et al. Intrathecal gene therapy corrects cns pathology in a feline model of mucopolysaccharidosis i. Mol Ther. 2014;22:2018–2027.
57. Hinderer C, Bell P, Louboutin JP, et al. Efficacy of intrathecal AAV9 delivery in canine MPS I and impact of neonatal tolerance induction to the transgene product. Hum Gene Ther. 2015:A64-A64.
58. Ciron C, Cressant A, Roux F, et al. Human α-iduronidase gene transfer mediated by adeno-associated virus types 1, 2, and 5 in the brain of nonhuman primates: vector diffusion and biodistribution. Hum Gene Ther. 2009. DOI:10.1089/hum.2008.155
    - **This manuscript shows the biodistribution of AAVs in monkeys.**
59. Belur LR, Temme A, Podetz-Pedersen KM, et al. Intranasal adeno-associated virus mediated gene delivery and expression of human iduronidase in the central nervous system: a noninvasive

and effective approach for prevention of neurologic disease in mucopolysaccharidosis type I. Hum Gene Ther. 2017;28:576–587.
60. B Hackett P, L Aronovich E, Hunter D, et al. Efficacy and safety of sleeping beauty transposon-mediated gene transfer in preclinical animal studies. Curr Gene Ther. 2012. DOI:10.2174/156652311797415827
61. Ivics Z, Kaufman CD, Zayed H, et al. The sleeping beauty transposable element: evolution, regulation and genetic applications. Curr Issues Mol Biol. 2004 Jan;6(1):43-55.
62. Aronovich EL, McIvor RS, Hackett PB. The sleeping beauty transposon system: A non-viral vector for gene therapy. Hum Mol Genet. 2011;20:R14–R20.
63. Narayanavari SA, Izsvák Z. Sleeping beauty transposon vectors for therapeutic applications: advances and challenges. Cell Gene Ther Insights. 2017;3:131–158.
64. Aronovich EL, Bell JB, Belur LR, et al. Prolonged expression of a lysosomal enzyme in mouse liver after sleeping beauty transposon-mediated gene delivery: implications for non-viral gene therapy of mucopolysaccharidoses. J Gene Med. 2007;9:403–415.
65. Aronovich EL, Bell JB, Khan SA, et al. Systemic correction of storage disease in MPS I NOD/SCID mice using the sleeping beauty transposon system. Mol Ther. 2009;17:1136–1144.
66. Matsumoto Martin PK, Stilhano RS, Samoto VY, et al. Mesenchymal stem cells do not prevent antibody responses against human α-L-iduronidase when used to treat mucopolysaccharidosis type i. PLoS One. 2014. DOI:10.1371/journal.pone.0092420
67. Tipanee J, Chai YC, VandenDriessche T, et al. Preclinical and clinical advances in transposon-based gene therapy. Biosci Rep. 2017;37. DOI:10.1042/bsr20160614
68. Stilhano RS, Martin PKM, de Melo SM, et al. α- l-iduronidase gene-based therapy using the phiC31 system to treat mucopolysaccharidose type I mice. J Gene Med. 2015;17:1–13.
69. Osborn MJ, McElmurry RT, Lees CJ, et al. Minicircle DNA-based gene therapy coupled with immune modulation permits long-term expression of α-L-iduronidase in mice with mucopolysaccharidosis type i. Mol Ther. 2011;19:450–460.
70. Gaj T, Sirk SJ, Shui SL, et al. Genome-editing technologies: principles and applications. Cold Spring Harb Perspect Biol. 2016;8:a023754.
71. Gupta SK, Shukla P. Gene editing for cell engineering: trends and applications. Crit Rev Biotechnol. 2017;37:672–684.
72. Carroll D. Genome engineering with zinc-finger nucleases. Genetics. 2011;188:773–782.
73. Bedell VM, Wang Y, Campbell JM, et al. In vivo genome editing using a high-efficiency TALEN system. Nature. 2012;491:114–118.
74. Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. Cell. 2014;157:1262–1278.
75. Tschaharganeh DF, Lowe SW, Garippa RJ, et al. Using CRISPR/Cas to study gene function and model disease in vivo. Febs J. 2016;22:3194–3203.
76. Lin J, Musunuru K. Genome engineering tools for building cellular models of disease. FEBS J. 2016;283:3222–3231.
77. Cox DBT, Platt RJ, Zhang F. Therapeutic genome editing: prospects and challenges. Nat Med. 2015;21:121–131.
78. Xu CL, Ruan MZC, Mahajan VB, et al. Viral delivery systems for crispr. Viruses. 2019;11:28.
79. Ain QU, Chung JY, Kim YH. Current and future delivery systems for engineered nucleases: ZFN, TALEN and RGEN. J Control Release. 2015;205:120–127.
80. Hoshijima K, Jurynec MJ, Grunwald DJ. Precise genome editing by homologous recombination. Methods Cell Biol. 2016. DOI:10.1016/bs.mcb.2016.04.008
81. Wechsler T, DeKelver R, Rohde M, et al. ZFN-mediated in vivo genome editing results in supraphysiological levels of lysosomal enzymes deficient in hunter and hurler syndrome and gaucher disease. Mol Ther. 2015;23:S190.
82. Sharma R, Anguela XM, Doyon Y, et al. In vivo genome editing of the albumin locus as a platform for protein replacement therapy. Blood. 2015;126:1777–1784.
•• **This study shows the principle of gene editing in a safe harbour locus.**
83. Ou L, DeKelver RC, Rohde M, et al. ZFN-mediated in vivo genome editing corrects Murine Hurler syndrome. Mol Ther. 2019;27:178–187.
84. Sadelain M, Papapetrou EP, Bushman FD. Safe harbours for the integration of new DNA in the human genome. Nat Rev Cancer. 2012;12:51–58.
85. Ou L, DeKelver R, Tom S, et al. ZFN-mediated correction of murine MPS I model by expression of the human IDUA cDNA from the albumin "safe harbor" locus. Mol Genet Metab. 2016;117:S89.
86. Pan, D. Preclinical studies of hematopoietic cell gene therapy for patients with mucopolysaccharidosis type I and type II by retroviral vectors. Ph.D. thesis, University of Minnesota; 1997.
87. Gomez-Ospina N, Scharenberg SG, Mostrel N, et al. Human genome-edited hematopoietic stem cells phenotypically correct mucopolysaccharidosis type I. Nat Commun. 2019 Sep 6;10(1):4045.
• **First description of an ex vivo gene editing in MPS.**
88. Campbell LA, Richie CT, Maggirwar NS, et al. Cas9 ribonucleoprotein complex delivery: methods and applications for neuroinflammation. J Neuroimmune Pharmacol. 2019;14:565–577.
89. Ciron C, Cressant A, Roux F, et al. AAV1-, AAV2- and AAV5-mediated human α-iduronidase gene transfer in the brain of nonhuman primate: vector diffusion and bio distribution. Hum Gene Ther. 2009. DOI:10.1089/hgt.2008.155
90. Rai R, Alwani S, Badea I. Polymeric nanoparticles in gene therapy: new avenues of design and optimization for delivery applications. Polymers (Basel). 2019;11:745.
91. Schuh RS, de Carvalho TG, Giugliani R, et al. Gene editing of MPS I human fibroblasts by co-delivery of a CRISPR/Cas9 plasmid and a donor oligonucleotide using nanoemulsions as nonviral carriers. Eur J Pharm Biopharm. 2018;122:158–166.
92. Schuh RS, Poletto E, Pasqualim G, et al. In vivo genome editing of mucopolysaccharidosis I mice using the CRISPR/Cas9 system. J Control Release. 2018;288:23–33.
• **First description of an in vivo gene editing in MPS I mice.**
93. Harmatz P, Lau HA, Heldermon C, et al. EMPOWERS: A phase 1/2 clinical trial of SB-318 ZFN-mediated in vivo human genome editing for treatment of MPS I (Hurler syndrome). Mol Genet Metab. 2019;126:S68.
94. Sheridan C. Sangamo's landmark genome editing trial gets mixed reception. Nat Biotechnol. 2018;36:907–908.

4.2 Artigo II: Brain and visceral gene editing of mucopolysaccharidosis I mice by nasal delivery of the CRISPR/Cas9 system.

Artigo submetido para a revista “The Journal of Gene Medicine”

4.3 Artigo III: Short and long term nasal administrations of liposomal/CRISPR-cas9 complex in MPS II mice.

Artigo para submissão ao Molecular Genetics and Metabolism Reports

5. DISCUSSÃO

As mucopolissacaridoses I e II são doenças monogênicas causadas por variantes patogênicas nos genes das enzimas IDUA e IDS, respectivamente. A ausência ou mal funcionamento de estas enzimas leva a um acúmulo progressivo de componentes da matriz extracelular chamados GAGs nos lisossomos, afetando consequentemente diferentes sistemas (Muenzer 2011). Embora hoje existam disponíveis alguns tratamentos para os pacientes, estes possuem diversas limitações (Marques and Saftig 2019). Na procura de novas alternativas, a terapia gênica aparece como uma metodologia potencialmente mais eficaz e com resultados que perduram ao longo do tempo (Schlander and Beck 2009; Brennan and Wilson 2014).

Dentro da vasta área da terapia gênica, a edição genômica é uma abordagem que visa a entrega de um material gênico exógeno na célula, ao mesmo tempo que integra este material em uma região específica do genoma (Ho et al. 2018). O grande desafio destas terapias reside na possibilidade de entregar esse material a um grande número de células e tecidos. Por isso, diversas estratégias de entrega como o uso de vetores virais e não virais tem sido estudadas(Ates et al. 2020). Os lipossomos são os vetores não-virais mais utilizados para a entrega de diferentes biomoléculas no organismo, devido a sua biocompatibilidade com as membranas biológicas. Eles diminuem o risco de citotoxicidade, possuem capacidade de proteção do DNA, são de fácil manipulação e baixo custo de manufatura (Elsana et al. 2019).

A linha de pesquisa de nosso laboratório tem se focado no desenvolvimento e estudo de possíveis terapias gênicas alternativas baseadas em vetores não-virais para o tratamento de doenças lisossômicas (Bidone et al. 2018; Schuh et al. 2018b). Mais recentemente, os nossos estudos têm utilizado o sistema CRISPR-Cas9 como ferramenta de edição genômica, buscando aumentar a eficiência do sistema. A proposta desta tese de doutorado parte de trabalhos prévios desenvolvidos pelo nosso grupo, onde foi possível comprovar o potencial dos vetores não-virais tipo lipossomos em complexar e entregar o sistema CRISPR-Cas9 em vários tecidos após de uma injeção hidrodinâmica em animais MPS I neonatos e induzir modificações metabólicas y fenotípicas relevantes na doença (Schuh et al. 2018b, 2020). A

maior limitação destes trabalhos foi a impossibilidade do sistema proposto atingir o cérebro, o qual é um dos tecidos maios afetados em pacientes MPS I e MPS II com fenótipo grave. Assim, nesta tese se propôs uma abordagem não-invasiva que permitisse a entrega do sistema CRISPR-Cas9 ao cérebro.

Visando fornecer uma alternativa para os pacientes MPSs com compromisso neurológico, a administração intranasal pressupõe uma terapia mais segura, uma vez que as terapias experimentais atuais direcionadas ao cérebro se baseiam na administração localizada de vetores virais por médio de injeções intracerebrais ou via líquido cefalorraquidiano (injeções intratecais / intraventriculares) (Fraldi et al. 2018).

Na primeira etapa do trabalho prático foi usado como modelo de estudo o camundongo knockout para o gene da IDUA. Aqui se analisou a produção e distribuição da enzima IDUA nos diferentes tecidos após a última administração intranasal dos complexos lipossomo/DNA utilizados previamente (plasmídeo CRISPR-Cas9/RNAg + plasmídeo doador IDUA) durante 30 dias consecutivos. Justificamos o uso do mesmo sistema plasmidial e sitio alvo de corte (ROSA26) considerando os resultados positivos observados em animais que receberam injeção hidrodinâmica, atingindo uma produção da enzima IDUA em torno do 2-10 % do valor normal em alguns tecidos editados (Schuh et al. 2018b). As 30 doses utilizadas no estudo piloto e no estudo de longo prazo foram propostas baseando-nos em uma limitação de carga do lipossomo e uma limitação correspondente ao volume máximo que pode reter a cavidade nasal. Em humanos, o volume máximo de líquido retido é de aproximadamente 200 µl e para camundongos é de 5µl por fossa nasal (Hong et al. 2019). Devido ao fato de que a capacidade de complexação do lipossomo é limitada, e existe uma quantia específica de µg de DNA que podem ser carregados em 1000 µl de nossos lipossomos, são necessárias múltiplas doses do complexo para poder entregar uma quantidade de DNA suficiente ao organismo, e assim poder garantir um bom funcionamento do sistema de edição. Enquanto uma dose de 120 µl de complexo (volume administrado em um dia) carrega 43ug de DNA (plasmídeo CRISPR-Cas9/RNAg + plasmídeo doador IDUA; 1:1), após 30 doses são administrados 1400ug de DNA.

No estudo piloto demostrou-se, por meio da dosagem de atividade enzimática, a presença da enzima IDUA nos diferentes tecidos avaliados após 30 administrações dos

complexos. Estes resultados serviram como base para determinar o possível alcance e distribuição dos complexos no animal fazendo uso desta via de administração.

A atividade enzimática da IDUA foi avaliada em tecidos viscerais e diferentes regiões do cérebro logo depois da última administração no dia 30. Partindo do fato de nosso sistema ser composto por dois plasmídeos, e um deles ser um plasmídeo doador, com um cassete de expressão independente para o gene da IDUA, ter detectado atividade enzimática em todas as regiões avaliadas pode ter sido efeito da expressão episssomal do plasmídeo doador na circulação e nos tecidos que foi entregue. Durante a constante administração dos plasmídeos (30 dias no caso) a produção da enzima pode ter sido mantida devido ao promotor constitutivo EF1-alfa usado no cassette. Este promotor promove uma expressão forte em diferentes tipos célulare de camundongos, e sua regulação não está limitada a tecidos específicos (Chung et al. 2002). Portanto, uma vez que o plasmídeo esteja dentro do organismo, a maquinaria necessária para sua regulação se encontra disponível e consequentemente a expressão dos genes regulados por ele é ativada. A vida média de um DNA plasmidial em circulação pode variar de horas a umas poucas semanas, dependendo do organismo, até ser degradado por DNases, ou absorvido pelo fígado (Liu et al. 2007; Woo et al. 2011). Junto com a proteção que outorga o lipossoma peguilado (PEG) ao DNA (Gabizon et al. 2003; Khargharia et al. 2014), é possível estender sua vida média por umas semanas mais. Assim, a produção constante da enzima IDUA por parte do plasmídeo doador pode ter garantido uma expressão da enzima por alguns dias.

Com isto tudo, a segunda fase do trabalho em MPS I avaliou a real ocorrência da edição genômica nos diferentes tecidos, meses após a última administração do vetor. Desta forma, garante-se um tempo suficiente para que o vetor doador seja degradado. Neste estudo, procedeu-se a comprovar os resultados anteriores em animais adultos (6 meses de idade) que receberam 30 doses dos complexos em seus primeiros meses de vida. Além do modelo knockout da MPS I também foi usando o camundongo knockout da MPS II. Para este último, o plasmídeo doador utilizado foi o mesmo, mudando unicamente o gene da IDUA pelo gene da IDS.

Após seis meses de idade realizou-se a eutanásia dos animais, e foram observados aumentos discretos em atividade enzimática em menos regiões, comparado com o estudo

piloto. Na região do cérebro houve aumento da IDUA no bulbo olfatório, córtex frontal e total, e dos órgãos viscerais no pulmão e coração. A quantificação da porcentagem de correção pela presença de DNA exógeno mostrou que além do coração e pulmão, só o bulbo olfatório sofreu edição genômica. Estes dados corroboram os resultados da biodistribuição, pois foram os mesmos órgãos que tiveram evidenciada a captura dos complexos marcados com sonda fluorescente. Isso sugere que os resultados obtidos no estudo piloto foram causados pela quantidade elevada de proteína produzida durante o tempo da administração, e que esta conseguiu se difundir e ser capturada pelos diferentes tecidos. Esta difusão e distribuição tem sido observada em plasmídeos e proteínas expressas a partir deles após 24 ou 48 horas da administração intranasal (Oh et al. 2001; Harmon et al. 2014; Schuh et al. 2018a) .

Também foram investigadas outras mudanças metabólicas e fenotípicas associadas à eficácia da edição. Os níveis de GAGs em soro, urina e tecidos, apesar da baixa porcentagem de correção e dos níveis de enzima, reduziram seus valores, incluindo em tecidos que não apresentaram edição, os quais provavelmente foram beneficiados pelo fenômeno de correção cruzada. No entanto, apesar da redução, não houve normalização dos GAGs.

Surpreendentemente, os animais tratados melhoraram certos parâmetros motores e de memória, embora tenha existido certa variação entre os animas de cada grupo, especialmente os grupos tratados. Cabe ressaltar que um grupo de animais recebeu apenas o plasmídeo doador como tratamento, e estes animais não tiveram nem aumento significativo da enzima aos 6 meses e tampouco melhora neurológica.

A diferença entre os tecidos que sofreram edição após a administração nasal e hidrodinâmica (Schuh et al. 2018b) reside possivelmente no diferente perfil de biodistribuição dos complexos após cada tratamento. A transferência gênica por meio da injeção hidrodinâmica faz com que pressão gerada nos capilares aumente a permeabilidade das células endoteliais e parenquimatosas ao longo do sistema circulatório, possibilitando a difusão do vetor em vários tecidos (Suda and Liu 2007). Entretanto, a via nasal encontra-se ligada diretamente às vias respiratórias inferiores e estas ao sistema cardíaco, portanto, não era esperado encontrar uma maior distribuição dos complexos administrados usando esta via de administração. A porcentagem relativamente baixa da correção obtida em estes tecidos foi

provavelmente o resultado de fatores inerentes ao sistema de edição utilizado. Para que a edição genômica ocorra é preciso induzir a quebra da fita dupla no local alvo por meio do complexo gerado pela enzima Cas9 e o RNA guia. O sistema plasmidial tende a ser menos eficiente comparado com outros formatos do CRISPR utilizados, pois o plasmídeo precisa transpassar membranas e entrar ao núcleo para que as sequências de DNA da Cas9 e o RNA guia consigam ser transcritas e posteriormente aconteça a clivagem (Li et al. 2018). Este aspecto diminui parcialmente a eficiência da edição e também induz o atraso na eficácia terapêutica.

Embora as administrações tenham começado desde uma idade precoce, a quantidade de células em divisão celular comparado com um camundongo neonato é diferente. Assim, o mecanismo de reparo por homologia pode ocorrer mais frequentemente em neonatos que em animais adultos jovens, pois este mecanismo é inerente a células em divisão. Isto pode explicar também a diferença de porcentagem de edição entre os animais que receberam injeção hidrodinâmica (neonatos) (Schuh et al. 2018b) com os que receberam administração intranasal (do primeiro ao segundo mês).

Ema relação à região olfatória, tem sido descrito que a neurogênese persiste em duas regiões do cérebro em mamíferos adultos: o hipocampo e o bulbo olfatório. A maioria dos neurônios granulares do bulbo olfatório (estes compreendem a maior população de neurônios no bulbo e se encontram distribuídos entre as camadas externa, medial e mais profunda, (Takahashi et al. 2018)) são gerados após o nascimento e continuam a ser adicionados na idade adulta (Petreanu and Alvarez-Buylla 2002). Ao mesmo tempo, para regular a neurogênese, durante o período neonatal e até em adultos também a morte celular e renovação são constantes (Lemasson et al. 2005). Tendo isso em conta, é possível assumir que uma grande proporção das células que sofreram edição gênica no bulbo olfatório durante o tratamento pode ter sofrido apoptose antes dos 6 meses de idade. O oposto acontece com os tipos celulares progenitores que formam o tecido cardíaco e pulmonar. A proporção da divisão celular e crescimento é limitada aos primeiros meses pós-nascimento e vai diminuindo drasticamente até chegar à idade adulta, onde estas células permanecem em estado quiescente e são induzidas a regeneração/proliferação unicamente em função de uma lesão (Amy et al. 1977; Senyo et al. 2013; Ali et al. 2014; Kotton and Morrisey 2014).

Partindo dos resultados anteriores, decidiu-se prolongar as administrações por 60 dias. O raciocínio para aumentar o número de administrações foi justamente melhorar a porcentagem de células editadas em os tecidos-alvo usando a mesma estratégia proposta. Assim, administrar por mais um mês o sistema (tempo onde ainda a divisão celular está ocorrendo, mesmo em uma taxa menor) poderia favorecer o processo cumulativo da edição – quanto mais tempo à célula está em contato com o sistema de edição, a probabilidade de que edição ocorra é maior.

Com relação aos nanocompostos, tem sido demonstrado que partículas lipídicas de até 100nm tem uma residência prolongada na cavidade nasal e são mais facilmente transportadas até o bulbo pelos nervos olfatórios e trigeminais, e moléculas maiores que essas tendem a serem depuradas mais facilmente (Bonferoni et al. 2019). Tendo-se em conta que o tamanho do nosso complexo é de 112 ± 11 nm, aumentando o número de administrações pode-se suprir o efeito negativo da possível depuração. Por último, como a administração intranasal é um método não-invasivo, pensando em extrapolar a proposta para os pacientes, 60 administrações seriam ainda plausíveis.

Esta última fase do trabalho foi realizada usando como modelo o camundongo MPS II. Foram administradas 60 doses dos complexos durante os primeiros meses de vida (até 3 meses de idade) e os animais foram acompanhados até os 6 meses de idade. Após eutanásia foram avaliados novamente os mesmos parâmetros metabólicos e fenotípicos realizados previamente. Os resultados obtidos mostraram que após 60 dias de administração não houve incremento na percentagem de correção nos tecidos alvo, comparado ao grupo tratado por 30 dias. Estes resultados sugeriram que a percentagem de correção foi similar à de 30 administrações, contrariando nossa hipótese. Com relação às mudanças metabólicas e fenotípicas, também não existiu uma diferença significativa nos níveis de atividade enzimática ou GAGs quantificados em tecidos e fluidos que denotasse uma melhora ou piora após 60 doses, comparado às 30 doses.

Os resultados obtidos nesta última fase são importantes para ponderar a eficácia da abordagem proposta. Após 30 administrações as mudanças induzidas nos níveis de enzima foram poucas e após 60 não se conseguiu melhorá-las. Assim, a nossa abordagem mostrou não ser completamente eficiente comparada com outras abordagens experimentais existentes

(Belur et al. 2017, 2021). Ainda assim, aquelas existentes têm como base o uso de vetores virais, os quais embora muito mais eficazes, também trazem consigo barreiras, que podem eventualmente vir a ser superadas com o desenvolvimento de novos vetores de entrega. Nossa proposta foi uma prova de conceito que demostrou ter espaço para otimização, pois a baixa eficiência observada pode ser o resultado de várias limitações presentes em nosso desenho experimental, desde a escolha do formato do sistema CRISPR-Ca9 até as possíveis respostas imunes aos diversos componentes, que fez com que a administração prolongada dos complexos não tivesse melhores resultados. Como mencionamos anteriormente, o formato plasmidial não é o mais eficiente para a edição genica in vivo, pois além de ter que superar mais barreiras biológicas do que os outros formatos, a produção constante da Cas9 e do RNA guia proporcionada pelo plasmídeo é propensa à ativação de respostas imunes contra o material exógeno (Wilbie et al. 2019) diminuindo a eficácia do sistema. Assim, por exemplo, a entrega limitada da Cas9 (como proteína) e o RNA guia pode prevenir estes efeitos, pois não permanecem muito tempo na célula.

O sistema encontra-se desenhado para gerar um reparo por homologia direta. O uso de uma estratégia que não utilize este tipo de reparo pode ser uma alternativa interessante para melhorar a eficiência da edição. Tem sido reportada a possibilidade de induzir correção em células em estado pós-mitótico mediante a integração direcionada independente de homologia (HITI, homology-independent targeted integration) (Suzuki et al. 2016; Bloomer et al. 2021). Aqui o DNA doador não precisa de braços de homologia, mas da mesma sequência alvo de reconhecimento da Cas9 para ser clivado, linearizado e consequentemente integrado no local que sofreu quebra de fita dupla, incrementando assim a eficiência de correção em células em estado mitótico e pós-mitótico. Este DNA doador pode ser fornecido por meio de plasmídeos tipo minicírculos. Como vantagens, este também diminui a resposta imune pois não possui arcabouço bacteriano. Logo, também é possível diminuir o tamanho da partícula do complexo lipossomal e assim aumentar seu transporte pelos nervos olfatórios e trigeminais até o bulbo olfatório.

Finalmente, o lipossoma também pode gerar respostas imunes e causar citotoxicidade. Partindo do fato do nosso protocolo implicar a administração consecutiva dos complexos durante um longo período de tempo, é possível ter ativado diversas respostas que não só

podem ser prejudiciais para o organismo, mas contribuem para a perda da eficácia da terapia. Um dos componentes especiais destas formulações é o PEG. Assim como ele aumenta a vida média do lipossoma, o protegendo da degradação rápida no sangue, também é possível que, após injeções repetidas de lipossomas peguilados, eles percam essa capacidade e sejam eliminados do sangue mais rápido devido a produção e secreção abundante de IgM, IgE e IgG anti-PEG (Inglut et al. 2020). O PEG não só funciona como protetor do lipossomo, mas também como agente de mucoadesão e mucopenetração (Bandi et al. 2021). Por isso, dispensá-lo da formulação seria uma opção, contanto que seja substituído por outros agentes que promovam a eficácia de entrega do lipossomo. Um exemplo seria o uso do sulfato de protamina na formulação, um policátion natural não tóxico que além de ser um agente condensador do DNA, pode fornecer atividades únicas de translocação de membrana e localização nuclear devido à sua abundante sequência de aminoácidos (Tao et al. 2016). Assim, melhorando a eficiência de entrega do lipossomo, poderia se reduzir também número de administrações ou prolongar o espaço entre elas.

Infelizmente, não foi possível avaliar a ativação destas prováveis respostas imunes geradas por nosso lipossoma e pelos plasmídeos utilizados. As interleucinas avaliadas neste trabalho foram selecionadas justamente por estarem associadas à doença. Porém, para estudos futuros é imperativo investigar em tempo real o perfil imunogênico dos componentes do sistema utilizado. Com todo o mencionado, é possível afirmar que uma otimização no sistema é necessária para melhorar a eficácia e eficiência em a entrega e edição usando a via nasal como via de administração.

Um dos grandes objetivos do uso da edição genômica como terapia é desenhar uma estratégia que permita e entrega intracelular e funcionamento eficiente do sistema na clínica (Mashel et al. 2020). Nesta tese se apresentou uma abordagem alternativa que servirá como base para o a melhoramento e desenvolvimento de vetores não-virais adequados para a entrega tanto do sistema CRISPR-Cas9 como outros sistemas de edição genômica, visando a procura de um tratamento para o comprometimento neurológico e sistêmico nas MPS. Sendo assim, um aporte importante para nossa linha de pesquisa.

6. CONCLUSÕES

-A principal conclusão deste trabalho é que é possível utilizar lipossomas como vetores não virais para a entrega do sistema CRISPR-Cas9 usando a via nasal como alternativa para o tratamento da MPS I e MPS II. Os vetores foram produzidos, caracterizados e aplicados in vivo.

- A biodistribuição dos complexos lipossomais após administração nasal foi avaliada e demostrou-se que os tecidos atingidos por esta via de administração são majoritariamente o bulbo olfatório, coração e pulmão.

- A atividade enzimática tecidual, o acúmulo tecidual de GAGs e melhora do comprometimento neurológico após 30 administrações dos complexos em animais MPS I foram estudados. Demostrou-se a presença de células editadas e corrigidas em três tecidos diferentes, os quais mostraram um incremento na atividade enzimática da IDUA que levou a uma redução nos níveis de GAGs em tecidos e fluidos, como também uma melhora no desempenho motor dos camundongos.

- O efeito de administrações nasais cumulativas (30 ou 60 administrações) sobre os mesmos parâmetros do objetivo anterior foi comparado no modelo animal de MPS II. Não foi observado efeito cumulativo de 60 administrações comparado a 30 em nenhum dos parâmetros avaliados, sugerindo uma necessidade de otimização o sistema proposto.

- As respostas inflamatórias a longo prazo aos tratamentos foram avaliadas mediante a dosagem de duas interleucinas pro-inflamatórias (Il-6 E Il-1b) em soro. Foi demostrado que a inflamação inerente à doença foi persistente nos animais após os tratamentos, mas sem incremento significativo aos 6 meses de idade causados pelos mesmos. Não obstante, é necessário avaliar em tempo real se as respostas de estas e outros marcadores pro-inflamatórios são induzidos pelos tratamentos.

7. REFERÊNCIAS

Ali SR, Hippenmeyer S, Saadat L V., et al (2014) Existing cardiomyocytes generate cardiomyocytes at a low rate after birth in mice. Proc Natl Acad Sci U S A. https://doi.org/10.1073/pnas.1408233111

Alroy J, Lyons JA (2014) Lysosomal Storage Diseases. J Inborn Errors Metab Screen 2:

Amy RWM, Bowes D, Burrit PH, et al (1977) Postnatal growth of the mouse lung

Anguela XM, High KA (2019) Entering the modern era of gene therapy. Annu. Rev. Med.

Ates I, Rathbone T, Stuart C, et al (2020) Delivery approaches for therapeutic genome editing and challenges. Genes (Basel).

Azambuja AS, Correa L, Gabiatti BP, et al (2018) Aversive and non-aversive memory impairment in the mucopolysaccharidosis II mouse model. Metab Brain Dis. https://doi.org/10.1007/s11011-017-0110-5

Baldo G, Giugliani R, Matte U (2014) Gene delivery strategies for the treatment of mucopolysaccharidoses. Expert Opin. Drug Deliv.

Baldo G, Mayer FQ, Martinelli BZ, et al (2013) Enzyme replacement therapy started at birth improves outcome in difficult-to-treat organs in mucopolysaccharidosis I mice. Mol Genet Metab. https://doi.org/10.1016/j.ymgme.2013.03.005

Baldo G, Quoos Mayer F, Burin M, et al (2012) Recombinant encapsulated cells overexpressing alpha-L-iduronidase correct enzyme deficiency in human mucopolysaccharidosis type i cells. Cells Tissues Organs. https://doi.org/10.1159/000327532

Bandi SP, Bhatnagar S, Venuganti VVK (2021) Advanced materials for drug delivery across mucosal barriers. Acta Biomater.

Barbi MDS, Carvalho FC, Kiill CP, et al (2015) Preparation and Characterization of Chitosan Nanoparticles for Zidovudine Nasal Delivery. J Nanosci Nanotechnol 15:865–874

Beck M (2018) Treatment strategies for lysosomal storage disorders. Dev Med Child Neurol 60:13–18. https://doi.org/10.1111/dmcn.13600

Belur L, Huber A, Mantone H, et al (2021) Comparative systemic and neurologic effectiveness of intravenous and intrathecal AAV9 delivered individually or combined in a murine model of mucopolysaccharidosis type I. Mol Genet Metab. https://doi.org/10.1016/j.ymgme.2020.12.025

Belur LR, Temme A, Podetz-Pedersen KM, et al (2017) Intranasal Adeno-Associated Virus Mediated Gene Delivery and Expression of Human Iduronidase in the Central Nervous System: A Noninvasive and Effective Approach for Prevention of Neurologic Disease in Mucopolysaccharidosis Type I. Hum Gene Ther. https://doi.org/10.1089/hum.2017.187

Bidone J, Schuh RS, Farinon M, et al (2018) Intra-articular nonviral gene therapy in mucopolysaccharidosis I mice. Int J Pharm. https://doi.org/10.1016/j.ijpharm.2018.06.049

Bigger BW, Begley DJ, Virgintino D, Pshezhetsky A V. (2018) Anatomical changes and pathophysiology of the brain in mucopolysaccharidosis disorders. Mol. Genet. Metab.

Bloomer H, Smith RH, Hakami W, Larochelle A (2021) Genome editing in human hematopoietic stem and progenitor cells via CRISPR-Cas9-mediated homology-independent targeted integration. Mol Ther. https://doi.org/10.1016/j.ymthe.2020.12.010

Bonferoni MC, Rossi S, Sandri G, et al (2019) Nanoemulsions for "nose-to-brain" drug delivery. Pharmaceutics. https://doi.org/10.3390/pharmaceutics11020084

Bouard D, Alazard-Dany N, Cosset FL (2009) Viral vectors: From virology to transgene expression. Br. J. Pharmacol.

Bourdenx M, Dutheil N, Bezard E, Dehay B (2014) Systemic gene delivery to the central nervous system using Adeno-associated virus. Front. Mol. Neurosci.

Bouxsein NF, McAllister CS, Ewert KK, et al (2007) Structure and gene silencing activities of monovalent and pentavalent cationic lipid vectors complexed with siRNA. Biochemistry. https://doi.org/10.1021/bi062138l

Braulke T, Bonifacino JS (2009) Sorting of lysosomal proteins. Biochim. Biophys. Acta - Mol. Cell Res.

Braunlin E, Miettunen K, Lund T, et al (2019) Hematopoietic cell transplantation for severe MPS I in the first six months of life: The heart of the matter. Mol Genet Metab. https://doi.org/10.1016/j.ymgme.2018.11.007

Brennan TA, Wilson JM (2014) The special case of gene therapy pricing. Nat Publ Gr. https://doi.org/10.1038/nbt.3003

Campos D, Monaga M, González EC, Herrera D (2014) Identification of mucopolysaccharidosis I heterozygotes based on biochemical characteristics of L-iduronidase from dried blood spots. Clin Chim Acta 430:24–27. https://doi.org/10.1016/j.cca.2013.12.035

Chen F, Vitry S, Hocquemiller M, et al (2006) α-L-iduronidase transport in neurites. Mol Genet Metab. https://doi.org/10.1016/j.ymgme.2005.11.017

Chen P, Kovesdi I, Bruder JT (2000) Effective repeat administration with adenovirus vectors to the muscle. Gene Ther. https://doi.org/10.1038/sj.gt.3301137

Chiesa R, Wynn RF, Veys P (2016) Haematopoietic stem cell transplantation in inborn errors of metabolism. Curr. Opin. Hematol.

Chung S, Andersson T, Sonntag K-C, et al (2002) Analysis of Different Promoter Systems for Efficient Transgene Expression in Mouse Embryonic Stem Cell Lines. Stem Cells. https://doi.org/10.1634/stemcells.20-2-139

Clarke LA (2011) Pathogenesis of skeletal and connective tissue involvement in the mucopolysaccharidoses: Glycosaminoglycan storage is merely the instigator. Rheumatology. https://doi.org/10.1093/rheumatology/ker395

Clarke LA, Giugliani R, Guffon N, et al (2019) Genotype-phenotype relationships in mucopolysaccharidosis type I (MPS I): Insights from the International MPS I Registry. Clin Genet. https://doi.org/10.1111/cge.13583

Concolino D, Deodato F, Parini R (2018) Enzyme replacement therapy: efficacy and limitations. Ital. J. Pediatr.

Cullen PJ, Steinberg F (2018) To degrade or not to degrade: mechanisms and significance of endocytic recycling. Nat Rev Mol Cell Biol 19:679–696. https://doi.org/10.1038/s41580-018-0053-7

D′avanzo F, Rigon L, Zanetti A, Tomanin R (2020) Mucopolysaccharidosis type II: One hundred years of research, diagnosis, and treatment. Int J Mol Sci 21:1258. https://doi.org/10.3390/ijms21041258

Den Tandt WR (1996) On the stability of human lysosomal enzymes at room temperature in normal and acidified plasma and serum. Clin Chim Acta. https://doi.org/10.1016/0009-8981(95)06204-1

Dhaliwal HK, Fan Y, Kim J, Amiji MM (2020) Intranasal Delivery and Transfection of mRNA Therapeutics in the Brain Using Cationic Liposomes. Mol Pharm. https://doi.org/10.1021/acs.molpharmaceut.0c00170

Doench JG, Fusi N, Sullender M, et al (2016) Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. Nat Biotechnol. https://doi.org/10.1038/nbt.3437

Drize N, Petinati N (2015) What do we know about the participation of hematopoietic stem cells in hematopoiesis? F1000Research

Du Z, Munye MM, Tagalakis AD, et al (2014) The Role of the helper lipid on the DNA transfection efficiency of lipopolyplex formulations. Sci Rep. https://doi.org/10.1038/srep07107

Eisengart JB, Rudser KD, Xue Y, et al (2018) Long-term outcomes of systemic therapies for Hurler syndrome: an international multicenter comparison. Genet Med. https://doi.org/10.1038/gim.2018.29

Elsana H, Olusanya TOB, Carr-wilkinson J, et al (2019) Evaluation of novel cationic gene based liposomes with cyclodextrin prepared by thin film hydration and microfluidic systems. Sci Rep. https://doi.org/10.1038/s41598-019-51065-4

Fecarotta S, Tarallo A, Damiano C, et al (2020) Pathogenesis of mucopolysaccharidoses, an update. Int. J. Mol. Sci.

Federhen A, Pasqualim G, de Freitas TF, et al (2020) Estimated birth prevalence of mucopolysaccharidoses in Brazil. Am J Med Genet Part A. https://doi.org/10.1002/ajmg.a.61456

Finn JD, Smith AR, Patel MC, et al (2018) A Single Administration of CRISPR/Cas9 Lipid Nanoparticles Achieves Robust and Persistent In Vivo Genome Editing. Cell Rep. https://doi.org/10.1016/j.celrep.2018.02.014

Foldvari M, Chen DW, Nafissi N, et al (2016) Non-viral gene therapy: Gains and challenges of non-invasive administration methods. J Control Release. https://doi.org/10.1016/j.jconrel.2015.12.012

Fraldi A, Serafini M, Sorrentino NC, et al (2018) Gene therapy for mucopolysaccharidoses: in vivo and ex vivo approaches. Ital. J. Pediatr.

Gabizon A, Shmeeda H, Barenholz Y (2003) Pharmacokinetics of Pegylated Liposomal Doxorubicin. Clin Pharmacokinet. https://doi.org/10.2165/00003088-200342050-00002

Ghatak S, Maytin E V., MacK JA, et al (2015) Roles of Proteoglycans and Glycosaminoglycans in Wound Healing and Fibrosis. Int J Cell Biol 2015:834893. https://doi.org/10.1155/2015/834893

Gonzalez EA, Visioli F, Pasqualim G, et al (2020) Progressive eye pathology in mucopolysaccharidosis type I mice and effects of enzyme replacement therapy. Clin Exp Ophthalmol. https://doi.org/10.1111/ceo.13713

Gupta D, Bhattacharjee O, Mandal D, et al (2019) CRISPR-Cas9 system: A new-fangled dawn in gene editing. Life Sci.

Hampe CS, Eisengart JB, Lund TC, et al (2020) Mucopolysaccharidosis Type I: A Review of the Natural History and Molecular Pathology. Cells

Harmon BT, Aly AE, Padegimas L, et al (2014) Intranasal administration of plasmid DNA nanoparticles yields successful transfection and expression of a reporter protein in rat brain. Gene Ther. https://doi.org/10.1038/gt.2014.28

Hattori Y, Tamaki K, Ozaki K ichi, et al (2019) Optimized combination of cationic lipids and neutral helper lipids in cationic liposomes for siRNA delivery into the lung by intravenous injection of siRNA lipoplexes. J Drug Deliv Sci Technol. https://doi.org/10.1016/j.jddst.2019.06.016

Ho BX, Loh SJH, Chan WK, Soh BS (2018) In vivo genome editing as a therapeutic approach. Int J Mol Sci 19:2721. https://doi.org/10.3390/ijms19092721

Hong SS, Oh KT, Choi HG, Lim SJ (2019) Liposomal formulations for nose-to-brain delivery: Recent advances and future perspectives. Pharmaceutics

Inglut CT, Sorrin AJ, Kuruppu T, et al (2020) Immunological and toxicological considerations for the design of liposomes. Nanomaterials

Joshi MU, Pittman HK, Haisch CE, Verbanac KM (2008) Real-time PCR to determine transgene copy number and to quantitate the biolocalization of adoptively transferred cells from EGFP-transgenic mice. Biotechniques. https://doi.org/10.2144/000112913

Kedmi R, Ben-Arie N, Peer D (2010) The systemic toxicity of positively charged lipid nanoparticles and the role of Toll-like receptor 4 in immune activation. Biomaterials.

https://doi.org/10.1016/j.biomaterials.2010.05.027

Keles E, Song Y, Du D, et al (2016) Recent progress in nanomaterials for gene delivery applications. Biomater. Sci.

Khargharia S, Baumhover NJ, Crowley ST, et al (2014) The uptake mechanism of PEGylated DNA polyplexes by the liver influences gene expression. Gene Ther. https://doi.org/10.1038/gt.2014.81

Khatri K, Goyal AK, Gupta PN, et al (2008) Plasmid DNA loaded chitosan nanoparticles for nasal mucosal immunization against hepatitis B. Int J Pharm 354:235–241. https://doi.org/10.1016/j.ijpharm.2007.11.027

Kiely BT, Kohler JL, Coletti HY, et al (2017) Early disease progression of Hurler syndrome. Orphanet J Rare Dis. https://doi.org/10.1186/s13023-017-0583-7

Kotton DN, Morrisey EE (2014) Lung regeneration: Mechanisms, applications and emerging stem cell populations. Nat. Med.

Kubaski F, de Oliveira Poswar F, Michelin-Tirelli K, et al (2020) Mucopolysaccharidosis type I. Diagnostics 10:131

Kubaski F, Mason RW, Nakatomi A, et al (2017a) Newborn screening for mucopolysaccharidoses: a pilot study of measurement of glycosaminoglycans by tandem mass spectrometry. J Inherit Metab Dis. https://doi.org/10.1007/s10545-016-9981-6

Kubaski F, Osago H, Mason RW, et al (2017b) Glycosaminoglycans detection methods: Applications of mass spectrometry. Mol. Genet. Metab.

Lallemand F, Daull P, Benita S, et al (2012) Successfully Improving Ocular Drug Delivery Using the Cationic Nanoemulsion, Novasorb. J Drug Deliv 2012:1–16. https://doi.org/10.1155/2012/604204

Laoharawee K, DeKelver RC, Podetz-Pedersen KM, et al (2018) Dose-Dependent Prevention of Metabolic and Neurologic Disease in Murine MPS II by ZFN-Mediated In Vivo Genome Editing. Mol Ther 26:1127–1136. https://doi.org/10.1016/j.ymthe.2018.03.002

Lemasson M, Saghatelyan A, Olivo-Marin JC, Lledo PM (2005) Neonatal and adult neurogenesis provide two distinct populations of newborn neurons to the mouse olfactory bulb. J Neurosci. https://doi.org/10.1523/JNEUROSCI.1114-05.2005

Li L, Hu S, Chen X (2018) Non-viral delivery systems for CRISPR/Cas9-based genome editing: Challenges and opportunities. Biomaterials

Li M (2018) Enzyme replacement therapy: A review and its role in treating lysosomal storage diseases. Pediatr Ann. https://doi.org/10.3928/19382359-20180424-01

Liu C, Zhang L, Liu H, Cheng K (2017) Delivery strategies of the CRISPR-Cas9 gene-editing system for therapeutic applications. J. Control. Release

Liu F, Shollenberger LM, Conwell CC, et al (2007) Mechanism of naked DNA clearance

after intravenous injection. J Gene Med. https://doi.org/10.1002/jgm.1054

Lochhead JJ, Thorne RG (2012) Intranasal delivery of biologics to the central nervous system. Adv. Drug Deliv. Rev.

LOWRY O, ROSEBROUGH N, FARR A, RANDALL R (1994) Protein measurement with Folin phenol reaegnt. Anal Biochem. https://doi.org/10.1016/0304-3894(92)87011-4

Lukashev AN, Zamyatnin AA (2016) Viral vectors for gene therapy: Current state and clinical perspectives. Biochem.

Ma Y, Zhang L, Huang X (2014) Genome modification by CRISPR/Cas9. FEBS J.

Marques ARA, Saftig P (2019) Lysosomal storage disorders – challenges, concepts and avenues for therapy: beyond rare diseases. J Cell Sci 132:221739. https://doi.org/10.1242/jcs.221739

Mashel T V., Tarakanchikova Y V., Muslimov AR, et al (2020) Overcoming the delivery problem for therapeutic genome editing: Current status and perspective of non-viral methods. Biomaterials 258:120282. https://doi.org/10.1016/j.biomaterials.2020.120282

Meikle PJ, Fuller M, Hopwood JJ (2005) Lysosomal degradation of heparin and heparan sulfate. In: Chemistry and Biology of Heparin and Heparan Sulfate

Melbouci M, Mason RW, Suzuki Y, et al (2018) Growth impairment in mucopolysaccharidoses. Mol. Genet. Metab.

Muenzer J (2011) Overview of the mucopolysaccharidoses. Rheumatology. https://doi.org/10.1093/rheumatology/ker394

Naldini L (2011) Ex vivo gene transfer and correction for cell-based therapies. Nat. Rev. Genet.

O'Neal WK, Zhou H, Morral N, et al (2000) Toxicity associated with repeated administration of first-generation adenovirus vectors does not occur with a helper-dependent vector. Mol Med. https://doi.org/10.1007/bf03402113

Oh YK, Kim JP, Hwang TS, et al (2001) Nasal absorption and biodistribution of plasmid DNA: An alternative route of DNA vaccine delivery. Vaccine. https://doi.org/10.1016/S0264-410X(01)00188-8

Ørstavik KH, Apold J (2001) Gene therapy for monogenic inherited diseases. Tidsskr den Nor laegeforening

Ou L, Herzog TL, Wilmot CM, Whitley CB (2014) Standardization of α-L-iduronidase enzyme assay with Michaelis-Menten kinetics. Mol Genet Metab. https://doi.org/10.1016/j.ymgme.2013.11.009

Ou L, Przybilla MJ, Ahlat O, et al (2020) A Highly Efficacious PS Gene Editing System Corrects Metabolic and Neurological Complications of Mucopolysaccharidosis Type I. Mol Ther 28:1442–1454. https://doi.org/10.1016/j.ymthe.2020.03.018

Pan G, Shawer M, Øie S, Lu DR (2003) In vitro gene transfection in human glioma cells using a novel and less cytotoxic artificial lipoprotein delivery system. Pharm Res 20:738-744. https://doi.org/10.1023/A:1023477317668

Parini R, Deodato F, Di Rocco M, et al (2017) Open issues in Mucopolysaccharidosis type I-Hurler. Orphanet J Rare Dis 12:112. https://doi.org/10.1186/s13023-017-0662-9

Parker H, Bigger BW (2019) The role of innate immunity in mucopolysaccharide diseases. J. Neurochem.

Parker H, Ellison SM, Holley RJ, et al (2020) Haematopoietic stem cell gene therapy with IL -1Ra rescues cognitive loss in mucopolysaccharidosis IIIA . EMBO Mol Med. https://doi.org/10.15252/emmm.201911185

Pasqualim G, Baldo G, De Carvalho TG, et al (2015) Effects of enzyme replacement therapy started late in a murine model of mucopolysaccharidosis type I. PLoS One. https://doi.org/10.1371/journal.pone.0117271

Patil S, Gao YG, Lin X, et al (2019) The development of functional non-viral vectors for gene delivery. Int J Mol Sci. https://doi.org/10.3390/ijms20215491

Pereirinha PRR, Figueiras ARR (2016) Desenvolvimento De Sistemas Mucoadesivos Para a Administração Controlada De Fármacos. nformative Geum Bull Bol 7:63–81

Petreanu L, Alvarez-Buylla A (2002) Maturation and death of adult-born olfactory bulb granule neurons: Role of olfaction. J Neurosci. https://doi.org/10.1523/jneurosci.22-14-06106.2002

Pimentel-Vera LN, Poletto É, Gonzalez EAEA, et al (2021) Genome editing in lysosomal disorders. In: Progress in Molecular Biology and Translational Science

Podolska K, Stachurska A, Hajdukiewicz K, Małecki M (2012) Gene therapy prospects - Intranasal delivery of therapeutic genes. Adv. Clin. Exp. Med.

Poletto E, Baldo G, Gomez-Ospina N (2020) Genome editing for mucopolysaccharidoses. Int J Mol Sci 21:500. https://doi.org/10.3390/ijms21020500

Ponder KP (2013) Mucopolysaccharidosis. In: Brenner's Encyclopedia of Genetics: Second Edition

Poswar F, Vairo F, Burin M, et al (2019) Lysosomal diseases: Overview on current diagnosis and treatment. Genet Mol Biol 42:165–177. https://doi.org/org/10.1590/1678-4685-gmb-2018-0159

Prakash V, Moore M, Yáñez-Muñoz RJ (2016) Current progress in therapeutic gene editing for monogenic diseases. Mol. Ther.

Rajawat YS, Humbert O, Kiem HP (2019) In-vivo gene therapy with foamy virus vectors. Viruses

Reynolds PN, Danilo SM (2002) Intravascular Re-Targeting of Viral Vectors. In: Biomedical Aspects of Drug Targeting. pp 173–190

Schlander M, Beck M (2009) Expensive drugs for rare disorders: To treat or not to treat? The case of enzyme replacement therapy for mucopolysaccharidosis VI. Curr. Med. Res. Opin.

Schuh RS, Bidone J, Poletto E, et al (2018a) Nasal Administration of Cationic Nanoemulsions as Nucleic Acids Delivery Systems Aiming at Mucopolysaccharidosis Type I Gene Therapy. Pharm Res. https://doi.org/10.1007/s11095-018-2503-5

Schuh RS, Gonzalez EAEA, Tavares AMVAMV, et al (2020) Neonatal nonviral gene editing with the CRISPR/Cas9 system improves some cardiovascular, respiratory, and bone disease features of the mucopolysaccharidosis I phenotype in mice. Gene Ther 27:. https://doi.org/10.1038/s41434-019-0113-4

Schuh RS, Poletto E, Pasqualim G, et al (2018b) In vivo genome editing of mucopolysaccharidosis I mice using the CRISPR/Cas9 system. J Control Release 288:23–33. https://doi.org/10.1016/j.jconrel.2018.08.031

Senyo SE, Steinhauser ML, Pizzimenti CL, et al (2013) Mammalian heart renewal by pre-existing cardiomyocytes. Nature 493:. https://doi.org/10.1038/nature11682

Shalaby K, Aouida M, El-Agnaf O (2020) Tissue-specific delivery of crispr therapeutics: Strategies and mechanisms of non-viral vectors. Int. J. Mol. Sci.

Simonaro CM (2016) Lysosomes, lysosomal storage diseases, and inflammation. J Inborn Errors Metab Screen. https://doi.org/10.1177/2326409816650465

Solano ML, Fainboim A, Politei J, et al (2020) Enzyme replacement therapy interruption in patients with Mucopolysaccharidoses: Recommendations for distinct scenarios in Latin America. Mol Genet Metab Reports. https://doi.org/10.1016/j.ymgmr.2020.100572

Stapleton M, Kubaski F, Mason RW, et al (2017) Presentation and treatments for Mucopolysaccharidosis Type II (MPS II; Hunter Syndrome). Expert Opin. Orphan Drugs

Suda T, Liu D (2007) Hydrodynamic gene delivery: Its principles and applications. Mol. Ther.

Suzuki K, Tsunekawa Y, Hernandez-Benitez R, et al (2016) In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. Nature. https://doi.org/10.1038/nature20565

Takahashi H, Yoshihara S, Tsuboi A (2018) The Functional Role of Olfactory Bulb Granule Cell Subtypes Derived From Embryonic and Postnatal Neurogenesis. Front. Mol. Neurosci.

Tan EY, Boelens JJ, Jones SA, Wynn RF (2019) Hematopoietic Stem Cell Transplantation in Inborn Errors of Metabolism. Front Pediatr 7:433. https://doi.org/10.3389/fped.2019.00433

Tao J, Ding WF, Che XH, et al (2016) Optimization of a cationic liposome-based gene delivery system for the application of miR-145 in anticancer therapeutics. Int J Mol

Med. https://doi.org/10.3892/ijmm.2016.2530

Trivedi PC, Bartlett JJ, Pulinilkunnil T (2020) Lysosomal Biology and Function: Modern View of Cellular Debris Bin. Cells 9:1131. https://doi.org/10.3390/cells9051131

Vera LNPLNP, Baldo G (2020) The potential of gene therapy for mucopolysaccharidosis type I

Villate-Beitia I, Puras G, Zarate J, et al (2015) First Insights into Non-invasive Administration Routes for Non-viral Gene Therapy. In: Gene Therapy - Principles and Challenges

Voznyi Y V., Keulemans JLM, Van Diggelen OP (2001) A fluorimetric enzyme assay for the diagnosis of MPS II (hunter disease). J Inherit Metab Dis. https://doi.org/10.1023/A:1012763026526

Wadell C, Bjork E, Camber O (2003) Permeability of porcine nasal mucosa correlated with human nasal absorption. Eur J Pharm Sci 18:47–53. https://doi.org/10.1016/S0928-0987(02)00240-3

Wang D, Gao G (2014) State-of-the-art human gene therapy: Part I. Gene delivery technologies. Discov Med

Wang H, La Russa M, Qi LS (2016) CRISPR/Cas9 in Genome Editing and beyond. Annu Rev Biochem. https://doi.org/10.1146/annurev-biochem-060815-014607

Wang T, Upponi JR, Torchilin VP (2012) Design of multifunctional non-viral gene vectors to overcome physiological barriers: Dilemmas and strategies. Int. J. Pharm.

Wang Z, Xiong G, Tsang WC, et al (2019) Nose-to-brain delivery. J. Pharmacol. Exp. Ther.

Wilbie D, Walther J, Mastrobattista E (2019) Delivery Aspects of CRISPR/Cas for in Vivo Genome Editing. Acc Chem Res. https://doi.org/10.1021/acs.accounts.9b00106

Wilkinson FL, Holley RJ, Langford-Smith KJ, et al (2012) Neuropathology in mouse models of mucopolysaccharidosis type I, IIIA and IIIB. PLoS One. https://doi.org/10.1371/journal.pone.0035787

Woo JG, Kim NY, Yang JM, Shin S (2011) Biodistribution and blood clearance of plasmid DNA administered in arginine peptide complexes. Genet Vaccines Ther. https://doi.org/10.1186/1479-0556-9-13

Wraith JE (2013) Mucopolysaccharidoses. In: Emery and Rimoin's Principles and Practice of Medical Genetics

Yi W, Kiara F B, Stephanie F, et al (2021) Is Viral Vector Gene Delivery More Effective Using Biomaterials? Adv Healthc Mater 10:e2001238. https://doi.org/doi:10.1002/adhm.202001238

Zhou J, Lin J, Leung WT, Wang L (2020) A basic understanding of mucopolysaccharidosis: Incidence, clinical features, diagnosis, and management. Intractable Rare Dis Res. https://doi.org/10.5582/irdr.2020.01011

Zincarelli C, Soltys S, Rengo G, Rabinowitz JE (2008) Analysis of AAV serotypes 1-9 mediated gene expression and tropism in mice after systemic injection. Mol Ther. https://doi.org/10.1038/mt.2008.76

8. ANEXOS

Produção cientifica relacionada: Neste item constam 4 artigos publicados durante o período do doutorado na área de estudo.

Genome editing in lysosomal disorders

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Contents

Abstract

Lysosomal disorders are a group of heterogenous diseases caused by mutations in genes that encode for lysosomal proteins. With exception of some cases, these disorders still lack both knowledge of disease pathogenesis and specific therapies. In this sense, genome editing arises as a technique that allows both the creation of specific cell lines, animal models and gene therapy protocols for these disorders. Here we

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explain the main applications of genome editing for lysosomal diseases, with examples based on the literature. The ability to rewrite the genome will be of extreme importance to study and potentially treat these rare disorders.

Abbreviations

AAV	adeno associated virus
AAVS1	AAV integration site
ABE	adenine base editor
ALB	albumin
BBB	blood-brain barrier
CBE	cystine base editors
CCR5	chemokine (C-C motif) receptor
CHO	Chinese hamster ovary
CMT	Charcot-Marie-tooth disease
CNS	central nervous system
CRISPR	clustered regularly interspaced short palindromic repeats
DNA	deoxyribonucleic acid
DSB	double-strand break
EMA	European Medicines Agency
eNOS	endothelial nitric oxide synthase
ERT	enzyme replacement therapy
FDA	American Food and Drug Administration
FTDALS	frontotemporal dementia and/or amyotrophic lateral sclerosis
GAA	acid α-glucosidase
GALC	galactosylceramidase
GBA	glucocerebrosidase
GLA	α-galactosidase
gRNA	guide RNA
HDR	homology directed repair
HEK	human embryonic kidney
hESC	human embryonic stem cells
HEX	β-hexosaminidases
HGSNAT	heparan-alpha-glucosaminide *N*-acetyltransferase
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplantation
IDS	iduronate-2-sulfatase
IDUA	alpha-L-iduronidase
IL2RG	interleukin-2 receptor gamma chain
iPSCs	induced pluripotent stem cells
LAL	lysosomal acid lipase
LDs	lysosomal disorders
ML	mucolipidosis
MPS	mucopolysaccharidosis
NBIA	neurodegeneration with brain iron accumulation
NCL	neuronal ceroid lipofuscinosis
NHEJ	non-homologous end joining
Nick	single-strand break

NSCs	neural stem cells
PAM	protospacer adjacent motif
PC	pharmacological chaperone
PEG	polyethylene glycol
PME	progressive myoclonic epilepsy
RNA	ribonucleic acid
RNP	ribonucleoprotein
SNV	single nucleotide variant
SRT	substrate reduction therapy
TALENs	transcription activator-like effector nucleases
UCB	umbilical cord blood
ZFNs	zinc-finger nucleases

1. Introduction: Lysosomal disorders

The lysosomal disorders (LDs) comprise a group of over 70 monogenic disorders involving genes encoding for acid hydrolases, membrane proteins, transporters, enzyme modifiers and activators, or other proteins affecting the function of the lysosome. Many LDs result from an impairment in the catabolism of complex molecules, which as result tend to store in cells and tissues. For instance, a defect in any of the 11 enzymes related to the degradation of glycosaminoglycans (formerly denominated mucopolysaccharides) implicates in mucopolysaccharidoses I-IX (MPS I-IX). Likewise, an impairment in the catabolism of sphingolipids due to either the deficiency of lysosomal enzymes or their activators is the cause of sphingolipidoses, such as Fabry, Gaucher, Niemann Pick types A/B, Tay-Sachs and Sandhoff diseases, and a defective degradation of oligosaccharide chains of glycoproteins causes oligosaccharidoses (also known as glycoproteinoses), including sialidosis, fucosidosis, α-mannosidosis, β-mannosidosis, and aspartylglycosaminuria.[1] Defects in other lysosomal components may also result in lysosomal dysfunction with or without storage.

Almost all LDs have an autosomal recessive inheritance, except for Danon disease, mucopolysaccharidosis II and Fabry disease, which are X-linked traits. LDs are rare, with some conditions, like MPS IX and β-mannosidosis, having less than 100 cases described. Collectively, the global birth prevalence of the LDs is estimated to be around 1:7000.[2] However, this number is likely to be underestimated, considering the results of newborn screening studies that suggest the existence of a large number of undiagnosed patients with later-onset phenotypes, especially for Fabry disease, presently considered the commonest LD.[3,4] Furthermore, a much

higher prevalence of certain LDs is observed in specific ethnicities, most likely as a result of bottleneck and founder effects.

In this chapter, we aim to discuss the clinical and molecular aspects of the LDs and how the use of genome editing tools may aid the development of better treatments, by overcoming the limitations of current therapeutical approaches.

2. Physiopathology, clinical manifestations and natural history

Lysosomes are involved in the degradation of several macromolecules through the action of more than 50 hydrolases. Those enzymes require the acidic environment of the lysosomes for their proper function and may be much less active in the cytosol. The final products (e.g., amino acids and monosaccharides) are released from the lysosomes and made available for other metabolic pathways outside the organelle. When a lysosomal enzyme is deficient, intra-lysosomal accumulation of undegraded or partially degraded substrates occurs, resulting in enlargement of lysosomes and promoting a cascade of secondary effects. Besides the storage of the substrate of the deficient enzyme, secondary storage may also occur in LDs, due to inhibition of other lysosomal degradation pathways,[5] as is the case of the accumulation of gangliosides in the mucopolysaccharidoses. Lysosomes are known to be involved in several cellular functions including autophagy,[6] cholesterol homeostasis[7] and cell death,[8] processes that may be either primarily or secondarily compromised in LDs.

Although lysosomes are ubiquitously present in the cells, the consequences of a deficiency of a lysosomal enzyme may result in different impacts in distinct cell types, according to the cell function and the tissue where a particular substrate is present. For instance, phagocytes play a major role in the engulfment and digestion of extracellular components and some lysosomal diseases may be accompanied by histopathological and functional changes in the cells of the mononuclear phagocyte system, particularly Gaucher and Niemann Pick diseases.[9] This results in a range of overlapping clinical manifestations including hepatosplenomegaly, osteolysis, interstitial lung disease and, in severe cases, neurological involvement.

For some LDs, clinical manifestations are nearly restricted to a few cells and tissues where the substrate is present, as is the case of Krabbe disease and metachromatic leukodystrophy. Both diseases result from impairments in the degradation of two major components of the myelin lipids, galactocerebroside

and its sulfated derivative, sulfatide, resulting mainly in demyelinating disease of the central nervous system.[10]

Without treatment, most LDs will have a progressive course, with worsening of symptoms, disability and early death. There is a wide range of presentation for each of the LDs, which may span from prenatal presentations such as hydrops fetalis (as in some patients with Niemann Pick type C and Gaucher Disease type 2, and a large proportion of patients with MPS VII) to a much more attenuated phenotype presenting in adulthood and without the full range of manifestations (as in some patients with the cardiac variant of Fabry disease).

The rate of the disease progression is, in general, faster in patients who have more severe, infantile-onset presentations, while patients with the adult-onset disease may have a much slower progression. These characteristics of LDs may complicate the assessment of treatment efficacy in clinical trials if the intervention is not expected to result in improvement but only in stabilization or slower progression of the symptoms.[11] Table 1 summarizes the main lysosomal storage disorders, their clinical manifestations and the available treatments.

3. Diagnosis and molecular genetics

Considering the rarity of the LDs, their absence in most public newborn screening programs, and the fact that many patients do not have a family history of the disease (as expected for the autosomal recessive inheritance), diagnostic delays are common, with many patients being diagnosed several months or even decades after the onset of the symptoms.[13] LDs manifestations may be misattributed to more common conditions. For example, patients with Fabry disease and white matter involvement were misdiagnosed as multiple sclerosis[14] and patients with late-onset GM2 gangliosidosis and psychiatric manifestations were considered to suffer from schizophrenia.[15]

When a LD is suspected, the traditional approach involves assessing enzyme activity and/or the levels of informative biomarkers, which are typically derived from the undegraded substrates (e.g., the glycosphingolipids psychosine, glucosylsphingosine and globotriaosylsphingosine for Krabbe, Gaucher and Fabry diseases, respectively). The biochemical diagnosis is then confirmed through a molecular test, including DNA sequencing and deletion/duplication analysis. Enzyme activity assessments may be less reliable in

Table 1 Lysosomal diseases groups, manifestations and treatment.

Group of LDs	Diseases	Clinical manifestations	Treatment
Sphingolipidoses	Fabry, Gaucher, Krabbe and Farber diseases; GM1 and GM2 gangliosidoses; multiple sulfatases deficiency, saposin deficiencies	Hepatosplenomegaly, chronic kidney disease, anemia, thrombocytopenia, cognitive impairment, neurological regression, cherry-red spots, white matter disease, ataxia	ERT, SRT, HSCT, PC
Mucopolysaccharidoses	MPS I, II, IIIA, IIIB, IIIC, IIID, IVA, IVB, VI, VII, IX, MPS Plus	Coarse facial features, corneal clouding, joint stiffness, dysostosis multiplex, hepatomegaly, valve disease	ERT, HSCT
Oligossacharidoses	Sialidosis, fucosidosis, α-mannosidosis, β-mannosidosis, galactosialidosis and aspartylglycosaminuria	Coarse facial features, ataxia, myopathy, myoclonus, hepatosplenomegaly	ERT
Mucolipidoses	ML-II, ML-III, ML-IV	Coarse facial features, dysostosis multiplex, hepatomegaly, hyperparathyroidism, valve disease	None available
Neuronal ceroid lipofuscinoses (Batten diseases)	NCL1-14	Epilepsy, brain atrophy, ataxia, optic nerve atrophy	ERT
Disorders of lysosomal transport	Cystinosis, sialic acid storage disease, EPM4	Chronic kidney disease, Fanconi syndrome, coarse facial features, cardiomegaly, dysostosis multiplex, hypopigmented skin, myoclonus	SRT

Disorders of lysosomal cholesterol metabolism	Niemann Pick type C, lysosomal acid lipase deficiency	Hepatomegaly, splenomegaly, ataxia, neurodegeneration, steatosis, hypercholesterolemia	ERT, SRT
Disorders of autophagy	Autosomal recessive spastic paraplegia 11, 15, 48 and 49, Vici syndrome, NBIA 5, FTDALS 4, CMT type 2B	Spastic paraplegia, thin corpus callosum, ocular albinism, cardiomyopathy, extrapyramidal signs, fasciculations, peripheral neuropathy	None available
Disorders of lysosomal protein degradation	Pycnodysostosis, Papillon-Lefevre syndrome	Bone dysplasia, periodontitis, hyperkeratosis of palms and soles	None available

Classification as proposed by Ferreira.[12] The treatment column denotes if there is an available treatment for any of the conditions cited in each group. Disease abbreviations: CMT, Charcot-Marie-tooth disease; EPM, epilepsy progressive myoclonic; FTDALS, frontotemporal dementia and/or amyotrophic lateral sclerosis; ML, mucolipidosis; MPS, mucopolysaccharidosis; NBIA, neurodegeneration with brain iron accumulation; NCL, neuronal ceroid lipofuscinosis. Treatment abbreviations: ERT, enzyme replacement therapy; HSCT, hematopoietic stem cell transplantation; PC, pharmacological chaperone; SRT, substrate reduction therapy.

some situations, especially when investigating females for Fabry disease and when pseudo-deficient alleles are present. Furthermore, for conditions caused by the deficiency of activator proteins and other nonenzymatic proteins, molecular tests are the only available method for establishing a definitive diagnosis, although clinical and biochemical biomarkers may play a role in raising the level of suspicion.

Targeted mutation analysis is a feasible approach in many situations for selected LDs, considering the existence of frequent variants. For instance, the *HEXA* gene variants c.1421 + 1G > C, c.1274_1277dupTATC, and c.805G > A (p.G269S) are found in 98% of the Ashkenazi Jews with Tay-Sachs disease and 35% of non-Ashkenazi patients. On the other hand, in ~80% of Japanese patients with Tay-Sachs disease the *HEXA* gene variant c.571–1G > T is found.[16] For Gaucher disease, the variant c.1226A > G (N370S) is known to be highly represented in the Ashkenazi Jewish population and is associated with a later-onset phenotype.[17]

With the incorporation of massive parallel sequencing to the clinical practice, a simultaneous molecular testing approach for more than one condition is been advocated in some cases, especially considering the existence of significant clinical overlap among some LDs.[18] Targeted next-generation sequencing pipelines, however, may face the challenges of overcoming technical difficulties imposed by the presence of high GC content and pseudogenes and for some LDs, which may also need to be addressed when designing genome-editing tools.[19]

Pseudogenes have been described in different types of lysosomal diseases. The gene mutated in Gaucher disease, *GBA*, has a highly homologous pseudogene *GBAP1*, and nonreciprocal homologous recombination is a common mechanism of mutation for this disease. The two most common disease-causing recombinant variants are RecNcil and Recdelta55, which result in the incorporation of the sequence of *GBAP1* to the *GBA* gene resulting in the introduction of missense variants or the deletion of 55 nucleotides, respectively.[20] Similarly, in MPS type II, a pseudogene located 20 kb telomeric to the *IDS* gene is responsible for a range of gene rearrangements and deletions.[21]

With the increased availability of next-generation sequencing, unexpected phenotypes are being more and more described, as a clinical picture dominated by retinitis pigmentosa in some patients with mutations in *HGSNAT*, which is usually related to Mucopolysaccharidosis IIIC[22]. It is expected that with the growing use of genomic-based diagnosis, findings like this will become more common.

Besides the diagnosis, identification of causal variants may be valuable for establishing the prognosis and the most appropriate therapies, as further discussed in the next section. As LDs are caused by a loss of function mechanism, null variants are predicted to be associated with more severe phenotypes or even fetal death in some cases. However, genotype-phenotype correlations are not perfect, and patients with the same genotype were described as having variations in disease severity. Enzyme activity may also not have a clear correlation with the phenotype. Genetic, epigenetic and environmental factors are expected to modulate the phenotype, but the magnitude of the effects of those factors is not always defined.[5]

4. Current treatments: Impact and limitations

Despite significant advance in the comprehension of the pathophysiology of LDs, most conditions do not have an approved therapy, although important progress has been observed in the last two decades. Currently available treatments for lysosomal disorders include hematopoietic stem cell transplantation, enzyme replacement therapy and small molecules (Fig. 1).

4.1 Hematopoietic stem cell transplantation

The first established treatment modality for the LDs was the hematopoietic stem cell transplantation.[23] The cells, transplanted from a healthy donor will produce resident cells of hematopoietic origin, including glial cells and macrophages, which may act as permanent sources of the deficient enzyme in the central nervous system and other parts of the body.[5] HSCT is currently the first treatment choice for infants with severe MPS I (Hurler syndrome). This treatment can halt the neurodegeneration and attenuate the overall phenotype, especially when performed early in the disease course. HSCT is also recommended for some LD patients with metachromatic leukodystrophy and Krabbe disease.[24] However, morbidity and mortality associated with the immunosuppression and graft vs host disease preclude its more generalized use in attenuated presentations of LDs. Furthermore, HSCT has not been proven to be successful in some of the LDs with CNS involvement, including MPS III and Niemann Pick disease type C.[24]

4.2 Enzyme replacement therapy

Another treatment modality for LDs is enzyme replacement therapy (ERT), which consists of the periodical administration of a therapeutic enzyme. The

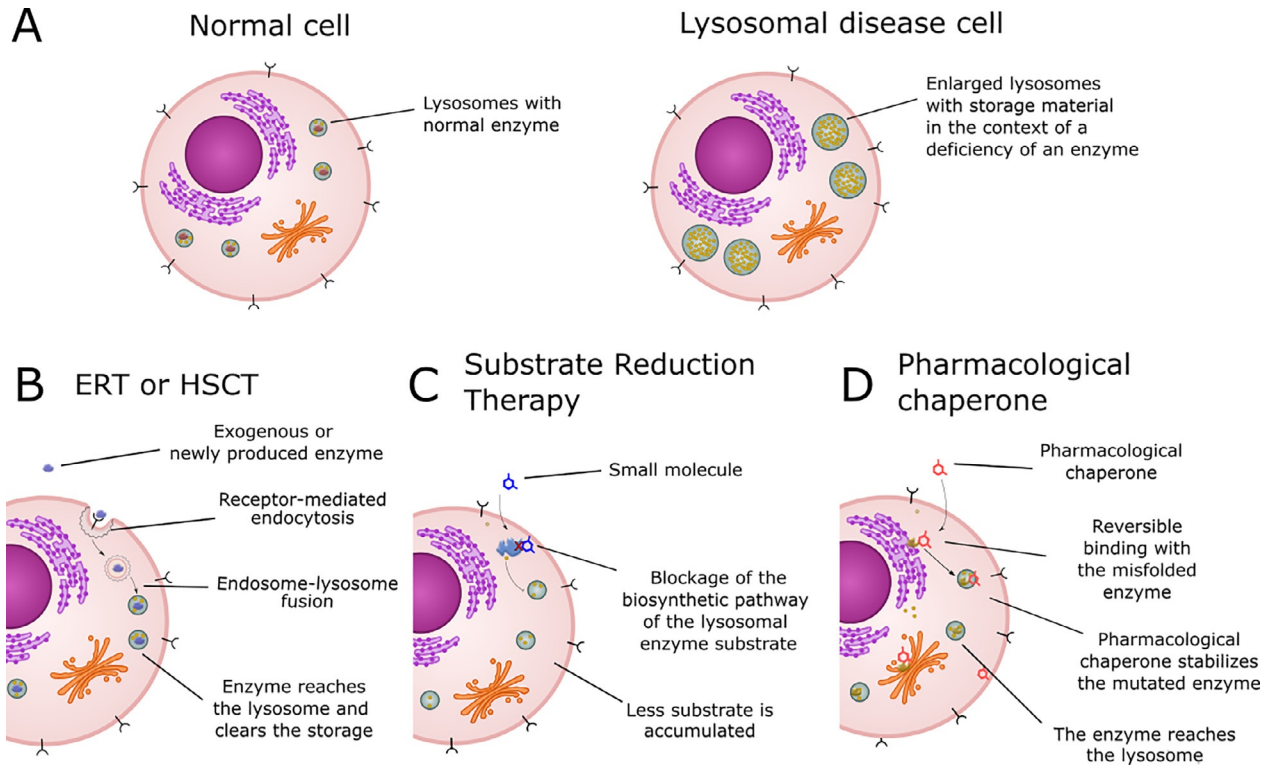

Fig. 1 See figure legend on opposite page.

first ERT for a LD, alglucerase, was approved in 1991 for Gaucher disease.[25] The product was extracted and purified from the human placenta, a method that was eventually replaced by the more scalable recombinant DNA technology. Enzyme replacement therapy is currently available for Gaucher, Fabry and Pompe diseases, as well as for MPS types I, II, IVA, VI and VII, lysosomal acid lipase deficiency, α-mannosidosis and CLN type 2. These biopharmaceuticals are produced in different cell or animal lineages, including Chinese hamster ovary (CHO) cells, human fibroblasts, carrot root cells and transgenic hens. In all cases, the enzymes are administered intravenously, except for CLN2, in which the approved product has an intracerebroventricular route of administration.

ERT was shown to promote a marked improvement in signs and symptoms of Gaucher disease type I, including anemia, thrombocytopenia, splenomegaly and bone pain, and this success was a key driver to develop new products for other LDs. Nevertheless, ERT does not seem to respond as well in other lysosomal diseases, where macrophages are not the target cells. For instance, worsening of cardiac and renal parameters has been observed for patients with Fabry disease on ERT, when treatment is started at older ages.[26] Likewise, some patients with late-onset Pompe disease on ERT have experienced worsening of strength and respiratory function after a period of improvement and stabilization of the disease.[27]

Intravenous ERT is, in general, of limited efficacy in addressing the CNS manifestations of LDs, since currently approved therapies are unable to cross the blood-brain barrier (BBB), a limitation that may be especially relevant for patients who have both somatic and neurological manifestations, such as those with Gaucher disease type III and severe MPS II. However, the demonstration of the efficacy of intracerebroventricular ERT for CLN type 2 indicates that intrathecal/intracerebroventricular ERT, either alone or in

Fig. 1 Mechanisms of action of the currently approved therapies for lysosomal diseases (LDs). (A) In the absence of a lysosomal enzyme, substrate accumulates in the lumen of the lysosomes with consequent lysosomal dysfunction. (B) Hematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT) are aimed to provide the missing enzyme either through the production by donor cells (HSCT) or by regular infusion of recombinant enzymes (ERT). The newly produced or recombinant enzyme is captured by the LD cell through receptor-mediated endocytosis and directed to the lysosome. (C) Substrate reduction therapy (SRT) may be employed to inhibit the biosynthesis of the lysosomal substrate not degraded in LD cells. (D) Pharmacological chaperones (PCs) may bind transiently to the active site of the mutated enzyme, facilitating the enzyme targeting to the lysosome.

combination with intravenous ERT, may also be a promising approach for other LDs.[28]

ERT usually elicits an immune response with the production of immunoglobulins, especially in patients with null mutations, considering the lack of negative selection to the enzyme antigens. Although the presence of high titers of anti-drug antibodies may not always be directly associated with loss of efficacy or harm to the patient, adverse events may occur, including moderate to severe infusion-related reactions and worsening of clinical parameters and biomarkers.[29] ERT also requires the patient to commit to a strict schedule of periodical, and frequently hospital-based infusions that may be difficult to adhere to.

4.3 Small molecules

Besides cell transplantation and biotherapeutic proteins, small molecules are also an option for the treatment of some LDs, like Fabry and Gaucher diseases. For other LDs, such as cystinosis and Niemann Pick disease type C, small molecules are, currently, the only available treatment. The mechanisms of action of the small molecules used for LDs include pharmacological chaperone therapy (e.g., migalastat for Fabry disease) and substrate reduction therapy (e.g., miglustat and eliglustat for Gaucher disease).

Substrate reduction therapy (SRT) aims to halt the biosynthesis of the accumulated substrate and has also the potential to treat more than one LD, as the first step of a common pathway of synthesis may be inhibited. For instance, N-butyl-deoxynojirimycin (miglustat), which inhibits the synthesis of glucosylceramide, is approved for the treatment of Gaucher and Niemann Pick type C diseases.[30,31] As glucosylceramide is also a precursor of gangliosides in the biosynthetic pathway, miglustat has also been administrated to patients with GM1 and GM2 gangliosidoses in clinical trials or as an off-label treatment, although the evidence is not conclusive for its efficacy for those conditions.[32,33] Eliglustat, another SRT, has also demonstrated efficacy for Gaucher disease type 1, for which it was approved as a first-line treatment.[34] However, being unable to cross the blood-brain barrier, eliglustat is not applicable for neurodegenerative LDs.

Migalastat is currently the only approved pharmacological chaperone for a LD. It binds and provides stabilization for amenable mutant forms (misfolded functional variants) of α-galactosidase A, the enzyme deficient in Fabry disease. The medication has been shown to stabilize renal function, reduce cardiac mass, and improve gastrointestinal symptoms in its phase 3

clinical trial.[35] Due to its recent approval and the limited number of eligible patients with amenable variants, there is less available data regarding the long-term effectiveness of the therapy, but results to date have confirmed its potential as an important therapeutic option.[36]

5. Genome editing tools

In the past few years, some gene-based therapies have been approved by the American Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of blindness, cancer, viral infections, blood, and neuromuscular disorders.[37] Nevertheless, gene therapy has still been considered as an experimental therapy for most monogenic disorders, including LDs. Among the many hurdles that this approach holds, the most significant ones are immune responses to some viral vectors, random integration of lentiviral vectors in non-desired regions, or dilution of episomal vectors, such as adenovirus and adeno-associated virus, leading to loss of the transgene expression.[38] To overcome these drawbacks, researchers have investigated the potential of genome editing technologies for developing new therapies.

Genome editing tools offer the possibility of editing a specific genome region with precision in any organism. Overall, these tools rely on programmable nucleases that work along with customized DNA-binding motifs or RNA molecules that serve as guides to target the region of interest. Once the specific site is recognized by the nucleases, they introduce a double-strand break (DSB) or a single-strand break (nick), depending on the nucleases type.[39] The editing process begins when endogenous DNA repair machinery is activated after the break. There are two distinct repair mechanisms: non-homologous end joining (NHEJ) and homology-directed repair (HDR). In the first, the DNA molecule may undergo the introduction of indels, frequently knocking-out the gene. On the other hand, HDR is less common and requires the presence of a homologous DNA template to repair the break in a precise manner, copying the sequence at the break site from the template. Moreover, the HDR pathway is active mostly in proliferating cells.[40]

Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR) are the genome editing tools most commonly employed. ZFNs are composed of a *Fok*I restriction endonuclease cleavage domain and an array of zinc-finger proteins. Each array is composed of

three or four DNA zinc-finger domains each recognizing a DNA triplet. Zinc-finger domains are in charge of recognizing different specific DNA sequences—18 base pairs each in distal parts of the target sequence. Then, *Fok*I cuts DNA within a five- to seven-bp between the two flanking zinc-finger targets as the FokI enzyme requires dimerization for DNA cleavage.[41] The TALENs also consists of a *Fok*I endonuclease, but this is paired with transcription activator-like effectors (TALE) proteins. As ZFNs, TALENs also work as modules and undergo dimerization. TALE-binding domain has a series of repeat domains that recognize only a single base. In total, these effectors recognize 12- to 20-bps of DNA each, and FokI cuts within a 12- to 19-bp between them.[41] Currently, the most used method for gene editing is, however, based on CRISPR-Cas, which is more simple and versatile. CRISPR machinery involves only two components: the Cas9 enzyme, responsible for DNA cleavage, and a guide RNA (gRNA). The gRNA can be customized to virtually recognize any specific 20 bp sequence. Both components form a complex together and, after gRNA binds the target region by base-pairing, Cas9 makes the DSB. However, this cleavage is restricted to the presence of three NGG nucleotides (called PAM site) in the target sequence.[39]

More recently, different variations to CRISPR-Cas9 system have been proposed, mainly focusing on base editing. The first developed was the cytosine base editor (CBE). This system uses a cytidine deaminase enzyme linked to a Cas9 that underwent inactivation of one of its cleavage domains, becoming a nickase (Cas9n). This enzyme complex can catalyze the conversion of C-G base pairs to T-A in a target sequence, by converting cytidine to uridine which, in turn, is converted to thymidine by DNA repair mechanisms.[42] Another variation of the base editor is the adenine base editor (ABE), which can convert A-T base pairs to G-C by adenosine deaminase. The last CRISPR-derived tool, the prime editing system,[43] relies on the fusion of the Cas9n to an engineered viral reverse transcriptase enzyme to create a modified nuclease. The other component is a pegRNA, a longer gRNA that also serves as a template for the repair. After binding to the specific site, Cas9n makes a nick and the reverse transcriptase domain generates a complementary DNA sequence by copying the pegRNA, restoring a segment of the nicked strand. Thus, it mediates all 12 possible base-to-base conversions due to pegRNA carrying the desired sequence.[39]

Due to their potential, these tools have been used to develop several cell and animal models, aiming not only to contribute to understanding diseases' physiopathology but also to develop different strategies for gene therapy.

6. Genome-edited cell models

Patient-derived cells have been an important resource for understanding disease mechanisms and the development of new therapies. The majority of basic research or proof-of-concept studies for lysosomal disorders was done in fibroblasts, as they are easy to collect without the need for surgical intervention. With the development of genome editing technologies, cell models became more accessible as one can choose the most relevant cell type and induce the modification of interest, without the need for complex biopsies and patient availability. Currently, these models based on genome editing are mainly developed using induced pluripotent stem cells (iPSCs) or cell lines.

Innumerous cell models using iPSCs have been developed for lysosomal disorders, mainly for the ones with neurological involvement.[44] This is justifiable since little is known about the pathophysiological processes involved in neurodegeneration and access to relevant models is generally scarce. Differentiation in cardiomyocytes is also a big hit, as some lysosomal disorders have major cardiovascular pathology[45] that is not reverted with approved or even experimental therapies, like gene therapy.[46] Lastly, hematopoietic stem cells have been targeted for differentiation of iPSCs as well, as they can become a source of cells for autologous transplantation.[44]

For developing these iPSCs models, fibroblasts or blood cells are collected from patients, reprogrammed in vitro and, then, differentiated into the cell of interest. This way, the newly created model carries the patient's pathogenic variants and will present the disease phenotype. For controlled studies, however, an isogenic healthy control is required, as the cells behave differently depending on the genetic background. To address this, genome editing tools can be used to correct the disease-causing mutation in the iPSCs population, before the differentiation protocol.[47] In the absence of a patient's cells, healthy iPSCs can be edited to present various genotypes and to mimic the disease of interest.[48,49] A complete review about using iPSCs for LD studies was published by Borger and colleagues.[44]

Although the possibilities are virtually endless, the use of iPSCs is still dispendious, limiting its broad use. Cell lines, on the other hand, are much more affordable—they are easy to culture and have high reproducibility of results. They are generally easy to transfect, making the editing process easier, and many different cell types are available—including endothelial, myeloid, lymphoid and neuron-like cells, for example, in case specific

morphologic characteristics are required. Here we reference some cell models for LD, created using genome editing tools, which became important resources for basic research and therapy development.

Fabry disease is caused by a deficiency of α-galactosidase A (GLA). The most common manifestations include renal and cardiovascular disease. Several cell lines for modeling Fabry disease have been developed using CRISPR-mediated genome editing. One of the first studies knocked-out *GLA* in the human embryonic kidney (HEK) 293 cells, to create a simple model for drug screening purposes.[50] In the same year, a more relevant cell type to study the disease was developed—immortalized podocytes were edited with CRISPR-Cas9 for microarray studies, identifying MAPK, VEGF and TGF-beta pathways as enriched in Fabry cells,[51] all of which are also involved in other glomerular diseases.

Fabry patients have increased vasculopathy incidence than healthy individuals due to the accumulation of substrate in endothelial cells. Thus, the endothelial cell line EA.hy926 was used to help understand some of the disease's mechanisms. GLA-deficient endothelial cells have lower endothelial nitric oxide synthase (eNOS) and treatment with substrate reduction therapy did not affect in this, suggesting that the absence of the enzyme rather than the accumulation of the substrate that affects eNOS activity.[52] Further investigation with the same model showed that decreased eNOS leads to increased secretion of von Willebrand factor and this is not altered neither by substrate reduction therapy nor administration of recombinant GLA.[53] Therefore, this aspect of the disease should be addressed separately. In a different approach, human embryonic stem cells (hESC) knocked-out for *GLA* were differentiated in cardiomyocytes, as cardiomyopathy is an important manifestation of Fabry disease. Proteome analysis of the new model showed downregulation of exocytotic vesicle release proteins, causing impaired autophagic flux and protein turnover and ultimately leading to increased apoptosis.[54] Altogether, these findings provide new insights about the disease pathophysiology and can help identify new targets for vasculopathy and cardiomyopathy in Fabry disease.

Macrophages are key cells in diseases of lipid metabolism, like atherosclerosis, acid lipase deficiency, Niemann-Pick and Gaucher diseases, in which they become foamy cells after an accumulation of lipids in their lysosomes. To explore the loss-of-function phenotype and accumulation of lipids in the macrophages, Zhang and colleagues[49] generated iPSCs knockout for lipase A and induced the differentiation of these cells to macrophages, observing the macrophage-specific response to lipase A deficiency. Alternatively,

macrophage-like cells can be obtained using the hematopoietic cell line THP-1 and submitting them to a differentiation protocol, as developed to model Gaucher disease.[55] An advantage of using this last instead of other approaches is the possibility of high-throughput studies, mainly for drug testing and screening, as cell lines are better suited for large scale production.

Besides the macrophage model aforementioned, a microglia-like cell model for Gaucher disease using the human U87 cell line was also developed by Pavan and colleagues,[55] in an attempt to generate a more relevant model to study the neuronal component of the disease. U87 *GBA1* mutant cells showed retention of misfolded GBA in the endoplasmic reticulum, increased production of interleukin-1-beta, α-synuclein accumulation and increased cell death compared to non-edited cells. Accordingly, α-synuclein accumulation was also observed in GBA-knockout HEK293[56] and heterozygote mutant for Cathepsin D gene (CTSD), responsible for a lysosomal hydrolase that causes neuronal ceroid lipofuscinosis when absent.[57] Even though these models were created with different cell types and targeting different genes, they all conversely contributed to understanding part of lysosomal function. These are not useful only for lysosomal disorders; GBA-knockout HEK293 and A549 cells were used to study endocytic processes in viral infections. It was found that endocytic trafficking of viruses and cellular cargos are impaired in GBA-knockout cells.[58]

There are few Niemann-Pick type C (NPC) cell models generated with CRISPR-Cas9. The simplest is a near-haploid human cell line (HAP1) edited to carry new or private mutations in the *NPC1* gene that have been found in patients and lacked characterization.[59] Because it is a near-haploid line, editing is simplified, making this model useful mainly to resolve clinical interpretation of new variants. The classic HeLa and the CHO-Ldl-D cell lines were also knocked-out to generate different NPC models: the first was created to be used in drug screening studies, as HeLa cells are easy to culture.[60] The second was used to study glycosylation inhibition and its effect on cholesterol accumulation, in an attempt to better understand the biology of lysosomes.[61]

Using genome editing to create LDs cell models is a useful tool for basic research, initial screenings and proof-of-concept studies. For example, one can observe the effect of different pathogenic variants all at once in the same cell type; or the effect of a single variant in many cell types (Fig. 2). Lysosome biology can be explored and, this way, pathogenic mechanisms of lysosomal diseases can be elucidated.

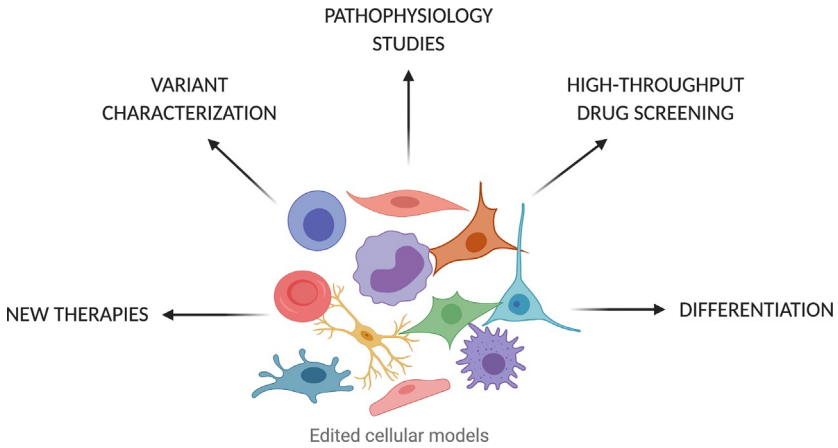

Fig. 2 Applications of genetically edited cell lines in disease modeling. Cell lines are easy to obtain and maintain in culture, thus being excellent models for initial pathophysiology studies, development of new therapies and high-throughput drug screening. They can also be used to characterize new variants observed in patients and, in cases where the line can be differentiated into other cell types, the process can be observed in the disease context.

7. Genome-edited animal models

As mentioned previously, cellular models play a role in several aspects, including proof-of-concept studies, for emergent therapeutic strategies. However, the use of multicellular transgenic model organisms is mandatory to study disease pathogenesis and treatment options, because they can more closely recapitulate the complex metabolic and cellular pathways occurring in patients. As a standard, the murine model has been the most used in this field; however, some of these do not manifest the clinical aspects of the LDs; as seen in the Gaucher, Fabry, Tay-Sachs, and cystinosis mouse models, compromising the understanding of experimental therapies.[62] Despite several mammalian species, including cats and dogs, naturally develop some of the LDs seen in humans, researchers have taken advantage of genome editing tools to develop models according to their research needs.

Zebrafish has become an interesting non-mammalian model, and its use in LDs studies has raised notably through the years. The reason is due to high comparable anatomy to humans, where the major systems and organs are present, and also its genetics—82% of all human disease-associated genes have orthologues in this model. Lately, a considerable number of stable

mutants fish strains have been generated with these approaches (TALENs: 6, CRISPR-Cas9: 10; combined TALENs/CRISPR-Cas9: 1).[63] Mutants generated for Gaucher and Sandhoff diseases displayed sphingolipid accumulation, microglial alterations, neuron loss, apoptosis, and impaired locomotion. For mucolipidosis IV, pathological signs in the muscle and eye were observed in the models. The Niemann-Pick type C1 models displayed hepatic disease features as lipid storage, vacuolated hepatocytes and also Purkinje cell defects. Finally, the MPS II model showed most of the clinical presentations seen in patients, as impaired development, abnormal heart morphogenesis, and bone alterations, like craniofacial defects, scoliosis, and kyphosis. An extended review of Zebrafish for LD studies was recently published by Zhang and Peterson.[63]

Lastly, an ovine model for infantile neuronal ceroid lipofuscinosis (NCL1) was developed aiming to better translate the human condition. The sheep model presented biochemical, morphological, and neurological alterations including loss of vision and reduced lifespan.[64] With this, the use of genome editing tools to develop more accurate animal models that resemble the human disease condition holds promising potential.

8. Therapies based on genome editing

Moving to therapeutic approaches, unlike the classical gene therapies, genome editing came up with the possibility of a precise and one-time long-term treatment. That also includes a significant decrease in off-target activities and immunogenic risks related to the former ones.

Some LDs are better candidates for genome editing based therapies than others. As indicated previously, the majority of them rely on the lack of production of a secreted protein. Editing tools can be employed to develop a unified platform that allows the production of any of these proteins since only one gene is the target for each condition. From in vitro to in vivo studies, researchers have been working on the choice of the proper features for the desired approach, and the majority of efforts have brought positive and promising results. Herein below are presented the leading studies that comprise proof of concepts in a cell model, development of ex vivo stem cell therapy platforms, and in vivo preclinical studies (Table 2).

8.1 In vitro mutation-specific genome editing

Most LDs have a vast number of genetic variants reported, resulting in a broad range of phenotypes. Depending on the complexity of the patient

Table 2 Genome editing studies for lysosomal disorders.

Preclinical studies in cell models

Disease	Affected gene	Targeted gene/locus	Platform	Cell type	Delivery method	Genetic modification	References
MPS I	*IDUA*	*IDUA*	CRISPR-Cas9	Patient fibroblasts	Plasmid-Liposome complex	SNV correction	65,66
MPS I	*IDUA*	*IDUA*	CRISPR-Cas9	Mouse iPSCs	Plasmids	Precise deletion	67
Tay-Sach	*HEXA*	*HEXA*	Prime editing	HEK293T cell	Plasmids	SNV correction	43
Fabry	*GLA*	*GLA*	CRISPR-Cas9	Patient fibroblast	NS	SNV correction	68
Fabry	*GLA*	*GLA*	CRISPR-Cas9	Patient iPSCs	RNP	Deletion correction	69

Preclinical studies in murine models

Disease	Affected gene	Targeted gene/locus	Platform	In vivo/ex vivo	Delivery method	Genetic modification	References
Gaucher	*GBA*	CCR5	CRISPR-Cas9	Ex vivo	RNP/AAV6	Knock-in	70
Pompe	*GAA*	AAVS1	CRISPR-Cas9	Ex vivo	Plasmids	Knock-in	71
Krabbe	*GALC*	IL2RG	CRISPR-Cas9	Ex vivo	RNP/AAV6	Knock-in	72
Wolman disease	*LAL*	α-globin	CRISPR-Cas9	Ex vivo	RNP/AAV6	Knock-in	73
Fabry	*GLA*	α-globin	CRISPR-Cas9	Ex vivo	RNP/AAV6	Knock-in	73
MPS I	*IDUA*	α-globin	CRISPR-Cas9	Ex vivo	RNP/AAV6	Knock-in	73

MPS I	*IDUA*	*CCR5*	CRISPR-Cas9	Ex vivo	RNP/AAV6	Knock-in	74
MPS I	*IDUA*	*ROSA26*	CRISPR-Cas9	In vivo	Liposome and plasmid vectors	Knock-in	75
MPS I	*IDUA*	*ALB*	ZFNs	In vivo	3 AAV2/8 vectors	Knock-in	76
MPS I	*IDUA*	*ALB*	CRISPR-cas9	In vivo	2AAV 8 vectors	Knock-in	77
MPS I	*IDUA*	*IDUA*	CRISPR-cas9	In vivo	2AAV9 vectors	Allelic Exchange/ SNV correction	78
MPS II	*IDS*	*ALB*	ZFNs	In vivo	3 AAV2/8 vectors	Knock-in	79
Gaucher	*GBA*	*ALB*	ZFNs	In vivo	2 AAV8 vectors	Knock-in	80
Fabry	*GLA*	*ALB*	ZFNs	In vivo	2 AAV8 vectors	Knock-in	80
Sandhoff/ Tay-Sach	*HEX*	*ALB*	CRISPR-Cas9	In vivo	2 AAV8 vectors	Knock-in	81
Niemman Pick C	*NPC1*	*NPC1*	CBE	In vivo	AAV9 vector	SNV correction	82

Summary of preclinical and clinical studies using genome editing for LDs. AAVS1, AAV integration site; ALB, albumin; CBE, cystine base editors; CCR5, chemokine (C—C motif) receptor 5; GAA, acid α-glucosidase; GALC, galactosylceramidase; GBA, glucocerebrosidase; GLA, α-galactosidase; HEX, β-hexosaminidases; IDS, iduronate 2 sulfatase; IDUA, iduronidase; IL2RG, interleukin-2 receptor gamma chain; LAL, lysosomal acid lipase; NS, not specified; RNP, ribonucleoprotein; SNV, single nucleotide variant.

variant, it is possible to "change" a single mismatched nucleotide or to correct a small indel with precision using genome editing tools. These edits have been assessed for different LDs using the CRISPR-Cas9 system in cellular models or even in patient's cells.

MPS I is one of the common LDs worldwide, with a prevalence of 1/100.000 births.[83] As well as other well-known MPS, it presents a broad spectrum of phenotypes that can be frequently associated with the genotype. The severe one (MPS I-H), is the most common among western patients and is the one with progressive neurologic impairment. This phenotype has been related to several pathogenic variants, but the most frequent is p. Trp402Ter.[83] Researchers evaluated the capability of the CRISPR-Cas9 system and a single-strand donor oligonucleotide complexed in commercial liposome to correct this variant in patient fibroblasts.[65] A similar approach was carried out later, but this time using cationic nanoemulsions as a non-viral delivery vector.[66] In both, IDUA activity was significantly increased (2–6% of normal) and the lysosomal mass was decreased in treated fibroblasts. The presence of correctly edited cells was confirmed by next-generation sequencing[65] and nanoemulsion/DNA complexes were well tolerated by cells (viability of about 80%),[66] demonstrating the potential of this approach for further studies. Nanomaterials such as nanoemulsions, liposomes, and nanoparticles have been studied in the past years as an alternative drug/gene delivery system for the treatment of many diseases and conditions.[84] The oil-water phase nanoemulsions have shown a notable cellular targeting ability due to their 1–100 nanometers (nm) droplet size, and their biocompatible and non-toxic materials that are commonly safe (GRAS) grade excipients approved by FDA.[85] Their nature allows them to protect the cargo (molecule to carry) and to bind and fuse with cell membranes since many of their components are lipids. Also, they have an increased half-life in circulation due to hydrophilic polymers such as PEG (polyethylene glycol) that avoid their interaction with plasma macrophages reducing their clearance.[86] Some nanoemulsion dosage forms, mostly carrying small molecules or active biopharmaceuticals, are already in the pharmaceutical market and several others are under development.[85] As well as we presented earlier for lysosomal storage diseases, nanoemulsions have been studied as nanocarriers to help the delivery of nucleic acids targeting brain tumors[86,87] and neuroinflammatory conditions,[88] with promising results, demonstrating reasonable transfection efficiency and lower cytotoxicity carrying RNA.

In the cardiac phenotype of Fabry disease, the vasculopathy is caused by the accumulation of Gb3 which, in turn, is caused by different pathogenic variants in *GLA*. Using CRISPR-Cas9 ribonucleoprotein (RNP) and single-stranded oligodeoxynucleotide (ssODN), the correction of a genomic deletion—c.803_806del, causing a frameshift in exon 6 and amino acid position 268—was performed in the patient's fibroblast-derived iPSCs. After differentiation into vascular endothelial cells, a significant improvement in GLA activity and enhanced expression of the angiogenic factors was observed when compared to non-differentiated iPSCs.[69] In a different study, a double sgRNA was employed to target the deletion IVS4 + 919 G > A in the *GLA* gene of FD patient's fibroblasts—a common variant in the cardiac type. This strategy succeeded in restoring the aberrant splicing, causing an increase in the enzyme activity of GLA and a decrease in the Gb3 accumulation.[68]

The CRISPR-Cas9 system was also studied to mediate gene insertion in cell models. The approach could be useful when complex pathogenic variants are present. As an example, the complete restoration of the exon VI in the *IDUA* gene was performed in iPSCs derived from knockout mouse embryonic fibroblasts.[67] The strategy consisted of removing the whole neomycin resistance gene placed into the exon VI of *IDUA* with double guide RNAs, and several donor templates, to allow complete coverage of the exon VI. The correction was successful without any undesired indels, and enzyme function was restored to levels equivalent to wild-type iPSC-derived fibroblasts; plus, the pluripotency of iPSCs was maintained.[67]

A more recent genome editing platform, called prime editing, is a more precise tool that allows modifying genes without the necessity of DSB and a long DNA donor.[43] As proof-of-concept, it was used to correct the most common mutation that causes Tay-Sachs disease, a 4-bp insertion in *HEXA* ($HEXA^{1278+TATC}$), in a HEK293T cell line model that was generated previously using the same approach. As a result, prime editing allowed the correction of the mutation with 33% efficiency and 0.32% indel formation, showing its potential to correct genetic variants associated with human diseases.[43]

8.2 Ex vivo genome editing targeting safe harbors loci

Besides these in vitro studies, where the goal was editing specific disease-causing mutations, other researchers have searched for different genomic regions as the target for editing. They use "safe harbors" loci

to integrate the entire cDNA of the functional gene to overcome the limitations of correcting each gene mutation individually. It is unlikely feasible that a specific therapeutic strategy targeting a dedicated mutation can reach the clinic when patients are very rare and there are a large number of disease-causing mutations. Moreover, the presence of pseudogenes hinders the design of gRNAs and donor templates and increase the risk of undesired genome recombination. To address these issues, studies using safe harbors, tissue-specific promoters and ex vivo or in vivo platforms have been conducted.

As mentioned previously in the introduction, allogeneic HSCT is to date the gold standard for the treatment of a few LDs. Ex vivo gene therapy using genome editing strategies allows for autologous transplantation, where the patient's cells are collected, edited outside the body and transplanted back into the patient. This approach removes the need for matching donors and reduces complications related to the procedure, like graft-vs-host disease. Besides hematopoietic stem cells (HSC), mesenchymal stromal cells and neural stem cells have been studied for gene therapy, due to their ability to engraft and migrate to the brain and bone.[89]

Pavani and colleagues proposed the use of the α-globin locus as a promising "safe harbor" candidate to integrate and express therapeutic transgenes.[73] The α-globin genes are present in four copies per cell and are expressed at an extremely high level by erythroid lineages. Also, it has been reported that the loss of three of the α-globin alleles does not represent a negative impact on the organism.[73] Since erythroid cells are the most abundant hematopoietic progeny, they hold the potential to secrete relevant amounts of therapeutic proteins as required for LDs. Using Cas9-gRNA ribonucleoprotein (RNP) and AAV6 donor vector, researchers targeted the α-globin locus in CD34+ cells, purified from human umbilical cord blood (UCB), to integrate lysosomal acid lipase (LAL), α-L-iduronidase (IDUA), or α-galactosidase (GLA) transgenes. As a result, mRNA and enzymes were detected post-transduction and at increased levels after erythroid differentiation. The enzyme produced by the erythroblasts derived from LAL-HSPCs presented cross-correction activity when co-cultured with patients' cells. Additionally, edited cells maintained their repopulation and multilineage potential after transplantation in immunodeficient NOD SCID gamma (NSG) mice.[73]

Another potential candidate as a safe harbor locus for LDs gene addition therapies is the chemokine (C-C motif) receptor 5 (CCR5), which is used in prospective therapies for HIV infection. This gene encodes an HIV-1

receptor, and when mutated, minimal consequences such as increased vulnerability to other virus infections (influenza or West Nile virus) have been described besides conferring resistance to HIV-1 infection in individuals.[90,91] However, as a matter of future concerns, it has been shown the potential risk of inducing a null CCR5 phenotype in bone development and immune system regulation in mice.[91]

For LDs emergent therapies, Gomez-Ospina and colleagues reported an efficient ex vivo genome editing approach using CRISPR-Cas9 and AAV6 to targeted α-L-iduronidase into CCR5 locus in human CD34+ cells under the control of synthetic promoters such as spleen focus-forming virus (SFFV) and 3-phosphoglycerate kinase (PGK). The rationale of the promoter choice was to exploit their constitutiveness nature and guarantee the expression of the transgene in all hematopoietic lineages to facilitate cross-correction.[74] After transplantation into immunodeficient NSG-MPS I mice, the engineered cells secreted supra-endogenous enzyme levels, improving phenotypical and biochemical features of MPS I, including neurological impairment. Edited cells maintained the self-renewal capacity and multilineage differentiation potential, but the engraftment of the edited cells was less efficient compared to unedited cells, 3.9% (0.8–9.7%) and 30.4% (7.7–48.2%) respectively. This lower engraftment was shown in cells modified at other loci as well.[92–94] Besides effective, this approach also proved to be safe, since the off-target activity of the CCR5 sgRNA was undetectable with the use of high-fidelity Cas9, p53 pathways were not altered and there was no evident tumorigenicity.[74]

More recently, the same platform was used with slight modifications. Human CD34+ cells were edited with a glucocerebrosidase expression cassette driven by a monocyte/macrophage-specific promoter (CD68) into the CCR5 locus.[70] The rationale of this strategy is related to the hallmark manifestations in Gaucher disease, which are mainly caused due to infiltration and inflammation by macrophages. While edited HSC did not express the enzyme, differentiated monocytes/macrophages had increased glucocerebrosidase activity both in vitro and 16 weeks post-transplantation, as measured in sorted cells from bone marrow, spleen and lung. The use of a specific promoter restricted enzyme expression to affected cells only and prevented ectopic (and possibly toxic) expression in hematopoietic stem cells.[70]

Different stem cells, such as neural stem cells (NSCs), can be a promising platform for the therapy of some LDs with CNS involvement. NSCs cells have shown a high capability of neural multilineage differentiation,

migration, and proliferation when they are transplanted into the brain or spinal cord of NSG mice.[95] NSCs are suitable for in vitro manipulation as they continue to express NSC markers after been isolated, propagated, and banked.[72] To date, only one proof-of-concept study has demonstrated CRISPR-Cas9 genome editing in NSCs, using the Krabbe disease as model.[72] In this study, the authors were able to edit human brain-derived NSCs to produce the enzyme galactocerebrosidase (GALC) under the regulation of the IL2RG promotor. This locus was described as a safe harbor in NSCs due to the lack of functional role in these cells. Edited cells cross-corrected fibroblasts from Krabbe patients. Moreover, after transplantation into the cerebellum of juvenile mice, cells engrafted and maintained biological properties.[72]

Finally, iPSCs are also suitable candidates for ex vivo therapy due to their pluripotency and self-renewal characteristics. It was demonstrated by Van de wal et al. that iPSCs from Pompe disease patients can be edited in vitro to introduce the cDNA for the enzyme acid α-glucosidase (GAA) into the AAVS1 locus, another human safe harbor. Myogenic progenitors were generated from edited iPSCs and their differentiation capability into myotubes was assessed. In these cells, the correction of the disease was evident since GAA activity was threefold higher compared to healthy controls. Also, the restoration of the enzyme activity led to the normalization of glycogen accumulation. To assess their therapeutical potential, edited myogenic progenitors were transplanted in the tibia muscle of pre-injured NSG mice, where they engrafted robustly and contributed to muscle regeneration at the injured-site.[71]

8.3 In vivo genome editing

In vivo systemic genome editing strategies for the treatment of LDs have also been studied. Some of these strategies managed to reach clinical trials, but several approaches using CRISPR-Cas9, zinc-finger nucleases, and other newly emerging systems have only been used in preclinical studies aiming for reliable and efficient therapies.

For in vivo genome editing approaches, liposomes and viral vectors are the most promising strategies. Liposomes are highly utilized for delivering different cargoes, from small therapeutic molecules to gene therapy products such as plasmids. This non-viral delivery was studied aiming for the correction of Hurler syndrome in the MPS I mouse model.[96] As a proof of concept, cationic liposomes carrying CRISPR-Cas9 and cDNA donor plasmids

targeting the ROSA26 locus were administrated in neonatal MPS I mice by a single hydrodynamic injection. At 6 months, treated mice presented an increase in enzyme activity in every visceral tissue, with the highest levels in the heart and lungs (~5% of normal activity). Indeed, the biodistribution of the complex showed a high affinity of the cationic liposome for these tissues.[96] The enzyme produced by edited cells also led to a significant reduction in the substrate (GAGs) accumulation and secretion in serum, urine and visceral organs, though still not to normalized levels. The effects of gene editing were also evidenced in the improvement of cardiovascular, respiratory and bone pathology.[75] Nevertheless, this approach failed to reach the CNS.

The albumin locus has also been shown to be a safe harbor in vivo. Researchers engineered a flexible platform where this locus is used for transgene expression without interrupting hepatic albumin production, exploiting the strong promoter activity.[97] Using AAV8 vectors (with a high tropism for hepatocytes) zinc-finger nuclease (ZFN), and cDNA donor templates were delivered systemically to target overexpression of α-galactosidase A, acid β-glucosidase, iduronate-2 sulfatase, or α-L-iduronidase in wild-type mice liver.[80] Donor DNA was designed to allow, after appropriate recombination, directed-splicing between intron 1 of the albumin locus and the transgene cDNA, to produce a functional hybrid therapeutic transcript. After treatment, the therapeutic enzymes were found in liver extracts and plasma, along with albumin protein.[80] To evaluate the clinical potential of the platform, more studies were held in MPS I and II mouse models, using AAV2/8 as delivery vectors.[76,79] In treated mice, supraphysiological levels of the enzyme were observed in serum, while GAGs content in urine was found significantly decreased in both disease models. The therapeutic enzymes were uptaken from the blood to other tissues with high enzyme activity levels detected in the spleen, heart, lung and muscle. As a remarkable finding, along with the correction of the metabolic disease, the strategy managed to prevent neurologic impairment in young MPS I and II mice.[76,79] These results suggested that supraphysiological levels of the enzyme produced by the liver, which is commonly achieved using this type of vector in animal models, allowed a fraction of IDUA/IDS to penetrate the BBB. Thus, even a small percentage of normal enzyme levels in the brain seems sufficient to prevent neurologic deficit.[79] This was different from what was observed previously from Schuh and colleagues,[96] where the levels of the enzyme compared to normal conditions were about 10% in liver and heart tissue and 2–5%

in the other visceral tissues. Thus, there was no sign of the enzyme in the brain.

This last strategy resulted in two clinical trials, which will be covered in the next section of this chapter. However, preliminary results showed a low expression of targeted enzymes in treated individuals. Aiming for an optimized outcome, the platform was recently redesigned using the CRISPR-Cas9 system instead of ZNF-nucleases.[77] Compared to the previous strategy, which required the co-transduction of three different AAV8 vectors to deliver the whole system, the new approach only uses two. The strategy succeeded in correcting both the metabolic and the neurological features of MPS I mice. Furthermore, no tumorigenesis or off-target activity was detected.[77] Additionally, the platform was tested recently to deliver a modified human Hex μ subunit (HEXM) into the albumin locus.[81] Since HEXM forms a homodimer that degrades GM2 gangliosides, the strategy represents a promising unified therapy for both Tay-Sachs and Sandhoff diseases.[81] Importantly, along with the increase in enzyme activity in the brain, memory was improved in treated mice when compared to untreated animals. Despite these promising results, more studies are needed before this new platform can reach clinical trials since this approach used a high volume/pressure injection to deliver the vectors, which is hard to scale up to humans.

Finally, the correction of some specific point mutations was also investigated in vivo using genome editing tools. In one study, allelic exchange was induced in a compound heterozygous MPS I mice model using the CRISPR-Cas9 system.[78] Here, a unique gRNA targeting an intronic site was used to create double-stranded DNA breaks in both chromosomal homologs aiming at switching arms between chromosomes in a chromosomal translocation-like mechanism. Researchers were able to restore IDUA levels (~0.5% of the wild-type level) in the heart of young treated mice accompanied by a considerable GAGs reduction. The results indicated the possibility of inducing allelic exchange in post-mitotic tissues. Nevertheless, chromosomal translocation occurs in low frequency compared to any other strategy based on NHEJ or HDR.[78] In another study, a full-length cytosine base editor was modified and split in a dual-AAV packaging vector.[82] After delivery into the organism, the components undergo reconstitution by trans-splicing machinery, overcoming the main limitation of reduced packaging capacity that AAV vectors have.[82] This dual strategy was tested in *Npc1*^{I1061T} (c.3182 T > C) homozygous mice, a model that reproduces the neurological pathology and also has reduced

lifespan. Low and high doses of the dual vectors were delivered by retro-orbital injections. Mice who received the higher dose survived longer than untreated and low-dose mice. Genomic DNA from the brain of surviving animals revealed that a total of 94% of alleles were edited—C-to-T—without any undesirable indels in the region.

9. Clinical trials for lysosomal disorders

Gene editing technology for LDs has been successfully applied in a variety of preclinical models, both ex vivo and in vivo.[75,77,80,98] The promising results from these studies have provided the basis for the development of clinical trials.

To date, two new therapies using gene editing, named SB-318 and SB-913, are being tested as a single treatment therapy for MPS I and MPS II patients, respectively. Both were designed using ZFN system to insert a therapeutic transgene into the albumin locus of the patient's liver cells.[77] Each one of these therapies consists of the intravenous infusion of two ZFNs (left and right arms), targeting the albumin locus and the corrective donor gene (*IDUA* or *IDS*) packaged into AAV2/6 vectors. This one-time therapy has the potential to provide permanent lifetime production of the impaired enzyme, to improve the current clinical outcome of ERT or HSCT, and the quality of life for patients.[99]

EMPOWERS and CHAMPIONS (clinicaltrials.gov, NCT02702115 and NCT03041324) are the two Phase 1/2 clinical trials, for respectively SB-318 and SB-913, that are ongoing in the U.S. They are multicenter, open-label, dose-escalating studies. Subjects received gene editing and initiated under protocol-specified schedule with monitoring of safety, IDS or IDUA activity and urinary glycosaminoglycan (GAG) levels, and functional assessments.[100,101] At present, the data analysis for both studies is still in progress.

The CHAMPIONS study started in 2017, being the first-ever clinical trial to attempt in vivo genome editing in patients with LDs. The purpose of this study is to assess the safety, tolerability and preliminary efficacy (changes from baseline in plasma IDS activity and urine GAG levels) of ascending doses of SB-913 in subjects with attenuated MPS II. MPS II patients receive a single-dose of SB-913 with 3 years of follow-up. A three-dose cohort with two subjects each is expected, along with three additional individuals who will receive the high dose, in an expanded cohort.[101] Interim data from the first three cohorts showed that SB-913

was generally well tolerated and no serious adverse events related to the drug were reported. Some adverse events related to the study drug were mild or moderate and were eventually resolved. The incidence of adverse events was assessed by Common Terminology Criteria for Adverse Events (CTCAE) and includes pruritus, flushing, erythema, increased serum transaminases, headache and pyrexia. Preliminary analysis of liver tissue biopsy, using RT-qPCR, showed evidence of albumin-IDS mRNA transcript in both subjects at the mid-dose, suggesting that genome editing had occurred. A substantial increase in plasma IDS activity was observed only in one patient (mid-cohort). Nevertheless, it decreased after the development of mild transaminitis, a known risk of AAV-based therapies.[101,102]

EMPOWERS is a clinical trial started in mid-2018 and designed, firstly, to assess the safety and tolerability of ascending doses of SB-318 in adult subjects with the mild form of MPS I and, secondly, to evaluate the changes from baseline in IDUA activity and urine GAG levels. The clinical design is similar to the CHAMPIONS study. Here, two-dose cohorts with two subjects each are conducted, along with an expansion of five additional subjects who will receive the high dose (total of nine subjects).[100] Interim data of the first 3 subjects across 2 dosing cohorts (1 patient with a low dose and 2 patients with high dose), showed that the administration of SB-318 was generally well tolerated without adverse events related to SB-318 treatment. Increases in leukocyte IDUA activity were observed in all three subjects and suggest a dose-dependent increase. On the other hand, plasma IDUA activity was not significantly changed from pre-treatment values.[100] The results provided so far indicate that efficacy needs to be improved since the levels of transgene expression are low.[77] This low expression seems to be the major obstacle and has already been described in other clinical trials for gene therapy.[77] As previously reported in animal studies, the low transgene expression issue may be improved by increasing the dose or through repeating administration, for example, using lipid nanoparticles delivery. Increasing the dose, however, could lead to a higher risk of toxicity, challenging vector production, and increased manufacture cost.[77] Preclinical studies indicated that CRISPR technology has higher efficiency than ZFN system with lower doses of AAV vector, which minimizes the risk of toxicity as well reduced complexities and challenges regarding viral vector manufacturing.[77] Otherwise, the use of lipid nanoparticle delivery enables repeated administrations, resulting in efficient in vivo genome editing, being a useful alternative tool.[103]

10. Conclusions

There are a myriad of applications for genome editing in LDs, including the development of cell and animal models to study disease pathogenesis, as well as permanent therapeutic approaches. Genome editing allows recreating the same mutation found in patients, which can be essential to develop individualized therapies. Therefore, we believe that the number of studies on LDs using techniques to rewrite the genome will grow exponentially in the next years.

References

1. Johnson WG. Disorders of glycoprotein degradation: sialidosis, fucosidosis, α-mannosidosis, β-mannosidosis, and aspartylglycosaminuria. In: *Rosenberg's Molecular and Genetic Basis of Neurological and Psychiatric Disease*. 5th ed. Elsevier; 2014:369–383. https://doi.org/10.1016/B978-0-12-410529-4.00033-4.
2. Waters D, Adeloye D, Woolham D, Wastnedge E, Patel S, Rudan I. Global birth prevalence and mortality from inborn errors of metabolism: a systematic analysis of the evidence. *J Glob Health*. 2018;8(2). https://doi.org/10.7189/jogh.08.021102, 021102.
3. Hsu T-R, Niu D-M. Fabry disease: review and experience during newborn screening. *Trends Cardiovasc Med*. 2018;28(4):274–281. https://doi.org/10.1016/j.tcm.2017.10.001.
4. Mechtler TP, Stary S, Metz TF, et al. Neonatal screening for lysosomal storage disorders: feasibility and incidence from a nationwide study in Austria. *Lancet*. 2012;379 (9813):335–341. https://doi.org/10.1016/S0140-6736(11)61266-X.
5. Marques ARA, Saftig P. Lysosomal storage disorders—challenges, concepts and avenues for therapy: beyond rare diseases. *J Cell Sci*. 2019;132(2):221739. https://doi.org/10.1242/jcs.221739.
6. Darios F, Stevanin G. Impairment of lysosome function and autophagy in rare neurodegenerative diseases. *J Mol Biol*. 2020;432(8):2714–2734. https://doi.org/10.1016/j.jmb.2020.02.033.
7. Pfeffer SR. NPC intracellular cholesterol transporter 1 (NPC1)-mediated cholesterol export from lysosomes. *J Biol Chem*. 2019;294(5):1706–1709. https://doi.org/10.1074/jbc.TM118.004165.
8. Petersen NHT, Olsen OD, Groth-Pedersen L, et al. Transformation-associated changes in sphingolipid metabolism sensitize cells to lysosomal cell death induced by inhibitors of acid sphingomyelinase. *Cancer Cell*. 2013;24(3):379–393. https://doi.org/10.1016/j.ccr.2013.08.003.
9. Platt FM, D'Azzo A, Davidson BL, Neufeld EF, Tifft CJ. Lysosomal storage diseases. *Nat Rev Dis Primers*. 2018;4(1):27. https://doi.org/10.1038/s41572-018-0025-4.
10. Maegawa GHB. Lysosomal leukodystrophies lysosomal storage diseases associated with white matter abnormalities. *J Child Neurol*. 2019;34(6):339–358. https://doi.org/10.1177/0883073819828587.
11. Lachmann RH. Treating lysosomal storage disorders: what have we learnt? *J Inherit Metab Dis*. 2020;43(1):125–132. https://doi.org/10.1002/jimd.12131.
12. Ferreira CR, van Karnebeek CDM, Vockley J, Blau N. A proposed nosology of inborn errors of metabolism. *Genet Med*. 2019;21(1):102–106. https://doi.org/10.1038/s41436-018-0022-8.

13. Reisin R, Perrin A, García-Pavía P. Time delays in the diagnosis and treatment of Fabry disease. *Int J Clin Pract*. 2017;71(1), e12914. https://doi.org/10.1111/ijcp.12914.
14. Berger JR. Misdiagnosis of multiple sclerosis in a female heterozygote with Fabry's disease. *Mult Scler Relat Disord*. 2019;30:45–47. https://doi.org/10.1016/j.msard.2019.01.040.
15. Cox TM. Lysosomal diseases and neuropsychiatry: opportunities to rebalance the mind. *Front Mol Biosci*. 2020;7:177. https://doi.org/10.3389/fmolb.2020.00177.
16. Mistri M, Mehta S, Solanki D, et al. Identification of novel variants in a large cohort of children with Tay–Sachs disease: an initiative of a multicentric task force on lysosomal storage disorders by Government of India. *J Hum Genet*. 2019;64(10):985–994. https://doi.org/10.1038/s10038-019-0647-8.
17. Beutler E, Nguyen NJ, Henneberger MW, et al. Gaucher disease: gene frequencies in the Ashkenazi Jewish population. *Am J Hum Genet*. 1993;52(1):85–88.
18. Málaga DR, Brusius-Facchin AC, Siebert M, et al. Sensitivity, advantages, limitations, and clinical utility of targeted next-generation sequencing panels for the diagnosis of selected lysosomal storage disorders. *Genet Mol Biol*. 2019;42(1):197–206. https://doi.org/10.1590/1678-4685-gmb-2018-0092.
19. Hanss Z, Boussaad I, Jarazo J, Schwamborn JC, Krüger R. Quality control strategy for CRISPR-Cas9-based gene editing complicated by a pseudogene. *Front Genet*. 2020;10:1297. https://doi.org/10.3389/fgene.2019.01297.
20. Zampieri S, Cattarossi S, Bembi B, Dardis A. GBA analysis in next-generation era. *J Mol Diagn*. 2017;19(5):733–741. https://doi.org/10.1016/j.jmoldx.2017.05.005.
21. Froissart R, Da Silva IM, Maire I. Mucopolysaccharidosis type II: an update on mutation spectrum. *Acta Paediatr*. 2007;96(455):71–77. https://doi.org/10.1111/j.1651-2227.2007.00213.x.
22. Haer-Wigman L, Newman H, Leibu R, et al. Non-syndromic retinitis pigmentosa due to mutations in the mucopolysaccharidosis type IIIC gene, heparan-alpha-glucosaminide N-acetyltransferase (HGSNAT). *Hum Mol Genet*. 2015;24(13):3742–3751. https://doi.org/10.1093/hmg/ddv118.
23. Beck M. Treatment strategies for lysosomal storage disorders. *Dev Med Child Neurol*. 2018;60(1):13–18. https://doi.org/10.1111/dmcn.13600.
24. Tan EY, Boelens JJ, Jones SA, Wynn RF. Hematopoietic stem cell transplantation in inborn errors of metabolism. *Front Pediatr*. 2019;7:433. https://doi.org/10.3389/fped.2019.00433.
25. Barton NW, Brady RO, Dambrosia JM, et al. Replacement therapy for inherited enzyme deficiency—macrophage-targeted glucocerebrosidase for Gaucher's disease. *N Engl J Med*. 1991;324(21):1464–1470. https://doi.org/10.1056/NEJM199105233242104.
26. Germain DP, Charrow J, Desnick RJ, et al. Ten-year outcome of enzyme replacement therapy with agalsidase beta in patients with Fabry disease. *J Med Genet*. 2015;52(5):353–358. https://doi.org/10.1136/jmedgenet-2014-102797.
27. Schoser B, Stewart A, Kanters S, et al. Survival and long-term outcomes in late-onset Pompe disease following alglucosidase alfa treatment: a systematic review and meta-analysis. *J Neurol*. 2017;264(4):621–630. https://doi.org/10.1007/s00415-016-8219-8.
28. Edelmann MJ, Maegawa GHB. CNS-targeting therapies for lysosomal storage diseases: current advances and challenges. *Front Mol Biosci*. 2020;7:291. https://doi.org/10.3389/fmolb.2020.559804.
29. Poswar F, Vairo F, Burin M, et al. Lysosomal diseases: overview on current diagnosis and treatment. *Genet Mol Biol*. 2019;42(1):165–177. https://doi.org/10.1590/1678-4685-gmb-2018-0159.
30. Giraldo P, Andrade-Campos M, Alfonso P, et al. Twelve years of experience with miglustat in the treatment of type 1 Gaucher disease: the Spanish ZAGAL project. *Blood Cells Mol Dis*. 2018;68:173–179. https://doi.org/10.1016/j.bcmd.2016.10.017.

31. Patterson MC, Mengel E, Vanier MT, Moneuse P, Rosenberg D, Pineda M. Treatment outcomes following continuous miglustat therapy in patients with Niemann-Pick disease Type C: a final report of the NPC Registry. *Orphanet J Rare Dis*. 2020;15(1):104. https://doi.org/10.1186/s13023-020-01363-2.
32. Leal AF, Benincore-Flórez E, Solano-Galarza D, et al. GM2 gangliosidoses: clinical features, pathophysiological aspects, and current therapies. *Int J Mol Sci*. 2020;21 (17):6213. https://doi.org/10.3390/ijms21176213.
33. Fischetto R, Palladino V, Mancardi MM, et al. Substrate reduction therapy with Miglustat in pediatric patients with GM1 type 2 gangliosidosis delays neurological involvement: a multicenter experience. *Mol Genet Genomic Med*. 2020;8(10):1371. https://doi.org/10.1002/mgg3.1371.
34. Peterschmitt MJ, Cox GF, Ibrahim J, et al. A pooled analysis of adverse events in 393 adults with Gaucher disease type 1 from four clinical trials of oral eliglustat: evaluation of frequency, timing, and duration. *Blood Cells Mol Dis*. 2018;68:185–191. https://doi.org/10.1016/j.bcmd.2017.01.006.
35. Germain DP, Hughes DA, Nicholls K, et al. Treatment of Fabry's disease with the pharmacologic chaperone migalastat. *N Engl J Med*. 2016;375(6):545–555. https://doi.org/10.1056/NEJMoa1510198.
36. Germain DP, Nicholls K, Giugliani R, et al. Efficacy of the pharmacologic chaperone migalastat in a subset of male patients with the classic phenotype of Fabry disease and migalastat-amenable variants: data from the phase 3 randomized, multicenter, double-blind clinical trial and extension study. *Genet Med*. 2019;21(9):1987–1997. https://doi.org/10.1038/s41436-019-0451-z.
37. Ma CC, Wang ZL, Xu T, He ZY, Wei YQ. The approved gene therapy drugs worldwide: from 1998 to 2019. *Biotechnol Adv*. 2020;40:107502. https://doi.org/10.1016/j.biotechadv.2019.107502.
38. Colella P, Ronzitti G, Mingozzi F. Emerging issues in AAV-mediated in vivo gene therapy. *Mol Ther Methods Clin Dev*. 2018;8:87–104. https://doi.org/10.1016/j.omtm.2017.11.007.
39. Broeders M, Herrero-Hernandez P, Ernst MPT, van der Ploeg AT, Pijnappel WWMP. Sharpening the molecular scissors: advances in gene-editing technology. *iScience*. 2020;23(1), 100789. https://doi.org/10.1016/j.isci.2019.100789.
40. Ho BX, Loh SJH, Chan WK, Soh BS. In vivo genome editing as a therapeutic approach. *Int J Mol Sci*. 2018;19(9):2721. https://doi.org/10.3390/ijms19092721.
41. Gaj T, Sirk SJ, Shui SL, Liu J. Genome-editing technologies: principles and applications. *Cold Spring Harb Perspect Biol*. 2016;8(12):a023754. https://doi.org/10.1101/cshperspect.a023754.
42. Porto EM, Komor AC, Slaymaker IM, Yeo GW. Base editing: advances and therapeutic opportunities. *Nat Rev Drug Discov*. 2020;19(12):839–859. https://doi.org/10.1038/s41573-020-0084-6.
43. Anzalone AV, Randolph PB, Davis JR, et al. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature*. 2019;576(7785):149–157. https://doi.org/10.1038/s41586-019-1711-4.
44. Borger DK, McMahon B, Lal TR, Serra-Vinardell J, Aflaki E, Sidransky E. Induced pluripotent stem cell models of lysosomal storage disorders. *Dis Model Mech*. 2017;10 (6):691–704. https://doi.org/10.1242/dmm.029009.
45. Nair V, Belanger EC, Veinot JP. Lysosomal storage disorders affecting the heart: a review. *Cardiovasc Pathol*. 2019;39:12–24. https://doi.org/10.1016/j.carpath.2018.11.002.
46. Poletto E, Pasqualim G, Giugliani R, Matte U, Baldo G. Effects of gene therapy on cardiovascular symptoms of lysosomal storage diseases. *Genet Mol Biol*. 2019;42 (1):261–285. https://doi.org/10.1590/1678-4685-gmb-2018-0100.

47. Allende ML, Cook EK, Larman BC, et al. Cerebral organoids derived from Sandhoff disease-induced pluripotent stem cells exhibit impaired neurodifferentiation. *J Lipid Res*. 2018;59(3):550–563. https://doi.org/10.1194/jlr.M081323.
48. Latour YL, Yoon R, Thomas SE, et al. Human GLB1 knockout cerebral organoids: a model system for testing AAV9-mediated GLB1 gene therapy for reducing GM1 ganglioside storage in GM1 gangliosidosis. *Mol Genet Metab Rep*. 2019;21:100513. https://doi.org/10.1016/j.ymgmr.2019.100513.
49. Zhang H, Shi J, Hachet MA, et al. CRISPR/Cas9-mediated gene editing in human iPSC-derived macrophage reveals lysosomal acid lipase function in human macrophages-brief report. *Arterioscler Thromb Vasc Biol*. 2017;37(11):2156–2160. https://doi.org/10.1161/ATVBAHA.117.310023.
50. Song HY, Chiang HC, Tseng WL, et al. Using CRISPR/Cas9-mediated GLA gene knockout as an in vitro drug screening model for Fabry disease. *Int J Mol Sci*. 2016;17(12):2089. https://doi.org/10.3390/ijms17122089.
51. Pereira EM, Labilloy A, Eshbach ML, et al. Characterization and phosphoproteomic analysis of a human immortalized podocyte model of Fabry disease generated using CRISPR/CAS9 technology. *Am J Physiol Renal Physiol*. 2016;311(5):F1015–F1024. https://doi.org/10.1152/ajprenal.00283.2016.
52. Kaissarian N, Kang J, Shu L, Ferraz MJ, Aerts JM, Shayman JA. Dissociation of globotriaosylceramide and impaired endothelial function in α-galactosidase-A deficient EA.hy926 cells. *Mol Genet Metab*. 2018;125(4):338–344. https://doi.org/10.1016/j.ymgme.2018.10.007.
53. Kang JJ, Kaissarian NM, Desch KC, et al. α-galactosidase A deficiency promotes von Willebrand factor secretion in models of Fabry disease. *Kidney Int*. 2019;95(1):149–159. https://doi.org/10.1016/j.kint.2018.08.033.
54. Song C, Yarmishyn, et al. Generation of GLA-knockout human embryonic stem cell lines to model autophagic dysfunction and exosome secretion in Fabry disease-associated hypertrophic cardiomyopathy. *Cells*. 2019;8(4):327. https://doi.org/10.3390/cells8040327.
55. Pavan E, Ormazabal M, Peruzzo P, Vaena E, Rozenfeld P, Dardis A. Crispr/cas9 editing for Gaucher disease modeling. *Int J Mol Sci*. 2020;21(9):3268. https://doi.org/10.3390/ijms21093268.
56. Kim MJ, Jeon S, Burbulla LF, Krainc D. Acid ceramidase inhibition ameliorates α-synuclein accumulation upon loss of GBA1 function. *Hum Mol Genet*. 2018;27(11):1972–1988. https://doi.org/10.1093/hmg/ddy105.
57. Bae EJ, Yang NY, Lee C, Kim S, Lee HJ, Lee SJ. Haploinsufficiency of cathepsin D leads to lysosomal dysfunction and promotes cell-to-cell transmission of α-synuclein aggregates. *Cell Death Dis*. 2015;6(10), e1901. https://doi.org/10.1038/cddis.2015.283.
58. Drews K, Calgi MP, Harrison WC, et al. Glucosylceramidase maintains influenza virus infection by regulating endocytosis. *J Virol*. 2019;93(12):e00017–e00019. https://doi.org/10.1128/jvi.00017-19.
59. Erwood S, Brewer RA, Bily TMI, et al. Modeling Niemann-Pick disease type C in a human haploid cell line allows for patient variant characterization and clinical interpretation. *Genome Res*. 2019;29(12):2010–2019. https://doi.org/10.1101/gr.250720.119.
60. Du X, Lukmantara I, Yang H. CRISPR/Cas9-mediated generation of Niemann–pick C1 knockout cell line. *Methods Mol Biol*. 2017;1583:73–83. https://doi.org/10.1007/978-1-4939-6875-6_7.
61. Li J, Deffieu MS, Lee PL, Saha P, Pfeffer SR. Glycosylation inhibition reduces cholesterol accumulation in NPC1 protein-deficient cells. *Proc Natl Acad Sci U S A*. 2015;112(48):14876–14881. https://doi.org/10.1073/pnas.1520490112.

62. Haskins M. Gene therapy for lysosomal storage diseases (LSDs) in large animal models. *ILAR J*. 2009;50(2):112–121. https://doi.org/10.1093/ilar.50.2.112.
63. Zhang T, Peterson RT. Modeling lysosomal storage diseases in the zebrafish. *Front Mol Biosci*. 2020;7:82. https://doi.org/10.3389/fmolb.2020.00082.
64. Eaton SL, Proudfoot C, Lillico SG, et al. CRISPR/Cas9 mediated generation of an ovine model for infantile neuronal ceroid lipofuscinosis (CLN1 disease). *Sci Rep*. 2019;9:9891. https://doi.org/10.1038/s41598-019-45859-9.
65. de Carvalho TG, Schuh R, Pasqualim G, et al. CRISPR-Cas9-mediated gene editing in human MPS I fibroblasts. *Gene*. 2018;678:33–37. https://doi.org/10.1016/j.gene.2018.08.004.
66. Schuh RS, de Carvalho TG, Giugliani R, Matte U, Baldo G, Teixeira HF. Gene editing of MPS I human fibroblasts by co-delivery of a CRISPR/Cas9 plasmid and a donor oligonucleotide using nanoemulsions as nonviral carriers. *Eur J Pharm Biopharm*. 2018;122:158–166. https://doi.org/10.1016/j.ejpb.2017.10.017.
67. Miki T, Vazquez L, Yanuaria L, et al. Induced pluripotent stem cell derivation and ex vivo gene correction using a mucopolysaccharidosis type 1 disease mouse model. *Stem Cells Int*. 2019;2019:6978303. https://doi.org/10.1155/2019/6978303.
68. Chang S-K, Lu Y-H, Chen Y-R, et al. Correction of the GLA IVS4 + 919 G > A mutation with CRISPR/Cas9 deletion strategy in fibroblasts of Fabry disease. *Ann Transl Med*. 2017;5(suppl. 2), AB043. https://doi.org/10.21037/atm.2017.s043.
69. Do HS, Park SW, Im I, et al. Enhanced thrombospondin-1 causes dysfunction of vascular endothelial cells derived from Fabry disease-induced pluripotent stem cells. *EBioMedicine*. 2020;52:102633. https://doi.org/10.1016/j.ebiom.2020.102633.
70. Scharenberg SG, Poletto E, Lucot KL, et al. Engineering monocyte/macrophage–specific glucocerebrosidase expression in human hematopoietic stem cells using genome editing. *Nat Commun*. 2020;11:3327. https://doi.org/10.1038/s41467-020-17148-x.
71. van der Wal E, Herrero-Hernandez P, Wan R, et al. Large-scale expansion of human iPSC-derived skeletal muscle cells for disease modeling and cell-based therapeutic strategies. *Stem Cell Rep*. 2018;10(6):1975–1990. https://doi.org/10.1016/j.stemcr.2018.04.002.
72. Dever DP, Scharenberg SG, Camarena J, et al. CRISPR/Cas9 genome engineering in engraftable human brain-derived neural stem cells. *iScience*. 2019;15:524–535. https://doi.org/10.1016/j.isci.2019.04.036.
73. Pavani G, Laurent M, Fabiano A, et al. Ex vivo editing of human hematopoietic stem cells for erythroid expression of therapeutic proteins. *Nat Commun*. 2020;11(1):4146. https://doi.org/10.1038/s41467-020-17552-3.
74. Gomez-Ospina N, Scharenberg SG, Mostrel N, et al. Human genome-edited hematopoietic stem cells phenotypically correct Mucopolysaccharidosis type I. *Nat Commun*. 2019;10(1):4045. https://doi.org/10.1038/s41467-019-11962-8.
75. Schuh RS, Gonzalez EA, Tavares AMV, et al. Neonatal nonviral gene editing with the CRISPR/Cas9 system improves some cardiovascular, respiratory, and bone disease features of the mucopolysaccharidosis I phenotype in mice. *Gene Ther*. 2020;27(1–2):74–84. https://doi.org/10.1038/s41434-019-0113-4.
76. Ou L, DeKelver RC, Rohde M, et al. ZFN-mediated in vivo genome editing corrects murine hurler syndrome. *Mol Ther*. 2019;27(1):178–187. https://doi.org/10.1016/j.ymthe.2018.10.018.
77. Ou L, Przybilla MJ, Ahlat O, et al. A highly efficacious PS gene editing system corrects metabolic and neurological complications of mucopolysaccharidosis type I. *Mol Ther*. 2020;28(6):1442–1454. https://doi.org/10.1016/j.ymthe.2020.03.018.

78. Wang D, Li J, Song CQ, et al. Cas9-mediated allelic exchange repairs compound heterozygous recessive mutations in mice. *Nat Biotechnol*. 2018;36(9):839–842. https://doi.org/10.1038/nbt.4219.
79. Laoharawee K, DeKelver RC, Podetz-Pedersen KM, et al. Dose-dependent prevention of metabolic and neurologic disease in murine MPS II by ZFN-mediated in vivo genome editing. *Mol Ther*. 2018;26(4):1127–1136. https://doi.org/10.1016/j.ymthe.2018.03.002.
80. Sharma R, Anguela XM, Doyon Y, et al. In vivo genome editing of the albumin locus as a platform for protein replacement therapy. *Blood*. 2015;126(15):1777–1784. https://doi.org/10.1182/blood-2014-12-615492.
81. Ou L, Przybilla MJ, Tăbăran AF, et al. A novel gene-editing system to treat both Tay–Sachs and Sandhoff diseases. *Gene Ther*. 2020;27(5):226–236. https://doi.org/10.1038/s41434-019-0120-5.
82. Levy JM, Yeh WH, Pendse N, et al. Cytosine and adenine base editing of the brain, liver, retina, heart and skeletal muscle of mice via adeno-associated viruses. *Nat Biomed Eng*. 2020;4:97–110. https://doi.org/10.1038/s41551-019-0501-5.
83. Poletto E, Pasqualim G, Giugliani R, Matte U, Baldo G. Worldwide distribution of common IDUA pathogenic variants. *Clin Genet*. 2018;94(1):95–102. https://doi.org/10.1111/cge.13224.
84. Ventola CL. Progress in nanomedicine: approved and investigational nanodrugs. *P T*. 2017;42(12):742–755.
85. Ganta S, Talekar M, Singh A, Coleman TP, Amiji MM. Nanoemulsions in translational research—opportunities and challenges in targeted cancer therapy. *AAPS PharmSciTech*. 2014;15(3):694–708. https://doi.org/10.1208/s12249-014-0088-9.
86. Pan G, Shawer M, Øie S, Lu DR. In vitro gene transfection in human glioma cells using a novel and less cytotoxic artificial lipoprotein delivery system. *Pharm Res*. 2003;20(5):738–744. https://doi.org/10.1023/A:1023477317668.
87. Azambuja JH, Schuh RS, Michels LR, et al. Nasal administration of cationic nanoemulsions as CD73-siRNA delivery system for glioblastoma treatment: a new therapeutical approach. *Mol Neurobiol*. 2020;57(2):635–649. https://doi.org/10.1007/s12035-019-01730-6.
88. Yadav S, Gandham SK, Panicucci R, Amiji MM. Intranasal brain delivery of cationic nanoemulsion-encapsulated TNFα siRNA in prevention of experimental neuroinflammation. *Nanomed Nanotechnol Biol Med*. 2016;12(4):987–1002. https://doi.org/10.1016/j.nano.2015.12.374.
89. Gowing G, Svendsen S, Svendsen CN. Ex vivo gene therapy for the treatment of neurological disorders. *Prog Brain Res*. 2017;230:99–132. https://doi.org/10.1016/bs.pbr.2016.11.003.
90. Samson M, Libert F, Doranz BJ, et al. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature*. 1996;382(6593):722–725. https://doi.org/10.1038/382722a0.
91. Xie Y, Zhan S, Ge W, Tang P. The potential risks of C-C chemokine receptor 5-edited babies in bone development. *Bone Res*. 2019;7:4. https://doi.org/10.1038/s41413-019-0044-0.
92. Dever DP, Bak RO, Reinisch A, et al. CRISPR/Cas9 β-globin gene targeting in human hematopoietic stem cells. *Nature*. 2016;539:384–389. https://doi.org/10.1038/nature20134.
93. Wang J, Exline CM, Declercq JJ, et al. Homology-driven genome editing in hematopoietic stem and progenitor cells using ZFN mRNA and AAV6 donors. *Nat Biotechnol*. 2015;33(12):1256–1263. https://doi.org/10.1038/nbt.3408.

94. Genovese P, Schiroli G, Escobar G, et al. Targeted genome editing in human repopulating hematopoietic stem cells. *Nature*. 2014;510(7504):235–240. https://doi.org/10.1038/nature13420.
95. Uchida N, Chen K, Dohse M, et al. Human neural stem cells induce functional myelination in mice with severe dysmyelination. *Sci Transl Med*. 2012;4(165), 165er7. https://doi.org/10.1126/scitranslmed.3005469.
96. Schuh RS, Poletto E, Pasqualim G, et al. In vivo genome editing of mucopolysaccharidosis I mice using the CRISPR/Cas9 system. *J Control Release*. 2018;288:23–33. https://doi.org/10.1016/j.jconrel.2018.08.031.
97. Wechsler T, DeKelver R, Rohde M, et al. ZFN-mediated in vivo genome editing results in supraphysiological levels of lysosomal enzymes deficient in hunter and hurler syndrome and Gaucher disease. *Mol Ther*. 2015;27(1):178–187. https://doi.org/10.1038/mt.2015.74.
98. Leal AF, Espejo-Mojica AJ, Sánchez OF, et al. Lysosomal storage diseases: current therapies and future alternatives. *J Mol Med*. 2020;98(7):931–946. https://doi.org/10.1007/s00109-020-01935-6.
99. Harmatz P, Muenzer J, Burton BK, et al. Update on phase 1/2 clinical trials for MPS I and MPS II using ZFN-mediated in vivo genome editing. *Mol Genet Metab*. 2018;123(2):S59–S60. https://doi.org/10.1016/j.ymgme.2017.12.143.
100. Harmatz P, Lau HA, Heldermon C, et al. EMPOWERS: a phase 1/2 clinical trial of SB-318 ZFN-mediated in vivo human genome editing for treatment of MPS I (Hurler syndrome). *Mol Genet Metab*. 2019;126(2):S68. https://doi.org/10.1016/j.ymgme.2018.12.163.
101. Muenzer J, Prada CE, Burton B, et al. CHAMPIONS: a phase 1/2 clinical trial with dose escalation of SB-913 ZFN-mediated in vivo human genome editing for treatment of MPS II (Hunter syndrome). *Mol Genet Metab*. 2019;126(2):S104. https://doi.org/10.1016/j.ymgme.2018.12.263.
102. Poletto E, Baldo G, Gomez-Ospina N. Genome editing for mucopolysaccharidoses. *Int J Mol Sci*. 2020;21(2):500. https://doi.org/10.3390/ijms21020500.
103. Conway A, Mendel M, Kim K, et al. Non-viral delivery of zinc finger nuclease mRNA enables highly efficient in vivo genome editing of multiple therapeutic gene targets. *Mol Ther*. 2019;27(4):866–877. https://doi.org/10.1016/j.ymthe.2019.03.003.

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REVIEW ARTICLE

In Situ Gene Therapy

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Abstract: Gene therapy is a technique that aims at the delivery of nucleic acids to cells, to obtain a therapeutic effect. *In situ* gene therapy consists of the administration of the gene product to a specific site. It possesses several advantages, such as the reduction in potential side effects, the need for a lower vector dose, and, as a consequence, reduced costs, compared to intravenous administration. Different vectors, administration routes and doses involving *in situ* gene transfer have been tested both in animal models and humans, with *in situ* gene therapy drugs already approved in the market. In this review, we present applications of *in situ* gene therapy for different diseases, ranging from monogenic to multifactorial diseases, focusing mainly on therapies designed for the intra-articular and intraocular compartments, as well as gene therapies for the central nervous system (CNS) and for tumors. Gene therapy finally seems to blossom as a viable therapeutic approach. The growth in the number of clinical protocols shown here is evident, and the positive outcomes observed in several clinical trials indicate that more products based on *in situ* gene therapy should reach the market in the next years.

Keywords: *In situ* gene therapy, intra-articular gene therapy, intraocular gene therapy, intracerebral gene therapy, intratumoral gene therapy, gene therapy clinical trials.

1. INTRODUCTION

According to the United States Food and Drug Administration (FDA), "human gene therapy seeks to modify or manipulate the expression of a gene or to alter the biological properties of living cells for therapeutic use" [1]. In this context, gene therapy can be used to treat both genetic and multifactorial diseases. Either way, it holds great promise for treatment of many diseases, with more than 600 studies registered on ClinicalTrials.gov and a few approved therapies so far [2].

Gene therapy is an approach in which modified cells (endogenous or exogenous) persistently produce therapeutic factors *in vivo* after genetic manipulation [3]. Gene therapy approaches are grouped as *in vivo* or *ex vivo*; the first consists of direct injection of the vector in the patient to target the endogenous cells, while the second is the modification of cells *in vitro* (from autologous or allogeneic origins) with subsequent transplantation to the patient. *In vivo* gene therapy has a higher risk of adverse events or immune reactions as the vector is injected directly into the body; plus, it relies on having efficient transduction of an appropriate number of cells so that a sufficient amount of therapeutical factor can be produced, a requirement that sometimes is not met [4]. In spite of that, it is relatively more accessible, as the gene therapy product can be stored frozen and administered to patients in a one-fits-all fashion. Contrarily, *ex vivo* gene therapy usually modifies autologous cells *in vitro*, requiring specialized facilities and at least two medicals procedures, one to collect the cells and another to implant them. However, *ex vivo* modified cells circumvent immune responses and

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do not have associated toxicity, which can increase the effectiveness of the therapy [2].

Gene delivery to cells can be done by viral vectors, as adenovirus (Ad), retrovirus (RV) lentivirus (LV) or adeno-associated virus (AAV), to name a few. These vectors are recombinant particles that lack the sequences for viral replication inside the host but carry the transgene of interest in their genome; this way, they can effectively transduce cells, without provoking their death. Once inside the cell, they can remain episomal (as Ad and AAV) or they can integrate into the host's genome and express the transgene permanently (as RV and LV), but at a cost of possibly causing insertional mutagenesis, since the integration is random. Although very efficient for gene delivery, viral vectors can elicit an important immune response, thus they should be used with caution [5].

Alternatively, non-viral vectors have reduced transfection efficiency, but are overall safer. These are mostly composed of episomal plasmid vectors or transposons containing the transgene and they strictly require a transfection method to enter the cell [6]. Physical methods of transfection (like electroporation and microinjection) are mostly used *in vitro*, while chemical methods have been extensively tested and include, among others, lipid- and polymer-based carriers [7]. In addition to these vectors, biomaterials have been used in some contexts; for example, for cartilage and bone repair. Collagen, fibrin, alginate and others can serve as scaffolds for cell growth (both endogenous and modified/transplanted) and consequent tissue repair. If coupled with a gene therapy vector, the biomaterial protects the vector and can even control its release, increasing the durability of the therapy [8].

Lately, another strategy for gene therapy has been extensively tested, genome editing, mostly by clustering regularly interspaced short palindromic repeat (CRISPR)-Cas9. In this approach, the genome of cells is edited to permanently produce a therapeutic factor through correction or induction of point mutations or inserting/deleting sequences of interest [9]. Independently of the vector used to deliver the CRISPR components, one major advantage of using this system is the controlled expression of the transgene, since only a limited number of copies (frequently only one) can be integrated per allele, as the editing is targeted to a sequence-specific locus in the genome. With other methods, each cell can receive innumerous episomal vectors or have many viral copies integrated into a single allele, making the "gene dose" hard to predict.

Regardless of the gene therapy design, the treatment can be either administered systemically or it can be applied to small, compartmentalized areas, aiming to treat specific cells or tissues. Examples of local administration routes and application sites include intra-articular (IA) [9], intra-ocular [10], intradermal [11], intracerebral or intrathecal (IT) [12], intracardiac [13, 14] intra-luminal for vessel targeting [15, 16] injection in the inner ear [17], in ligaments [4] and in the periodontal area [18], thus contemplating a vast list of potential targets for localized gene delivery. The administration to a specific site is called *in situ* gene therapy.

In situ gene thrapy has many advantages compared to systemic interventions. Firstly, it requires smaller doses of the product, as it remains localized in the compartment where it is injected, as opposed to systemic administration that dilutes the vector. Contained administration also reduces drastically the possibility of the immune reaction (excluding the case of mRNA vaccines, which are meant to trigger an immune response), which increases the overall efficacy and reduces the possibility of adverse effects. Moreover, for some tissues, injecting the vector directly in the compartment of interest avoids physiological barriers that it would have to surpass (as penetrating a dense extracellular matrix or getting to poorly vascularized tissues) to reach the target [5]. *In situ* injection also reduces the off-target effects and the toxicity of the therapy, as systemic administrations can lead to hepatotoxicity, depending on the vectors and doses used [5]. Evidently, *in situ* gene therapy is not applicable to treat multisystemic disorders at once, though it can be used as a complement in hard-to-treat tissues where the systemic therapy cannot act efficiently [19].

In this review, we present applications of *in situ* gene therapy for different diseases, ranging from monogenic to multifactorial diseases, focusing mainly on therapies designed for the intra-articular and intra-ocular compartments, as well as gene therapies for the central nervous system (CNS) and for tumors (Fig. **1**).

2. DIRECTED GENE THERAPY TO THE CENTRAL NERVOUS SYSTEM

Gene therapy for neurodegenerative disorders is a promising therapeutic alternative to currently approved pharmacological therapies, as it can be a single-dose and long-lasting approach. Targeting the CNS is frequently achieved using AAV vectors, as several serotypes (1, 2, 4, 5, 7, 8 and 9) have the ability to successfully transduce large areas in the nervous tissue [20]. They present well-known advantages, as low immunogenicity, long-lasting gene expression, and efficient and scalable production. Moreover, AAV vectors are capable of transducing mitotic and non-mitotic cells, the latter being the most affected cell type in CNS disorders [20]. With a few differences in transduction pattern and efficiency, the majority of these serotypes transduce solely neurons; while AAV serotypes 1, 5 and 9 transduce both glial and neuronal cells [20, 21], serotype 4 targets mostly ependymal cells [20].

Delivery of gene therapy products targeting the CNS can be done either by the systemic or local administration. Intravascular (IV) administration appears as a non-invasive option in studies for CNS disease, with good vector distribution in the tissue when using AAV9 vector [22]. However, biodistribution of AAV9 is still not a consensus, as animal age might influence the transduction efficiency in both neurons and glial cells [23-25]. Despite the brain tissue tropism, AAV9 as well as other AAV serotypes also have a high tropism for the heart and the liver, which makes the vector prone to off-site delivery. Additionally, systemic administration might require higher doses to deliver enough vector titer to the CNS, which can result in toxicity and death [26]. Indeed, the incidence of hepatocellular carcinoma in mice after IV administration with high vector dosage was demon-

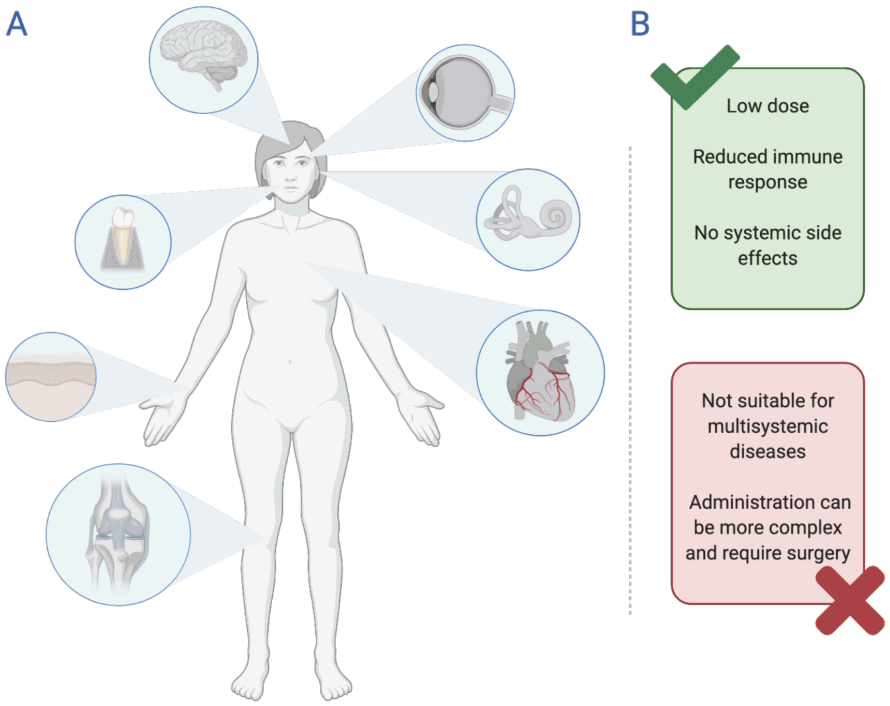

Fig. (1). Overview of *in situ* gene therapy. **A**) Local delivery of gene therapy products can be performed *via* different routes, including intracerebral, intraocular, in the inner ear, in the periodontal area, intracardiac, intradermal and intra-articular administrations. **B**) Advantages of *in situ* gene therapy include the lower dose necessary to reach the therapeutic effect at the site and reduced side effects, including the ones related to immune response; disadvantages are mainly the limited application for multisystemic diseases and invasive procedures for administration. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

strated [27]. Another disadvantage is the presence of neutralizing antibodies that can reduce therapy efficiency, since the majority of the population have had previous contact with AAVs [26, 28]. Therefore, systemic delivery of gene therapy vectors still has important drawbacks and *in situ* administration in the brain constitutes an interesting alternative.

The use of a localized administration approach has addressed some of those drawbacks listed hereinabove. Direct CNS delivery of viral vectors can increase the dissemination in the brain tissue; plus, it may also reduce the toxicity (as lower doses are required) and avoid humoral response triggered by systemic vector distribution [29, 30]. Direct cerebral delivery can be done in the brain parenchyma (intraparenchymal injection) or in the cerebrospinal fluid (CSF) by intrathecal (IT), intracerebroventricular (ICV), or intracisternal injection – the choice will depend on the cell type and the region of interest. Compared to IV, *in situ* administration has shown better outcomes in gene delivery to the brain [31].

Intraparenchymal injection, also known as an intracranial injection, is a direct administration into the brain parenchyma, which is the functional tissue made up of neurons and glial cells. This approach was the first applied to humans and, to date, it is the most commonly used in preclinical and clinical studies in the field [29, 31, 32]. In small animals, the procedure consists of a stereotaxic surgery where holes are made in the crane, and vectors are delivered by a localized injection in certain regions – mostly cortex, striatum, hippocampus, thalamus, ventral tegument and cerebellum [29, 33]. For large animals as non-human primates (NHP) and humans, guidance systems are used for increased accuracy [29]. In spite of being the most employed route, intraparenchymal injection is highly invasive and has important associated risks, as possible hemorrhages, tissue damage, or pathogen infections [20]. Frequently, vectors are poorly distributed, remaining near the site of injection [34, 35]. To overcome this, the convection-enhanced delivery (CED) method has been used lately; with this method, a constant flow is applied to the catheter to allow the vector dispersion through the interstitial fluid in the brain, enhancing viral particle distribution [34, 36].

Despite the expected restricted distribution in the tissue, AAV2, AAV5 and AAV9 have shown better dissemination from the injection site, reaching regions as the midbrain and deeper cortical layers. Increased dispersion relies on their ability to undergo axonal anterograde or retrograde transport [32]. Anterograde transport occurs when viral particles are disseminated through axons from the site of injection after transducing neural cell bodies to a distal area where the axon ends, transducing cells located there. On the other hand, retrograde transport occurs when viral particles are taken up by axonal terminals in the injection site and are then transported back to the neuronal cell soma, where they subsequently transduce the neuron [37].

Among all serotypes, AAV2 was the first studied and, due to its specificity for neurons and clinical safety profile, it has been considered the gold standard for neurosurgical

gene therapy for neurodegenerative diseases. As was demonstrated in other studies, AAV2 presents axonal transportation, by which is possible to assume that, after intraparenchymal injection, other regions with axonal projection will express the transgene [36]. In preclinical studies for Parkinson's disease (PD) which mainly affects dopaminergic cells of the substantia nigra (SN), AAV2 vector has shown a stable and long-term production of different neurotransmitters in the striatum and in the SN after intrathalamic administration [36].

Another similar vector, AAV5, had its brain biodistribution assessed in mice, rats and NHP [38], indicating high levels of transduction in the midbrain and several other distal parts. In rats, intrastriatal injection resulted in the transduction of several neurons in the cortex, striatum, thalamus, and hippocampus. Similarly, with intrathalamic administration, the transduction was observed at cortical, striatal, thalamic, hippocampal and cerebellar areas, plus the brainstem [38]. AAV5, as well as the AAV2, is also suited for targeting specific CNS sites whenever convenient. In a study on Canavan disease, for example, AAV5 could transduce cortex, brainstem and cerebellum [38]. This vector has been also assessed for Huntington's disease that affects predominantly basal ganglia and deep layers of the cerebral cortex. In this study, microRNAs (miRNAs) designed to block the production of the huntingtin protein were delivered *via* intrastriatal administration, resulting in lower huntingtin expression in both the cortex and striatum [39]. Finally, the distribution of AAV9 serotype in the NHP brain also showed axonal transport, transducing cells at axon terminals after a single injection into the ventral tegmental area. That provides support for its use in the treatment of neurological diseases with a substantial cortico-striatal pathology, such as Alzheimer's [40].

Lysosomal storage diseases (LSD) that develop brain impairment and rely on the cross-correction phenomenon are also good candidates for gene therapy. Regardless of the injection site, transduced cells in the treated area can produce and secrete the missing enzyme, which, in turn, can be captured by enzyme-deficient cells in more distal areas [29, 41]. This was demonstrated in a preclinical study for mucopolysaccharidosis IIIA (MPS IIIA) and for metachromatic leukodystrophy (MLD), using an AAVrh10 vector – a rhesus macaque serotype, that also transduces neurons [42, 43]. Additionally, different AAV serotypes (1, 2, 5, 9, rh8) have been tested in other LSD mouse models. In diseases such as gangliosidoses (both GM1 and GM2), globoid cell leukodystrophy (GCL), Niemann-Pick disease (NPD) type A, mucopolysaccharidoses (MPS) types I and VII, it was observed biochemical and histological correction in large regions of the brain, improving behavioral symptoms, motor function and life span [33].

Not all CNS diseases are limited to brain tissue though. Many neurological disorders affect both brain and spinal cord cells; therefore, an effective treatment will require delivery routes capable of widespread transduction throughout the CNS, while also minimizing off-site delivery (Fig. **2**). An alternative delivery approach to the CNS is CSF administration, since CSF is present in cerebral ventricles, cisternal spaces, and the spinal canal connecting the entire CNS [44]. As an advantage, this delivery method is less invasive than the intraparenchymal injection, though also dodging the blood-brain barrier (BBB)-related issues [34].

IT administration is performed at the bottom of the spinal cord, the lumbar region. This delivery is considered the least risky of all CSF delivery routes, since in humans, it can be accessed *via* lumbar puncture in a less invasive procedure. This method has shown extensive spinal cord transduction in animal models for amyotrophic lateral sclerosis (ALS) and also axonal neuropathy [29, 44]. Studies using the IT route were also conducted for LSDs that have broad CNS impairment, such as Pompe disease, where neurologic, neuromuscular, and cardiac functions are affected, and Krabbe disease, where upper motor neurons are also affected [29]. A single IT administration of either AAV9 or AAVrh10 achieved a significant improvement in neurologic function and neuromuscular aspects of these diseases [29], suggesting that lumbar IT delivery does not limit biodistribution to the spinal cord parenchyma.

The intra-cisterna magna (ICM) administration, at the cerebellomedullary cistern, occurs in the space between the cerebellum and the spinal cord. Using the ICM route, the vector spreads along the length of the spinal cord but often prevails in the brainstem and cerebellum [32]. ICM administration, though easily accessible in animal models, could be more problematic to translate to humans [16]. To overcome this limitation, a new method has been developed: it employs an intravascular microcatheter that advances from the lumbar puncture site up to cisterna magna through the spinal canal [32, 44]. The safety and biodistribution pattern of this new method has been assessed in sheep, achieving broad distribution of AAV9 gene expression in the CNS. Recently, this approach was scaled to treat Tay-Sachs disease patients using an AAVrh8 vector [44].

The last CSF-delivery route, ICV, refers to the delivery of AAV into ventricles or into large brain cavities that hold CSF. The largest and most common ventricles to target are the lateral ventricles, left and right, *via* stereotaxic surgery. This method is as invasive as intraparenchymal administration but it allows a broad distribution of the vector in the brain tissue and spinal cord, similarly to ICM administration [29]. Both routes, ICM and ICV, have been studied in neonates and adult mouse models for LSD, as MPS I, II, IIIA, VII and MLD, and also in neuromotor diseases as spinal muscular atrophy (SMA). Results showed a significant correction of neurological and motor symptoms using AAV1, -8 and -9, displaying a widespread AAV transduction across the cerebral tissue and the spinal cord [29].

Intranasal and intramuscular delivery are additional strategies for CNS targeting. A non-invasive intranasal instilling of AAV9 has been applied recently to treat MPS I. It resulted in alfa-L-iduronidase (IDUA) activity levels up to 50-fold of normal mice in the olfactory bulb. Additionally, due to IDUA trafficking to the CNS *via* the olfactory and trigeminal pathway, a reduction of tissue glycosaminoglycan accumulation in all brain tissue was observed [45]. Alternatively, by injecting AAV into the muscles, motor neuron diseases as amniotrofic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) can be treated. That is possible due to the axonal transport that some AAV serotypes under-

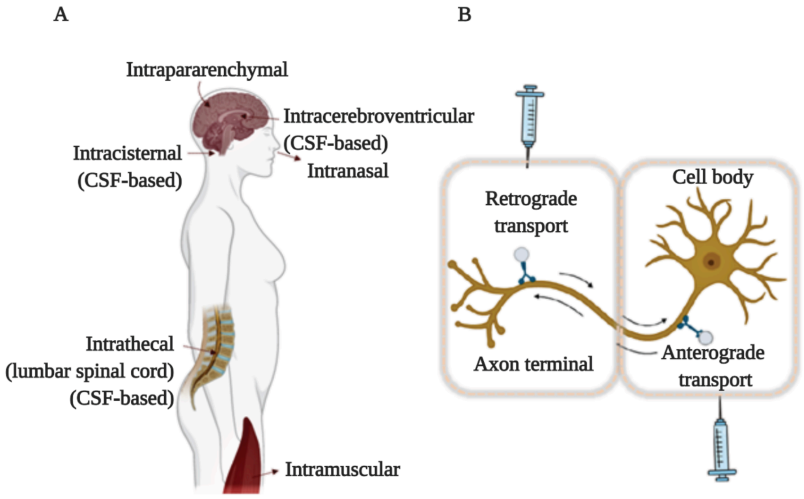

Fig. (2). Overview of intracerebral delivery routes and axonal transport. **A**) Vectors can be introduced into the central nervous system (CNS) directly into the brain parenchyma or *via* the cerebrospinal fluid (CSF). Intramuscular and intranasal injections can also lead to the transduction of CNS cells. **B**) Cargos are transported along axons in the anterograde direction, from the cell body to the axonal terminal, or in the retrograde direction, toward the cell body. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

go, as previously mentioned, traveling along neural innervations in the muscles. Nevertheless, axonal transport machinery may be dysfunctional in some of these diseases, ending in limited vector spreading in restricted areas of the spinal cord [29].

2.1. Clinical Trials

To date, many of the preclinical studies described hereinabove have managed to scale up to clinical trials (Table **1**). Neurodegenerative and LSD are the main groups in which directed brain therapy has been used.

A lot of effort has been addressed for PD and there are some promising results. The VY-AADC01 therapy (NCT01973543) is based on a direct delivery of AAV2 vector encoding the L-amino acid decarboxylase (*AADC*) protein into the striatum, which converts L-dopa into dopamine. In a 3-year follow-up, the procedure was well tolerated, improved motor function, and enhanced response to levodopa. Currently, long-term responses are being evaluated (NCT03733496). ProSavin, developed by Oxford Biomedica, encodes two more enzymes involved in the biosynthesis of dopamine besides *AADC* and uses a lentiviral vector for intrastriatal delivery (NCT00627588). In a one-year follow-up, a significant improvement of motor function was recorded, and a long-term analysis is also ongoing (NCT01856439). Moreover, the glial cell line-derived neurotrophic factor (*GDNF*) gene is being used in two other studies, both based on the intraparenchymal injection of AAV2 (NCT04167540, NCT01621581).

For Alzheimer's Disease, an AAV2 vector was used for intraparenchymal gene delivery of nerve growth factor (*NGF*), a protein with protective effects on cholinergic neurons (NCT00876863). The procedure was safe and well-tolerated; however, it did not affect cognitive outcomes in the phase II trial and future studies are required to determine if the therapy was accurately targeted to the brain area called nucleus basalis of Meynert. Two novel approaches using AAV are currently recruiting participants for a phase I trial: Libella Gene Therapy proposes the delivery of the telomerase gene (*TERT*) intravenously and intrathecally (NCT04133454), while the Weill Cornell Medicine study proposes intracisternal administration of apolipoprotein E2 (*APOE2*) (NCT03634007). Furthermore, a phase I/II trial in Huntington's Disease is also being performed, aiming at intrastriatal delivery of AAV5 encoding the huntingtin (*HTT)* gene (NCT04120493).

LSD have also a high representation in clinical trials. For neuronal ceroid lipofuscinoses or Batten's Diseases, three of the well-described causal genes are being assessed in clinical trials – Neuronal ceroid lipofuscinosis protein (*CLN* 2, 3 and 6). The AT-GTX-501 treatment is based on IT delivery of an AAV9 vector encoding the *CLN6* gene (NCT02725580), and AT-GTX-502 encoding the *CLN3* gene (NCT03770572). The Weill Cornell Medicine trial, on the other hand, used an AAV2 vector for intraparenchymal delivery of the *CLN2* gene (NCT00151216). Preliminary results demonstrated a small impact on the progression of the disease, and a delivery system based on AAV10 is currently being evaluated (NCT01161576).

For mucopolysaccharidosis type IIIA, the Lysogene phase I/II trial (NCT01474343) showed good safety data and a moderate improvement of behavioral and sleep disorders in the patients after the delivery of an AAV10 vector carrying both *SGSH* and sulfatase-modifying factor 1 (*SUMF1)* genes. A phase II/III study using only the N-sulfoglycosamine sulfohydrolase (*SGSH)* gene is currently ongoing (NCT03612869).

RegenxBio is running two trials on MPS I and II: the RGX-111 therapy (NCT03580083) is designed to deliver the *IDUA* gene for patients with MPS I through intracisternal injection of an AAV9 vector, while the RGX-121 therapy (NCT03566043) delivers the *IDS* gene for patients with MPS II using the same approach.

Table 1. Clinical trials using intracerebral gene therapy.

ID	Phase	Disease	*Gene*	Vector	Administration Route	Status	Sponsor
NCT01973543	I	PD	*AADC*	AAV2	Intraparenchymal (striatum)	Completed	Neurocrine Biosciences
NCT00627588	I/II	PD	*AADC, TH, CH1*	Lentiviral	Intraparenchymal (striatum)	Completed	Oxford BioMedica
NCT00195143	I	PD	*GAD*	AAV	Intraparenchymal (subthalamic nucleus)	Completed	Neurologix, Inc.
NCT00252850	I	PD	*NTN*	AAV2	Intraparenchymal (putamen)	Completed	Ceregene
NCT04167540	I	PD	*GDNF*	AAV2	Intraparenchymal (putamen)	Recruiting	Brain Neurotherapy Bio
NCT01621581	I	PD	*GDNF*	AAV2	Intraparenchymal (putamen)	Active, not recruiting	National Institute of Neurological Disorders and Stroke
NCT04127578	I/II	PD	*GBA1*	AAV9	Intracisternal	Recruiting	Prevail Therapeutics
NCT03634007	I	AD	*APOE2*	AAV10	Intracisternal	Recruiting	Weill Medical College of Cornell University
NCT00876863	II	AD	*NGF*	AAV2	Intraparenchymal	Completed	Sangamo Therapeutics (Ceregene)
NCT04133454	I	AD	*TERT*	AAV	Intrathecal and intravenous	Recruiting	Libella Gene Therapeutics
NCT04120493	I/II	HD	*HTT*	AAV5	Intraparenchymal (striatum)	Recruiting	UniQure Biopharma B.V.
NCT02362438	I	GAN	*GAN*	AAV9	Intrathecal	Recruiting	National Institute of Neurological Disorders and Stroke
NCT03727555	I/II	X-ALD	*ABCD1*	Lentiviral	Intraparenchymal	Recruiting	Shenzhen Geno-Immune Medical Institute
NCT03580083	I/II	MPS I	*IDUA*	AAV9	Intracisternal	Recruiting	Regenxbio Inc.
NCT03566043	I/II	MPS II	*IDS*	AAV9	Intracisternal	Recruiting	Regenxbio Inc.
NCT01474343	I/II	MPS IIIA	*SGSH, SUMF1*	AAV10	Intraprenchymal	Completed	LYSOGENE
NCT03612869	II/III	MPS IIIA	*SGSH*	AAV10	Intraparenchymal	Active, not recruiting	LYSOGENE
NCT03300453	I/II	MPS IIIB	*NAGLU*	AAV5	Intraparenchymal	Completed	UniQure Biopharma B.V.
NCT00151216	I	NCL	*CLN2*	AAV2	Intraparenchymal	Completed	Weill Medical College of Cornell University
NCT01161576	I	NCL	*CLN2*	AAV10	Intraparenchymal	Active, not recruiting	Weill Medical College of Cornell University
NCT03770572	I/II	NCL	*CLN3*	AAV9	Intrathecal	Active, not recruiting	Amicus Therapeutics
NCT02725580	I/II	NCL	*CLN6*	AAV9	Intrathecal	Active, not recruiting	Amicus Therapeutics
NCT03725670	I/II	MLD	*ARSA*	Lentiviral	-	Recruiting	Shenzhen Geno-Immune Medical Institute
NCT04273269	I/II	GM1	*GLB1*	AAV10	Intracisternal	Not yet recruiting	LYSOGENE

Abbreviations: AAV,adeno-associated vírus; PD, Parkinson's Disease; AD, Alzheimer's Disease; HD, Huntington Disease; X-ALD, X-linked Adrenoleukodystrophy; GAN, Giant Axonal Neuropathy; MPS, Mucopolysaccharidosis, MLD, Metachromatic Leukodystrophy; NCL, Neuronal Ceroid Lipofuscinosis (also known as Batten Disease); *AADC*, aromatic L-amino acid decarboxylase; *TH*, tyrosine hydroxylase; CH1, GTP-cyclohydrolase-1; *GAD*, glutamic acid decarboxylase; *NTN*, neurturin; GDNF, glial derived neurotrophic fator; GBA, glucocerebrosidase; APOE2, apolipoprotein; NGF, nerve growth fator; *TERT*, telomerase reverse transcriptase; *HTT*, huntingtin; *GAN*, gigaxonin; *ABCD1*, peroxisomal ATP-binding cassette transporter; *IDUA*, alpha-L-iduronidase; *IDS* = iduronate 2-sulfatase; *SGSH*, N-sulfoglycosamine sulfohydrolase; *SUMF1*, sulfatase-modifying factor 1; *NAGLU*, N-acetyl-alpha-glucosaminidase; *CLN*, neuronal ceroid lipofuscinosis protein; *ARSA*, arylsulfatase A; *GLB1*, beta-galactosidase.

3. INTRAOCULAR GENE THERAPY

Monogenic disorders, mostly inherited retinal diseases (IRD), are one of the most common causes of untreatable sight loss, while age-related macular degeneration (AMD) may cause untreatable blindness overall. The identification of genetic factors in eye diseases provides an array of potential targets for gene replacement, knockdown, and editing therapies [46].

3.1. Site of Action

The ocular route is often the first where new technologies are tested, since the eye is a relatively isolated and immunologically privileged organ. In this sense, drugs based on gene therapy were first approved for the ocular route, such as the antisense oligonucleotide Vitravene® [47], the aptamer Macugen® [48], and Luxturna® [49].

Taken as a whole, the eye is a structure with low permeability, having a hemato-ocular barrier system, which prevents the passage of blood substances and cells to the tissues [50]. This system is formed by two main barriers: the blood-aqueous barrier (BAB) and the blood–retinal barrier (BRB). The BAB is situated in the anterior part of the eye and is formed by endothelial cells of the blood vessels within the iris and the non-pigmented cell layer of the ciliary epithelium. The BRB is situated in the posterior part of the eye and is composed of the retinal capillary endothelial (RCE) cells and retinal pigment epithelial (RPE) cells which form the inner and outer BRB, respectively [51]. It has a protective function, with a biochemical structure and mechanisms to ensure its impermeability. On the other hand, it also hinders the passage of molecules of interest [52]. Fig. **3** illustrates the structure of the eye, detailing the cornea and some of the most common routes used in ocular gene therapy.

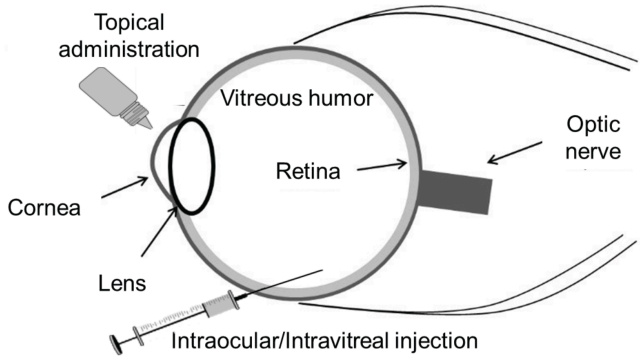

Fig. (3). Schematic representation of intraocular/ intravitreal injection and topical administration.

Ocular gene delivery may be accomplished through a series of administration routes, including topical drops (for surface corneal epithelium), subconjunctival, intracameral, intravitreal, suprachoroidal injection, or subretinal delivery. In practice, the particular disease, the exact target cell and the vector delivery system dictate the route of administration. Gene delivery for topical drops is limited to cells lining the anterior segment. This approach can be used to treat corneal diseases [53]. Intravitreal injection and subretinal delivery are the most common routes used for viral-based gene therapy for retinal diseases. Intravitreal injection is an established and quite safe route of administration where the vector is injected directly into the vitreous humor [54]. Intravitreal injection of viral vectors is the preferred route for targeting the retina, although there are anatomic barriers that prevent diffusion of viruses, especially the inner limiting membrane [55, 56]. A strategy to enhance the transduction of AAV is the design of second and third-generation AAV vectors [57]. Numerous national boards have defined guidelines on how to perform safe intravitreal injections in an operating room or in an examining office under sterile conditions [58].

Over the past 4 decades, several vector systems have been employed for gene therapy, including both nonviral strategies like liposomes, nanoemulsions, and nanoparticles, and modified viral vectors, mostly lentivirus, adenovirus, and adeno-associated viruses [54].

Regarding nonviral gene therapy, nanotechnology-based nonviral carriers have gained attention due to their ability to overcome the limitations inherent to both gene therapy and the administration of drugs to the eye. Naturally, the effectiveness of a system depends on its characteristics, but mainly on the biomaterials used. In this sense, a series of systems, both lipid (liposomes, nanoemulsions, nanostructured lipid carriers) and polymeric (nanoparticles, nanocapsules, dendrimers) have been proposed [50]. The biomaterials include phospholipids, cholesterol, cationic lipids, oils, surfactants, solid lipids, and pegylated lipids, while polymers may include poly (acrylic acid) derivatives (polyalquilcyanocrylates), albumin, poly-ε-caprolactone, chitosan, protamine, polyethyleneimine, polyamidoamine, cationized proteins, and hyaluronic acid [59, 60].

3.2. Applications

Retinal gene therapy has advanced considerably in the last years. Efforts have been devoted to optimize the transduction abilities of gene delivery vectors, to define the intraocular administration route and to obtain efficacy in animal models of IRD. Successful translation in clinical trials of the initial promising proof-of-concept studies led to the important milestone of the first approved product for retinal gene therapy in both US and Europe [10], and that is why research and publications on the subject are so important. In this sense, some of the latest publications on ocular gene therapy are listed in Table **2**.

3.3. Clinical Trials and Approved Drugs

Currently, there are more than 60 clinical studies (clinicaltrials.gov) [10] involving gene therapy for retinal diseases, such as Achromatopsia, Choroideremia, Leber congenital amaurosis (LCA), Leber's hereditary optic neuropathy, Neovascular/age-related macular degeneration, Retinitis pigmentosa, Stargardt disease, Usher syndrome, X-linked retinitis pigmentosa, and X-linked retinoschisis.

The most successful ocular gene therapy to date is the subretinal administration of AAV for the treatment of LCA type 2 (LCA2). LCA2 is caused by mutations in Retinal pigment epithelium-specific 65 (*RPE65*), which encodes an essential enzyme of the visual cycle. The treatment restored

Table 2. Recent approaches on ocular gene therapy.

Disease	Therapy	Vector	Model	Outcome	Refs.
Retinal degeneration	Electrotransfection of pEYS611, a plasmid encoding human transferrin, into the ciliary muscle evaluated in several rat models of retinal degeneration	Electrotransfection of the pEYS611 plasmid	Rat and rabbit models of retinal degeneration	Protected both retinal structure and function, reduced microglial infiltration in the outer retina and preserved the integrity of the outer retinal barrier.	[61]
Retinal ganglion cell loss after optic nerve injury or laser-induced ocular hypertension	Intravitreal delivery of brain-derived neurotrophic factor (*BDNF*) by injection of gene therapy	AAV adeno-associated virus (AAV) 2-BDNF	Rat microbead trabecular occlusion model of glaucoma	In models of glaucoma, *BDNF* therapy can delay or halt Retinal ganglion cell (RGC) loss, but this protection is time-limited	[62]
Retinal ganglion cell (RGC) loss after optic nerve injury or laser-induced ocular hypertension	Intravitreal delivery of brain-derived neurotrophic factor (*Bdnf*) and *Bdnf* receptor (TrkB) genes	AAV2 TrkB-2A-mBDNF	Mouse model of optic nerve injury and rat model of chronic intraocular pressure (IOP) elevation	Neuroprotective efficacy of AAV2 TrkB-2A-mBDNF in optic nerve injury. Neuroprotection of RGCs and axons in the rat model.	[63]
Wet age-related macular degeneration (AMD)	Single-injection of recombinant adeno-associated virus (rAAV)-based gene therapy treatment to prevent choroidal neovascularization formation	rAAV-based gene therapy	Mouse model of wet AMD	Incorporating riboswitch elements into the rAAV expression cassette allows protein expression levels to be modulated *in vivo* through oral supplementation of an activating ligand (*e.g*. tetracycline).	[64]
Leber congenital amaurosis (LCA) caused by Mutations in the Retinal pigment epithelium-specific 65 (*RPE65*) gene	Subretinal delivery of AAV5-IRBP/GNAT2-hDIO3 to investigate the effects of overexpression of DIO3 (iodothyronine deiodinases) to suppress TH (thyroid hormone) signaling and thereby modulate cone death/survival.	AAV5-IRBP/GNAT2-hDIO3	LCA model Rpe65 -/-/Nrl -/- mice	Subretinal delivery of AAV5-IRBP/GNAT2-hDIO3 induced robust expression of DIO3 in the mouse retina and significantly reduced the number of TUNEL-positive cells.	[65]
Age-related macular degeneration (AMD)	Anti- Vascular endothelial growth factor (*Vegf*) short hairpin RNAs (shRNA), and based on the most potent shRNAs, microRNA (miRNA)-mimicked hairpins expressed from vectors based on adeno-associated virus (AAV) or lentivirus (LV)	AAV-encoded interfering RNA (iRNA)	Laser-induced choroidal neovascularization (CNV) mouse model and cells	Results show co-expression of functional anti-*VEGF*-miRNAs in cell studies, and *in vivo* studies reveal an efficient retinal pigment epithelium (RPE)-specific gene expression.	[66]
Glaucoma	AAV-mediated gene delivery of sFasL (Fas Ligand) to the retina	AAV2.sFasL	Mouse models of glaucoma, the spontaneous genetic-based D2 (DBA/2J) mouse model and the microbead-induced mouse model	Data reveal the pleotropic effects of sFasL on glial activation, inflammation, and apoptosis of RGCs and show that AAV2.sFasL can provide complete and sustained neuroprotection of RGCs in both mouse models of glaucoma	[67]

vision in a large dog model of LCA2 and then clinical trials in patients were initiated [68-70]. All trials showed that AAV-mediated gene therapy was safe and effective [71]. Spark Therapeutics launched an advanced phase III clinical

trial, in which bilateral subretinal administrations of AAV2-RPE65 in LCA2 patients confirmed the safety and efficacy of the therapy [71], and provided market authorization granted initially by the Food and Drug Administration [72].

After years of extensive preclinical investigation, research on intraocular gene therapy has entered a very productive translational phase. Innovations on gene therapy platforms have been introduced allowing the effective delivery of large genes or to edit deleterious mutations, which may enable the identification of treatment options for many blinding diseases.

4. INTRA-ARTICULAR GENE THERAPY

IA gene therapy has the potential to treat diseases affecting the joint, such as osteoarthritis (OA) and rheumatoid arthritis (RA) [73]. Besides, it can be used to treat or prevent alterations that arise after joint and/or cartilage damage, such as post-traumatic osteoarthritis (PTOA) [74, 75] or limited range of motion (ROM) [76]. Also, it can be applied to treat hemophilic arthropathy (HA) [77-79] or even joint alterations present in multisystem diseases such as Mucopolysaccharidoses (MPS) [19].

Joint diseases have multiple etiologies, including mechanical, biochemical, and genetic factors. Therefore, genes to be target by gene therapy can vary depending on the pathophysiology of the disease. Targets used include anti and pro-inflammatory cytokines, matrix-degrading enzymes, membrane repair proteins, transcription factors, and even non-coding RNA molecules (Table **3**).

The IA gene delivery in animals occurs by injection in the knee through the infrapatellar ligament, in the ankles through the proximal interphalangeal joint, or in the paws through the proximal metacarpophalangeal/carpal/interphalangeal joints, because these regions are affected by joint diseases in humans. Thus, IA gene therapy is usually offered directly in the synovial fluid for fibroblast-like synoviocytes (FLS), macrophages and chondrocytes, osteocytes and joint structures such as tendons and ligaments [8].

A relevant question for clinical translation is the biodistribution of therapeutic agents in the intra-articular space. Although the joints are isolated spaces, there is a concern about vector leakage and adverse effects in non-target organs. A substantial literature has demonstrated a favorable safety profile, indicating that the transduction occurs *in situ* without systemic effect. As an example, a study used an IA self-complementary adeno-associated (scAAV) vector containing the interleukin-1 receptor antagonist (*Il1a*) gene to assess the local and systemic distribution of vector in healthy and OA (late-stage, naturally occurring) horses. In both groups, 99.7% of the vector was located *in situ*, and a consistent treatment effect was observed [95]. Another study applied a single IA injection of a cationic nanoemulsion complexed with a plasmid encoding the IDUA protein in MPS I mice. The treatment resulted in increased enzyme activity and gene expression in synovial fluid cells and joints without significant activity in the kidney, liver, lung, and spleen [19]. Additionally, IA studies that performed gene silencing with small-interfering RNA (siRNA) confirmed that the alteration in gene expression occurs only in the joint, without altering expression in blood and other organs [85, 90]. A pre-clinical study evaluated the biodistribution, safety, and initial efficacy of a recombinant adeno-associated vector of type 5 (rAAV5) expressing the human interferon B (*IFNB*) in rhesus monkeys with arthritis induced by collagen. No adverse events were observed after the evaluation of all organs. The *IFNB* expression and the highest number of copies of the vector were observed in the synovial tissue of the joint and in the adjacent lymph node [80]. Another study that used the same vector, tested different doses in Wistar rats with mono-iodoacetate-induced OA (MIA). The results were consistent with previous work cited in monkeys. In the group with higher doses, limited leakage of the vector to the circulation of the animals occurred, but after 7 days, no quantifiable vector was found in the blood. No local or systemic toxicity has been described [93].

IA gene therapy can be performed *ex vivo* or *in vivo* (Fig. **4**), nevertheless, the benefit-risk relationship of each approach should be considered for clinical translation. In general, joint diseases are not lethal, hence, there are some restrictions on the types of vectors that are used *in vivo*. To date, gene delivery was performed by lentiviral and adenoviral vectors only to study the importance of target genes in synovial joint physiology and the therapeutic effects of knocking down these genes [82, 83, 86, 91]. The use of adenovirus recombinant vectors type 5 (HAdV5) associated with baculovirus or helper-dependent adenoviruses is also very limited [84, 102].

The AAV vectors have emerged as the most used viral vector for IA applications due to their safety profile and improvements in vector design and manufacturing [79, 101] The rAAV vectors with AAV2 or AAV5 capsid have been shown to efficiently transduce synovial tissue in the joints of mice, rats and dogs [103]. The self-complementary AAV (scAAV), a modified AAV that bypasses the required second-strand DNA synthesis to achieve transcription of the transgene, has been used to achieve sustained protein drug delivery to joints of human proportions [75, 94]. However, a study detected limited neutralizing antibody (Nab) against AAV capsids in serum and synovial fluid samples from vector-treated dogs [103] and also in rhesus monkeys with collagen-induced arthritis [80]. Furthermore, some monkeys that received the highest dose developed a rAAV5-specific T-cell response. Considering that although the viral gene delivery is more efficient, these findings threaten its clinical application, and studies to investigate the immune response to viral vectors are being conducted to this day [87].

Alternatively, a variety of non-viral vectors have been used. Although not considered gene therapy products, nucleic acids such as siRNA were delivered by polycationic nanoparticles covalently conjugated [90], by a hydrogel-based in sericin (SC), by lipid-polymer hybrid nanoparticles (LPNs) and stable nucleic acid-lipid particles (SNALPs) [87]. Also, mRNA was delivered using nanomicelles [92]. Besides that, the injection of naked miRNAs [104] was performed, and results suggested that the duration of the siRNA effect lasts for at least 1 week [96]. Naked Ribbon-type decoy oligonucleotides (ODNs) were utilized to modulate transcriptional regulation of a target gene [76] and ultrasound-targeted microbubble destruction (UTMD) technique

Table 3. Target genes used in different intra-articular gene therapy approaches.

Application	Target Gene	Refs.
Arthritis	*i*nterferon beta (*IFNB*)	[80]
	transforming growth factor beta (*Tgfb1*) and SMAD family member 7 (*Smad7*)	[81]
	tryptases	[82]
	calcium release-activated calcium channel protein 1 (*Cracm1*)	[83]
	pro-apoptotic gene (*Puma*)	[84]
RA	proto-oncogene, nf-kb subunit (*Rela*)	[85]
	transforming growth factor β-activated kinase-1 (*Tak1*)	[86]
	proinflammatory cytokine tumor necrosis factor (*Tnf*)	[87]
	tumor necrosis factor alpha (*Tnfa*)	[88]
	insulin like growth factor 1 (*IGF1*)	[89]
	hypoxia-inducible factor-2 alpha (*HIF2A*)	[90]
	vascular endothelial growth factor a (*VEGFA*)	[91]
	runt-related transcription factor (*RUNX1*)	[92]
OA	Interleukin 1alpha (*Il1a*)	[93-95]
	Mmp13	[96]
	tnf receptor-associated factor 3 (*Traf3*)	[97]
	transcription factor sox-9 (*Sox9*)	[98]
	IL10	[99]
	Il4	[100]
OA and PTOA	matrix metalloproteinase 13 (*MMP13*), interleukin-1beta (*IL1B*) and nerve growth factor (*NGF*).	[101]
PTOA and ROM	hypoxia-inducible factor-1 (*Hif1*)	[76]
PTOA	*IL10*	[75]
	Il 1 and promoting chondroprotection using lubricin (*Prg4*)	[102]
HA	interleukin-4 (*IL4*) and interleukin-10 (*IL10*)	[77]
	coagulation factor VIII (F8)	[78, 79]
MPS	alfa-L-iduronidase (*IDUA*)	[19]

RA, rheumatoid arthritis; OA, osteoarthritis; PTOA, post-traumatic osteoarthritis; ROM; limited range of motion; HA, hemophilic arthropathy; MPS, mucopolysaccharidoses.

was used to improve *in vivo* transfection efficiency of a reporter plasmid [105]. In addition, plasmid DNA was delivered by D-mannose [99] and cationic nanoemulsions [19].

Presently, combining the advantages of viral vectors and biomaterials, an injectable and thermosensitive hydrogel based on poloxamers, capable of controlled release of a therapeutic rAAV vector overexpressing a transcription factor was used. This protocol allows a controlled and minimally invasive delivery of gene vectors in a spatially precise manner, reducing the intra-articular spread of the vector and possible loss of therapeutic gene product [98].

Mesenchymal stem cells (MSCs) have been genetically modified *ex vivo* using lentiviral vectors and have been applied for OA therapy because they can secrete chondroprotective and anti-inflammatory factors [88, 106]. In a preclinical study, bone-marrow-derived MSCs were used for hemophilic arthropathy in non-human primates [78].

The latest publications have shown that IA gene editing using the CRISPR/Cas9 tool can be used to silence different genes and has the potential to discover new disease targets [101] (Table **4**). Furthermore, although various viral vectors have been investigated and improved, AAV caused the greatest interest in musculoskeletal research due to their ability to transduce the cells located in regions with thick extracellular matrix (ECM), plus the other characteristics already mentioned [100]. Another tendency is the use of combined approaches. A recent study used a liposome to overexpress *Il4* in MSCs assembled in spheroids to treat OA. The results showed chondroprotective, and anti-inflammatory effects, relieving pain after intra-articular implantation in OA rats [100].

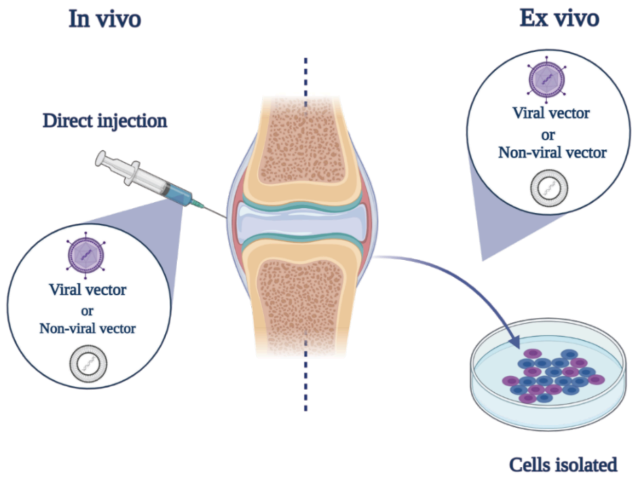

Fig. (4). Schematic representation of intra-articular gene therapy administration. The intra-articular gene therapy approach can be performed *in vivo* or *ex vivo* using viral and non-viral vectors. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 4. Latest studies published on intra-articular gene therapy.

Application	Summary of Therapy	Vector	Model	Main Results	Reference
Chondral defect	Injection of a thermosensitive biomaterial-guided delivery of recombinant adeno-associated virus (rAAV) vector to produce transcription factor SOX-9 (*Sox9*).	rAAV	Minipig	Improvement in cartilage repair and protection of the subchondral bone.	[98]
OA	Injection of a plasmid to overexpress interleukin 10 (*Il10*).	Plasmid	Dogs with naturally occurring OA	Reduced pain without toxicologic effects.	[99]
OA	Intra-Articular adeno-associated virus (AAV), AAV5-Il-10 administration.	AAV	Healthy horses	Rapid transduction and sustained expression of the transgene without inflammatory response.	[75]
OA	*Ex vivo*: interleukin-4 (*Il4*)-transfected Mesenchymal stem cells (MSCs) in spheroids (Il-4 MSC spheroid).	Liposome	OA rats	Better cartilage protection and pain relief compared to naïve MSCs.	[100]
OA and PTOA	CRISPR / Cas9 gene-mediated knockout of *n*erve growth factor (*Ngf*), matrix metalloproteinase-13 (*Mmp13*) or interleukin 1 beta (*Il1b*).	AAV	OA mouse model	Ablation of *Ngf* alleviates OA pain, and deletion of *Mmp13-Il1b* or *Il1b* attenuates structural damage. Multiplex ablation have synergistic effect.	[101]
PTOA	Combinatorial gene therapy approach to overexpress promoting chondroprotection using lubricin (*Prg4*) and interleukin-1 *receptor antagonist (Il1Ra).*	HDVs	PTOA animal models	Better effect than either monotherapy.	[102]
PTOA	IA injection of an adenovirus to silence TNF receptor-associated factor 3 (*Traf3*).	adenoviruses	*IL17a*$^{-/-}$ and *TRAF3* transgenic mice (T3TG)	Silencing *Traf3* through adenoviruses worsened cartilage degradation.	[97]
HA	Intravenous (IV) or intraarticular (IA) injection of AAV- recombinant human factor VIII *(rhFVIII).*	AAV	*FVIII*-/- mice	IA *rhFVIII* provided better protection from synovitis compared with IV *rhFVIII.*	[79]

Abbreviations: HA, hemophilic arthropathy; HDV- helper-dependent adenovirus; OA, osteoarthritis; PTOA, post-traumatic osteoarthritis; RA, rheumatoid arthritis.

4.1. Clinical Trials

As of November 2020, there are 12 clinical studies (clinictrials.gov) (Table **5**) involving IA gene therapy. However, no IA gene therapy product has been approved so far. Among the most advanced studies, the TissueGene-C (TG-C) protocol evaluates INVOSSA™, a product composed of allogeneic (donor) cells combined with a cell line transduced to overexpress the therapeutic growth factor (*TGFB1*), to treat OA. The phase I clinical trial evidenced that treatment offered sustained improvement in pain and function for more than 1 year with a single injection [107]. The phase II study showed that an injection improved pain and function for up to 24 months [108]. However, the sample size was not sufficiently large to be conclusive. Currently, clinical phase III is being performed for the United States [109].

Also, another clinical phase I study is currently recruiting patients to evaluate the safety and tolerability of FX201. It uses a helper-dependent adenovirus vector based on human serotype 5, designed to transfer the gene to produce an anti-inflammatory protein, interleukin-1 receptor antagonist (*IL1RN*), under the control of a promoter sensitive to inflammation (NCT04119687).

Moreover, another phase I study is currently ongoing, to assess the safety and tolerability of a single intra-articular administration of ART-I02 (AAV5.NF-kB.IFN-β), a recombinant adeno-associated virus type 5 vector in subjects with RA and active arthritis of a wrist. The primary outcomes are assessment of serious adverse events (vector DNA in whole peripheral blood, urine, feces, saliva, semen, an important humoral immune response against AAV and *IFNB1* (NCT03445715).

5. *IN SITU* ANTI-CANCER GENE THERAPY

Cancer refers to a set of distinct diseases that share similar fundamental properties [110], and is the second cause of death globally [111]. Cancer cells evolve with the accumulation of genetic and epigenetic alterations. Along this evolution, several subpopulations of tumor cells are selected according to the largest number of descendants generated and the greatest adaptability to the stress commonly present in the tumor microenvironment [112, 113]. Due to these characteristics, advances in the diagnosis and treatment of several types of cancer are still limited, resulting in poor or no improvement in patients' prognosis [114].

The post-genome era and technical advances have made it possible to improve the understanding of tumor biology, allowing the development of new strategies of therapy [110, 115, 116]. Along with surgery and radiotherapy, systemic chemotherapies are the main treatment strategy for cancer. However, the understanding of tumor microenvironment and advances in biotechnology have allowed *in situ* gene therapy, which has demonstrated various advantages considering both efficacy and safety [117]. The main strategies of intratumoral gene therapy consist of the introduction of exogenous nucleic acids, such as genes, gene segments, oligonucleotides, miRNAs or siRNAs into cancer cells, aiming to: (a) edit one or multiple genes, (b) affect endogenous gene expression or (c) interfere with the expression of exogenous protein [118-121]. In addition to this, advanced techniques of DNA editing such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR/Cas RNA-guided endonuclease system [122-124] have shown to be powerful strategies to treat cancer [125]. In general, the strategies of administration involve the direct injection of exogenous genetic material [126], which can be guided by imaging [127] or electroporation [128].

Table **6** summarizes the most important studies related to intratumoral gene therapy. As shown, the most prevalent vectors used for gene delivery are replication-deficient Adenoviral (ADd) vectors. In general, ADs have been extensively used as gene delivery tools for cancer gene therapy (20). They are double-stranded, non-enveloped DNA viruses that infect quiescent and dividing cells. ADs also have a large cloning capacity and do not integrate with the host genome. Besides, the use of therapy based on Herpes Simplex Virus-1 Thymidine Kinase (HSV-TK) has been the most evaluated method, as detailed below. Considering cancer types, prostate cancer has been the most studied neoplasia both in pre-clinical (Table **6**) and clinical trials (Table **7**). Among other tumor types, studies with frequent and/or aggressive human cancers like glioma, breast cancer, liver cancer and pancreatic cancer stand out. The next paragraphs discuss the most promising strategies for *in situ* gene therapy in cancer.

5.1. Strategies for Intratumoral Gene Therapy

Mechanisms underlying the effect of *in situ* gene therapy in cancer cells include triggering cell death, evoking the repair of DNA or sensitizing cancer cells to other therapies. Indeed, *in situ* gene therapy could also be used in combination with classic anti-cancer therapies like irradiation and chemotherapy, or with immunotherapy. This could lead to a synergistic increase in antitumor effects and decreased systemic toxicity [129-132].

Depending on the targeted gene/pathway, intratumoral gene therapy may trigger the death of cancer cells (*e.g.* suicide gene), impair cell growth or reactivate the anti-cancer immune response (*e.g.* gene silencing or the modulation of gene expression). According to this, three main strategies have been used in *in situ* gene therapy:

5.1.1. Suicide Gene

Through this, the products of transgenes' expression can trigger the death of cancer cells. The first application of suicide gene therapy was published in the early 1980s and was performed through the insertion of the Herpes Simplex Virus-1 Thymidine Kinase (HSV-TK) gene into murine BALB/c cell lines. The transformation of lymphocytes into lymphoblast cells was achieved through the infection with Epstein-Barr virus. Tumorigenesis inhibition was observed after the treatment with Ganciclovir (GCV), which was metabolized by cells infected with the HSV-TK, resulting in the formation of toxic metabolites that interrupted DNA replication and triggered cell death [133]. More recently, a pre-clinical study with rat orthotopic liver tumors has also

Table 5. Clinical trials using intra-articular gene therapy as of November 2020.

NCT Number	Title	Status	Conditions	Interventions	Phase	Sponsor/ Collaborators
NCT00126724	Study of Intra-articular Delivery of tgAAC94 in Inflammatory Arthritis Subjects	Completed	Arthritis, Rheumatoid Arthritis, Psoriatic Ankylosing Spondylitis	Genetic: tgAAC94 gene therapy vector Genetic: tgAAC94 placebo	Phase: Phase 1 Phase 2	Targeted Genetics Corporation
NCT00617032	Phase 1 Dose Escalation Study of Intra-Articular Administration of tgAAC94	Completed	Rheumatoid Arthritis	Genetic: tgAAC94 gene therapy vector Genetic: tgAAC94 placebo	Phase: Phase 1	Targeted Genetics Corporation
NCT02341378	Efficacy and Safety Study of TissueGene-C to Degenerative Arthritis	Completed	Degenerative Arthritis	Biological: TissueGene-C (Low dose) Biological: TissueGene-C (High dose)	Phase: Phase 2	Kolon Life Science
NCT02341391	Safety and Biological Efficacy Study of TisssueGene-C to Degenerative Arthritis	Completed	Degenerative Arthritis	Biological: TissueGene-C (Low dose) Biological: TissueGene-C (Medium dose) Biological: TissueGene-C (High dose)	Phase: Phase 1	Kolon Life Science
NCT00599248	Safety Study of TissueGene-C in Degenerative Joint Disease of the Knee	Completed	Osteoarthritis, Knee	Biological: TissueGene-C Biological: Placebo	Phase: Phase 1	Kolon TissueGene, Inc.
NCT02727764	A Single Dose Clinical Trial to Study the Safety of ART-I02 in Patients With Arthritis	Active, not recruiting	Arthritis, Rheumatoid Osteo Arthritis	Genetic: ART-I02	Phase: Phase 1	Arthrogen Centre for Human Drug Research (CHDR)
NCT04119687	Study to Evaluate the Safety and Tolerability of FX201 in Patients with Osteoarthritis of the Knee	Recruiting	Osteoarthritis, Knee	Biological: FX201	Phase: Phase 1	Flexion Therapeutics, Inc.
NCT02072070	Efficacy and Safety Study of TissueGene-C to Degenerative Arthritis	Completed	Degenerative Arthritis	Biological: TissueGene-C Drug: Placebo	Phase: Phase 3	Kolon Life Science
NCT03383471	The Efficacy and Safety of Invossa K Injection in Patients Diagnosed With Knee Osteoarthritis	Unknown status	Osteoarthritis	Biological: Invossa K Inj. Drug: Placebo	Phase: Phase 3	Kolon Life Science
NCT01671072	Efficacy and Safety Study of TissueGene-C to Degenerative Arthritis	Completed	Degenerative Arthritis	Biological: TissueGene-C Drug: Normal Saline	Phase: Phase 2	Kolon Life Science
NCT01782885	Comparison of Acetaminophen and PRP Therapy for Knee OA	Completed	Knee Osteoarthritis	Procedure: Intra-articular injection of PRP Drug: Acetaminophen	Phase: Not Applicable	Hospital Universitario Dr. Jose E. Gonzalez
NCT03445715	ART-I02 in Patients With Rheumatoid Arthritis With Inflamed Wrists	Unknown status	Rheumatoid Arthritis	Genetic: ART-I02	Phase: Phase 1	Arthrogen

Table 6. Literature review of pivotal studies related to *in situ* gene therapy found in Pubmed.

Refs.	Year	Article Type	Model	Method	Vector	Target	Cancer Type
Chen *et al*	1994	Research articles	Nude mouse	Intra-tumoral injection	Adenovirus: ADV/HSV-tk	The GCV-TK gene selectively induce apoptosis	Glioma
Yang *et al*	1996	Research article	Nude mouse	Intra-tumoral injection	Retroviral: GlTkSvNa.7	The GCV-TK gene selectively induce apoptosis	Pancreatic
Hull *et al*	2000	Research articles	Mouse	Intra-tumoral injection	Adenovirus: AdmIL-12	Administration of cytokines systemically	Prostate
Qin *et al*	2001	Research article	Mouse	Intra-tumoral injection	Vaccinia virus: RVV-IL-2	Induce strong nonspecific immunity and secretion of cytokines for the clonal expansion of precursor T cells.	Head and neck
Ma *et al*	2002	Research articles	Rat	Intra-tumoral injection	AAV: AAV-angiostatin	Cyclin-D1 expression	Head and neck
Hillman *et al*	2003	Research articles	Mouse	Intra-tumoral injection	pcDNA: pCIITA, pIFN-□, Ad-Ii-RGC: pIi-RGC	Upregulation of MHC class I1/class II1/Ii2 phenotype, cancer vaccine	Prostate
Satoh *et al*	2003	Research article	Mouse	Intra-tumoral injection	Adenovirus: ADmRTVP-1	*TP53*	Prostate
Subra-maniam *et al*	2007	Research articles	Mouse	Intra-tumoral injection	Non-Viral Vector: pSilenc-er 4.1 -CMV	CD44 siRNA	Colon
Baliaka *et al*	2013	Research articles	Mouse	Intra-tumoral injection	Non-Viral Vector: pSicop53	Regulation of p21 and *CDK4*/ Cyclin-D1 expression	NSCLC
Luo *et al*	2016	Research articles	Nude mouse	Intra-tumoral injection	Lentivirus: HSV-TK/GFP, PHSP-TK	The GCV-TK gene selectively induce apoptosis	Breast
Shi *et al*	2016	Research articles	Nude rat	Intra-tumoral injection	Lentivirus: HSV-TK/GCV	Ganciclovir, tumor suicide gene therapy	ESCC
Ariyoshi *et al*	2016	Research article	Mouse	Intra-tumoral injection	Adenovirus: AD/REIC/Dkk-3	Mediates simultaneous induction of cancer-selective apoptosis and augmentation of anti-cancer immunity	Lympho-ma
Xiong *et al*	2017	Research articles	Rat	RF elec-trodein	Lentivirus: HSV-TK/GFP	Ganciclovir, tumor suicide gene therapy	Hepato-cellular carcinoma
Jung *et al*	2017	Research article	Syrian hamster	Intra-tumoral injection	Oncolytic adenovirus: oAD-TRAIL/gel	T-cell-mediated antitumor immune response	Pancreas
Taeyoung *et al*	2017	Research articles	Nude mouse	Intra-tumoral injection	Adenovirus: AD/Cas9 + AD/sgEGFR	*EGFR* mutated	NSCLC
Liu *et al*	2018	Research article	Nude mouse	Intra-tumoral injection	Lentivirus: LV-H72-HIF-1α	Hypoxic pathways	Hepato-cellular carcinoma

(Table 6) contd….

Refs.	Year	Article Type	Model	Method	Vector	Target	Cancer Type
Kim *et al*	2018	Method	Nude mouse	Intra-tumoral injection	Adenovirus: CRISPR/Cas9-KRAS	Using Cas9 and guide RNAs that specifically recognize the mutant sequences	Colon
Jin *et al*	2019	Research article	Nude Mouse	Intra-tumoral injection	Lentivirus: HSV-TK/GCV + RFH	Activation of anti-tumor immunity + activation of apoptosis	Ovarian
Chen *et al*	2020	Research article	Mouse	Intra-tumoral injection	Non-Viral Vector: den-drimer/pDNA polyplexes (p53)	Regulation of p21 and *CDK4*/ Cyclin-D1 expression	Cervical
Kauczor *et al*	1999	Clinical Trial	Human - phase I	CT-guided intra-tumoral	Adenovirus: wt p53 cDNA	Regulation of p21 and *CDK4*/	NSCLC
Boulay *et al*	2000	Clinical Trial	Human - phase I/II	Intra-tumoral Injection	Adenovirus: rAd-p53	To restore a loss of p53 function	NSCLC
Ayala *et al*	2000	Clinical Trial	Phase I/II	Intrapros-tatic injections	Adenovirus: ADV/HSV-tk	The GCV-TK gene selectively induce apoptosis	Prostate
Teh *et al*	2001	Clinical Trial	Phase I/II	Intrapros-tatic injections	Adenovirus: ADV/HSV-TK	The GCV-TK gene selectively induce apoptosis	Prostate
Miles *et al*	2001	Clinical Trial	phase I/II	Intrapros-tatic injections	Adenovirus: ADV/HSV-TK	The GCV-TK gene selectively induce apoptosis	Prostate
Satoh *et al*	2004	Clinical Trial	Human	Intrapros-tatic injections	Adenovirus: ADV/HSV-TK	Systemic T-cell responses	Prostate
Fujita *et al*	2006	Clinical Trial	Human	Intrapros-tatic injections	Adenovirus: ADV/HSV-TK	The GCV-TK gene selectively induce apoptosis	Prostate

Abbreviatures: ADV, Adenovirus; TK, thymidine kinase; HSV, herpes simplex virus; NSCLC, Non-small Cell Lung Cancer; ESCC, Esophageal Squamous Cell Carcinoma.

demonstrated that the combination of intratumoral gene therapy using HSV-TK/GCV associated with radiofrequency hyperthermia reduced tumor growth in comparison to those therapies alone. This demonstrates that it is feasible to combine HSV-TK therapy with other therapies [134]. Another strategy of *in situ* gene therapy based on the HSV-TK/GCV system involves the control of HSV-TK expression by the promoter of the human telomerase reverse transcriptase (hTERT), which is frequently overexpressed in human cancer cells. After GCV treatment, this suicide gene system decreased the viability of human renal carcinoma cells, but not normal fibroblasts, illustrating the efficacy and the specificity of this strategy [135, 136]. Finally, repeated cycles of *in situ* HSV-TK plus GCV gene therapy in patients with prostate cancer resistant to radiotherapy led to the radiosensitization of tumor cells and delayed tumor recurrence, accompanied by increased anti-cancer immune response [137].

5.1.2. Exogenous Gene Expression

Another strategy of *in situ* gene therapy involves the electroporation of plasmids containing genes that, when expressed, may increase tumor immunogenicity. The most advanced strategy in this context involves the intratumoral cytokine gene therapy for IL-12, a pro-inflammatory molecule that links innate and adaptive immune responses [138]. Intratumoral electroporation-mediated IL-12 gene therapy (IT-pIL12/EP) has been tested in both animal models and in clinical trials [139] (see Table **7**). This strategy yielded a regression of melanoma lesions in 50% of patients as monotherapy [140], confirming data from several studies using animal models of melanoma, colorectal and renal cancer [141-143]. Importantly, the electroporation protocol, as well as the plasmid vector and other parameters have been modified in order to improve the effectiveness of this strategy, as shown in a murine melanoma model [144].

5.1.3. Gene Editing/repair

Allowing or triggering specific DNA repair in the driver genes involved in carcinogenesis is, in theory, one of the most promising strategies for antitumor gene therapy. Recent advances have increased the feasibility of this strategy, mainly due to the versatility of site-specific nucleases

Table 7. Clinical trials using *in situ* gene therapy in cancer, organized by date of initiation.

Title and Number of Registration	Status	Phase	Results	Condition	Interventions	Starting	Completion
Gene Therapy for the Treatment of Brain Tumors Using Intra-Tumoral Transduction With the Thymidine Kinase Gene and Intravenous Ganciclovir (NCT00001328)	Completed	1	No Results Available	Brain metastasis	Drug: Cytovene (Ganciclovir). Device: G1TKSVNa.53	1992	2010
Phase I Clinical Trial Of Gene Therapy For Hepatocellular Carcinoma By Intratumoral Injection Of TK99UN (An Adenoviral Vector Containing The Thymidine Kinase Of Herpes Simplex Virus) (NCT00844623)	Completed	1	No Results Available	Hepatocellular carcinoma	Genetic: TK99UN	2000	2004
Protocol IL-2001: A Multi-Center, Open-Label, Randomized Study of the Efficacy and Safety of Multiple Intratumoral Injections of hIl-2 Plasmid (1.8 mg) Formulated With DOTMA/Cholesterol [Ratio 1:0.5(-/+)] Liposomes in Patients With Unresctable or Recurrent/Refractory Squamous Cell Carcinoma of the Head and Neck (NCT00006033)	Completed	2	No Results Available	Head and Neck Cancer	Biological: Interleukin-2 gene; Drug: methotrexate	2002	2008
A Single Arm, Phase II Study of TNFerade™ Biologic Gene Therapy + Radiation + 5-FU and Cisplatin in Locally Advanced, Resectable, Esophageal Cancer (NCT00051480)	Completed	2	No Results Available	Esophageal Cancer	Genetic: TNFerade	2003	2011
Phase I Trial of Intratumoral pIL-12 Electroporation in Malignant Melanoma (NCT00323206)	Completed	1	No Results Available	Malignant Melanoma	Biological: IL-12p DNA; Procedure: Intratumoral Electroporation	2004	2008
A Phase II Study of the Efficacy, Safety and Immunogenicity of OncoVEX^GM-CSF in Patients With Stage IIIc and Stage IV Malignant Melanoma (NCT00289016)	Completed	2	Has Results	Melanoma	Drug: Talimogene Laherparepvec	2005	2009
Phase I Trial of Adenoviral Vector Delivery of the Human Interleukin-12 cDNA by Intratumoral Injection in Patients With Metastatic Breast Cancer (NCT00849459)	Completed	1	No Results Available	Breast Cancer	Biological: adenovirus-mediated human interleukin-12	2008	2011
A Phase I Trial of CCL21 Gene Modified Dendritic Cells In Non-Small Cell Lung Cancer (NCT00601094)	Completed	1	No Results Available	Lung Cancer	Biological: autologous dendritic cell-adenovirus CCL21 vaccine	2009	2017
Phase 1/2a, Dose-Escalation, Safety, Pharmacokinetic, and Preliminary Efficacy Study of Intratumoral Administration of DTA-H19 in Patients With Unresectable Pancreatic Cancer (NCT00711997)	Completed	2	Has Results	Pancreatic Neoplasms	Biological: DTA-H19	2009	2010
A Phase 1 Ascending Dose Trial of the Safety and Tolerability of Toca 511 in Patients With Recurrent High Grade Glioma (NCT01156584)	Completed	1	No Results Available	Glioma	Biological: Toca 511 vector|Drug: Toca FC	2010	2016

(Table 7) contd….

Title and Number of Registration	Status	Phase	Results	Condition	Interventions	Starting	Completion
A Pilot Feasibility Study of Oral 5-Fluorocytosine and Genetically-Modified Neural Stem Cells Expressing E.Coli Cytosine Deaminase for Treatment of Recurrent High Grade Gliomas (NCT01172964)	Completed	1	No Results Available	Glioma	Drug: flucytosine; Biological: *E. coli* CD-expressing genetically modified neural stem cells	2010	2015
A Phase II Study of Intratumoral Injection of Interleukin-12 Plasmid and *in Vivo* Electroporation in Patients With Merkel Cell Carcinoma (NCT01440816)	Completed	2	Has Results	Merkel Cell Carcinoma	Biological: Tavokinogene Telseplasmid (tavo); Device: OncoSec Medical System (OMS)	2012	2015
A Multicenter Phase II Trial of Intratumoral pIL-12 Electroporation in Advanced Stage Cutaneous and in Transit Malignant Melanoma (NCT01502293)	Completed	2	Has Results	Melanoma	Biological: Tavokinogene Telseplasmid (tavo); Device: OncoSec Medical System (OMS)	2012	2016
A Phase 1 / 2a Study of In-situ REIC/Dkk-3 Therapy in Patients With Localized Prostate Cancer (MTG-REIC-PC003) (NCT01931046)	Completed	2	No Results Available	Localized Prostate Cancer	Drug: Ad5-SGE-REIC/Dkk3	2013	2020
Evaluation of Pharmacodynamic Effects of Intratumoral Delivery of Plasmid IL-12 Electroporation in Patients With Triple Negative Breast Cancer (NCT02531425)	Completed	1	No Results Available	Breast Cancer	Biological: IT-pIL12-EP	2015	2018

[123, 125]. Gene editing/repair can be achieved using one of the three main nucleases (ZFN, TALEN or CRISPR/Cas) attached to a lentiviral vector. Once the viral vector enters the nucleus, it binds to a specific *locus* in the double-stranded DNA, leading to DNA strand breaks and subsequent endogenous repair mechanisms, which create a newly edited double-stranded DNA [145]. Recently, a novel class 2/type V CRISPR RNA guided endonuclease – using oncolytic AD as a vector – was succeed on targeting and editing EGFR gene in human lung cancer cells and in a murine xenograft model, triggering high levels of apoptosis and tumor growth arrest [146]. Importantly, this effect was cancer-specific, without detectable off-target nuclease activity.

5.1.4. Nanoformulations

The success of gene therapy depends, among other factors, on efficient delivery systems. Naked therapeutic nucleic acids are very susceptible to nuclease attack or phagocytosis, in addition to the difficulty of accessing biological barriers. Thus, the development of stable carriers of genetic material should contribute to the effectiveness and safety of these therapies [145], with several advantages over viral vectors or naked strategies [147, 148]. Among the alternatives, nanoparticles carrying the genetic material have been proved as a promising strategy of delivery, despite being tested mainly through systemic or pre-systemic routes of administration [149, 150]. Many studies have proposed nanoformulations carrying gene therapy to: (a) correct oncogenes/tumor suppressor genes [151, 152], (b) trigger cell death through activating cell death pathways [153, 154] or (c) reactivate anti-tumor immune cells [155]. As an example, a single-arm study investigated the treatment of glioblastoma multiforme with 66 patients. They combined the treatment of radiotherapy with intratumoral instillation of magnetic iron-oxide nanoparticles, and reported a notable increase in overall survival [156]. Besides, a phase 1 clinical study with 26 patients with advanced or metastatic solid tumor aimed to assess the efficacy of anti-EGFR-immunoliposomes intravenously infused. Authors found one complete response, one partial response, and ten stable disease lasting 2-12 months (NCT01702129) [157]. Another promising therapy includes the use of nanoencapsulated TNF-related apoptosis-inducing ligand (TRAIL) leading to the caspase-dependent apoptosis in glioblastoma *in vitro* and in an animal model [158]. Indeed, intratumoral TRAIL delivery is very promising both alone or in combination with other therapies, but clinical tests are necessary [159]. Owing to its short half-life *in vivo*, a cationic lipid was designed to

evaluate antitumor efficacy in a mice NSCLC model. They received treatment through tail veins, and both *in vitro*/*in vivo* showed intrinsic antitumor activity with no significant off-target toxicities to major organs and tissues [160]. These results demonstrate the potential of nanomedicine to *in situ* anti-cancer therapy, but also show that intravenous administration is still the most common strategy. Despite the results of intratumoral distribution suggest an improvement in the therapeutic response to pharmacological treatments, more advances are needed in terms of *in situ* gene therapy. Notwithstanding, the plasticity of nanoformulations, including variables such as particle size, charge and surface, can contribute to the development of more effective and safe delivery strategies for genetic material.

5.2. Tumor Microenvironment and Intratumoral Gene Therapy

Tumor microenvironment is comprised of tumor cells, tumor stroma, blood vessels, infiltrating immune cells, among other components. In order to favor their survival and progression, cancer cells modulate several of these components and processes, like angiogenesis and immune activity [161].

Systemic therapies targeting angiogenesis or aiming to reactivate the immune system against cancer have skyrocketed in last decades, but their efficacy was limited in several cancer types [162, 163]. In this regard, *in situ* gene therapies have shown some advantages over systemic administration once they allow high local concentrations of the treatment to be applied, while reducing the risk of immune-related toxicities. In addition, *in situ* gene therapy increases the bioavailability of immunostimulatory molecules, which may increase the therapeutic effectiveness [139, 164].

One of the strategies with the greatest therapeutic potential is the modulation of angiogenesis, which is essential for tumor growth and metastases [165]. Compared to the recombinant anti-Vascular endothelial growth factor (VEGF) antibody Bevacizumab, intratumoral gene therapy represents an attractive alternative [166]. Indeed, using anti-angiogenic genes such as angiostatin and endostatin, delivered by electroporation of an adeno-associated virus vector, has led to tumor regression in an animal xenograft model of colon cancer, with minimal side effects [167]. Related therapies, such as the intratumoral administration of vesicles containing siRNA for VEGF significantly reduced VEGF expression and suppressed the growth of prostate cancer in an animal tumor model [168]. Importantly, this effect was not accompanied by adverse reactions.

Immunotherapy, like anti-cancer vaccines, and immune stimulatory therapies, can reactivate the host immune system against tumor-specific antigens [169]. Cancer cells escape from the immune system by a plethora of mechanisms including the downregulation of antigenic proteins and the overexpression of negative regulators of anti-cancer immunity. Immune-based therapy may combine one or more of the above-mentioned tactics, also in combination with other modalities of cancer therapy [170]. One of the strategies to obtain a vaccine is through the transduction of tumor cells with a viral vector that contains multiple costimulatory molecules to enhance their immunogenicity. These modified cancer cells can, then, be administrated in the tumor microenvironment as a vaccine, as shown in a phase 1 clinical trial of prostate cancer [171]. An alternative approach is the direct administration of a poxviral vector into the tumor. Such an approach enhances the antigenicity and the subsequent antigen-specific T-cell response, leading to an anti-tumor response and tumor regression in a murine model of melanoma using B16-F10 mouse cells [172].

Despite presenting several genomic alterations, cancer cells usually do not express sufficient levels of these genes to trigger an anti-cancer immune response [173]. With this in mind, a cell-based vaccination strategy involves the insitu administration of vectors containing tumor neoantigens to directly augment the intratumoral expression of oncogenes and the presentation of tumor antigens. In animal models, this strategy strongly increased T cell infiltration in the tumor microenvironment, leading to the clearance of melanoma and pancreatic tumors in mice [174]. In humans, this strategy has been tested as a co-administration of virus-based vectors containing heterologous neoantigens with immune checkpoint inhibitors. It has been tested as a proof-of-concept in 5 patients with gastroesophageal adenocarcinoma, lung and colorectal cancers. To date, all patients produced a consistent CD8 T-cell response specific for predicted neoantigens, while not showing significant side effects [175].

Finally, studies from the last decade have shed some light on other key players of cancer progression also present in the tumor microenvironment, like cancer stem cells (CSCs) [113]. However, despite growing evidence showing that these cells are involved in cancer resistance and recurrence [176], no pharmacological therapy specific to this subpopulation of cells is available. In this context, Subramanian and colleagues showed a strong suppression of colon cancer growth in mice after intratumoral gene therapy with a polyethylenimine/siRNA CD44 plasmid DNA complex suppression [177]. CD44 is a classic marker of colon CSCs, so that intratumoral gene therapy emerges as a promising strategy to specifically reduce CSCs.

5.3. Clinical Trials for *In situ* Gene Therapy

The development of precision therapy in the early 2000s was focused on cancers with very poor prognoses. Considering *in situ* gene therapy, we found almost 20 completed trials in clinical trials (phase 1: 11 trials; phase 2: 9 trials) involving various cancer types including melanoma, pancreatic cancer, head and neck cancer, and glioma (Table **7**). Only 6 out of these trials showed results, which have been discussed in the above sections. Most of them started between the 1990s and 2010, but almost half of them started between 2010 and 2020. Despite a plethora of approaches and even combined radio-gene-hormonal therapy [178], only a few phase I and II clinical trials were completed. From all trials evaluating gene therapy to treat cancer, only 2.7% of them involve intratumoral strategies [137, 179].

5.4. Challenges and Perspective

In the last two decades, gene therapy has advanced as an alternative for several diseases, while its clinical use in cancer has encountered barriers related both to the biology of

the disease and to technological limitations. Recent years, however, have experienced considerable progress in three aspects that are crucial to the success of intratumoral gene therapy in cancer: 1) the best understanding of tumor molecular biology, 2) the best understanding of the tumor microenvironment; 3) the development of new biotechnological tools. Indeed, the post-genome era is revolutionizing cancer therapy, from nonspecific cancer treatments to more customized strategies based on patients' characteristics and the genetics of the disease [125]. All this knowledge brought many answers and, at the same time, raised new questions when considering intratumoral gene therapy. For example, it is necessary to understand how vectors or formulations interact with the different components of the extracellular matrix and with the other cells of the tumor microenvironment; how tumor heterogeneity may require multiple gene therapy to correct changes in the main drivers of each tumor, and the best manner to design these multi-target strategies; and how to avoid off-target effects as much as possible, since in many tumors the tumor stroma cells are very abundant

Despite these challenges, intratumoral therapies have several advantages in relation to systemic therapies, as proved by increased efficacy and reduced adverse effects after intratumoral chemotherapy in comparison to systemic therapies [167, 168]. This may also be the case for gene therapies, since using this strategy *in loco* should reduce the risk of exposure of normal cells to therapy, increase the likelihood that therapy will reach the target cell, and protect genetic material from degradation, increasing its half-life.

In addition to these biological aspects, biotechnology has advanced in several aspects including new delivery tools, new vectors, more accurate techniques and more accurate assessment methods. As highlighted in this section, nanoformulations carrying therapeutic nucleic acids, for instance, should guarantee greater protection as well as greater delivery efficiency and less risk of an immunological reaction to therapy. New surgical strategies may also allow better access for intratumor application of gene therapies with a reduced risk of adverse effects. Finally, new molecular tools and vectors can guarantee a lower risk of incorrect or off-target gene editing, improving efficiency and reducing the risk of side effects.

Finally, other translational questions have been raised: how to ensure that gene editing occurs in as many cells as possible? How to target tumor heterogeneity? How to protect therapeutic genetic material? What are the most effective and safe combinations considering surgery, pharmacological therapies and gene therapy *in situ*? How and when to apply intratumoral gene therapy in the context of clinical management? All these questions are currently being tested from cells to animal models and clinical trials. We believe that all these advances will allow the rational development of more effective and safe strategies for intratumoral gene therapy, alone or in combination with other therapeutic approaches.

CONCLUSION

As a field of science, Gene Therapy has come a long way. From initial tests to important drawbacks, it finally seems to blossom as a viable therapeutic approach, with the first gene therapy products been approved in the last years. The growth in the number of clinical protocols is evident, and many more products should reach the market in the next years. This increase is also evidencing new challenges, such as the high dose of vectors needed to produce a significant therapeutic effect when applied intravenously, the risks and costs associated with this approach. In this sense, *in situ* gene therapy allows the use of lower doses of vector, reducing the cost of the therapy and providing a safer treatment alternative. Considering these aspects, the preclinical and clinical studies summarized here add valuable information to the medical literature in the field, and certainly will allow the design of new gene therapy products and procedures in the years to come.

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES

[1] Drug AF and. What is gene therapy? 2020. Available from: https://www.fda.gov/vaccines-blood-biologics/cellu

[2] High KA, Roncarolo MG. Gene Therapy. N Engl J Med 2019; 381(5): 455-64. http://dx.doi.org/10.1056/NEJMra1706910 PMID: 31365802

[3] Hu WW, Ting JC. Gene immobilization on alginate/polycaprolactone fibers through electrophoretic deposition to promote *in situ* transfection efficiency and biocompatibility. Int J Biol Macromol 2019; 121: 1337-45. http://dx.doi.org/10.1016/j.ijbiomac.2018.09.043 PMID: 30201562

[4] Evans CH, Huard J. Gene therapy approaches to regenerating the musculoskeletal system. Nat Rev Rheumatol 2015; 11(4): 234-42. http://dx.doi.org/10.1038/nrrheum.2015.28 PMID: 25776949

[5] Wang D, Tai PWL, Gao G. Adeno-associated virus vector as a platform for gene therapy delivery. Nat Rev Drug Discov 2019; 18(5): 358-78. http://dx.doi.org/10.1038/s41573-019-0012-9 PMID: 30710128

[6] Sato M, Inada E, Saitoh I, Watanabe S, Nakamura S. *piggy*bac-based non-viral *in vivo* gene delivery useful for production of genetically modified animals and organs. Pharmaceutics 2020; 12(3): 12. http://dx.doi.org/10.3390/pharmaceutics12030277 PMID: 32204422

[7] Mashel TV, Tarakanchikova YV, Muslimov AR, *et al.* Overcoming the delivery problem for therapeutic genome editing: Current status and perspective of non-viral methods. Biomaterials 2020; 258: 120282. http://dx.doi.org/10.1016/j.biomaterials.2020.120282 PMID: 32798742

[8] Cucchiarini M, Madry H. Biomaterial-guided delivery of gene vectors for targeted articular cartilage repair. Nat Rev Rheumatol 2019; 15(1): 18-29. http://dx.doi.org/10.1038/s41584-018-0125-2 PMID: 30514957

[9] Poletto E, Baldo G, Gomez-Ospina N. Genome editing for mucopolysaccharidoses. Int J Mol Sci 2020; 21(2): 1-20. http://dx.doi.org/10.3390/ijms21020500 PMID: 31941077

[10] Trapani I, Auricchio A. Has retinal gene therapy come of age? From bench to bedside and back to bench. Hum Mol Genet 2019; 28(R1): R108-18. http://dx.doi.org/10.1093/hmg/ddz130 PMID: 31238338

[11] Lwin SM, Syed F, Di WL, *et al.* Safety and early efficacy outcomes for lentiviral fibroblast gene therapy in recessive dystrophic epidermolysis bullosa. JCI Insight 2019; 4(11): 4. http://dx.doi.org/10.1172/jci.insight.126243 PMID: 31167965

[12] Giugliani R, Vairo F, Kubaski F, *et al.* Neurological manifestations of lysosomal disorders and emerging therapies targeting the CNS. Lancet Child Adolesc Health 2018; 2(1): 56-68. http://dx.doi.org/10.1016/S2352-4642(17)30087-1 PMID: 30169196

[13] Ishikawa K, Weber T, Hajjar RJ. Human cardiac gene therapy. Circ Res 2018; 123(5): 601-13. http://dx.doi.org/10.1161/CIRCRESAHA.118.311587 PMID: 30355138

[14] Poletto E, Pasqualim G, Giugliani R, Matte U, Baldo G. Effects of gene therapy on cardiovascular symptoms of lysosomal storage diseases. Genet Mol Biol 2019; 42(1)(Suppl. 1): 261-85. http://dx.doi.org/10.1590/1678-4685-gmb-2018-0100 PMID: 31132295

[15] Eslami MH, Gangadharan SP, Sui X, Rhynhart KK, Snyder RO, Conte MS. Gene delivery to *in situ* veins: Differential effects of adenovirus and adeno-associated viral vectors. J Vasc Surg 2000; 31(6): 1149-59. http://dx.doi.org/10.1067/mva2000.106951 PMID: 10842152

[16] Rezaie ES, Visser NJ, Friedrich PF, Shin AY, Bishop AT. Intraluminal gene therapy in the porcine artery using a recombinant adeno-associated virus 9. Gene 2017; 618: 24-7. http://dx.doi.org/10.1016/j.gene.2017.03.019 PMID: 28322993

[17] Omichi R, Shibata SB, Morton CC, Smith RJH. Gene therapy for hearing loss. Hum Mol Genet 2019; 28(R1): R65-79. http://dx.doi.org/10.1093/hmg/ddz129 PMID: 31227837

[18] Goker F, Larsson L, Del Fabbro M, Asa'ad F. Gene delivery therapeutics in the treatment of periodontitis and peri-implantitis: A state of the art review. Int J Mol Sci 2019; 20(14): 20. http://dx.doi.org/10.3390/ijms20143551 PMID: 31330797

[19] Bidone J, Schuh RS, Farinon M, *et al.* Intra-articular nonviral gene therapy in mucopolysaccharidosis I mice. Int J Pharm 2018; 548(1): 151-8. http://dx.doi.org/10.1016/j.ijpharm.2018.06.049 PMID: 29940298

[20] Saraiva J, Nobre RJ, Pereira de Almeida L. Gene therapy for the CNS using AAVs: The impact of systemic delivery by AAV9. J Control Release 2016; 241: 94-109. http://dx.doi.org/10.1016/j.jconrel.2016.09.011 PMID: 27637390

[21] Griffin JM, Fackelmeier B, Fong DM, Mouravlev A, Young D, O'Carroll SJ. Astrocyte-selective AAV gene therapy through the endogenous GFAP promoter results in robust transduction in the rat spinal cord following injury. Gene Ther 2019; 26(5): 198-210. http://dx.doi.org/10.1038/s41434-019-0075-6 PMID: 30962538

[22] Zhang H, Yang B, Mu X, *et al.* Several rAAV vectors efficiently cross the blood-brain barrier and transduce neurons and astrocytes in the neonatal mouse central nervous system. Mol Ther 2011; 19(8): 1440-8. http://dx.doi.org/10.1038/mt.2011.98 PMID: 21610699

[23] Foust KD, Nurre E, Montgomery CL, Hernandez A, Chan CM, Kaspar BK. Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. Nat Biotechnol 2009; 27(1): 59-65. http://dx.doi.org/10.1038/nbt.1515 PMID: 19098898

[24] Gray SJ, Matagne V, Bachaboina L, Yadav S, Ojeda SR, Samulski RJ. Preclinical differences of intravascular AAV9 delivery to neurons and glia: A comparative study of adult mice and nonhuman primates. Mol Ther 2011; 19(6): 1058-69. http://dx.doi.org/10.1038/mt.2011.72 PMID: 21487395

[25] Duque S, Joussemet B, Riviere C, *et al.* Intravenous administration of self-complementary AAV9 enables transgene delivery to adult motor neurons. Mol Ther 2009; 17(7): 1187-96. http://dx.doi.org/10.1038/mt.2009.71 PMID: 19367261

[26] Hanlon KS, Meltzer JC, Buzhdygan T, *et al.* Selection of an efficient AAV vector for robust CNS transgene expression. Mol Ther Methods Clin Dev 2019; 15: 320-32. http://dx.doi.org/10.1016/j.omtm.2019.10.007 PMID: 31788496

[27] Chandler RJ, LaFave MC, Varshney GK, *et al.* Vector design influences hepatic genotoxicity after adeno-associated virus gene therapy. J Clin Invest 2015; 125(2): 870-80. http://dx.doi.org/10.1172/JCI79213 PMID: 25607839

[28] Boutin S, Monteilhet V, Veron P, *et al.* Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: Implications for gene therapy using AAV vectors. Hum Gene Ther 2010; 21(6): 704-12. http://dx.doi.org/10.1089/hum.2009.182 PMID: 20095819

[29] Hudry E, Vandenberghe LH. Therapeutic AAV gene transfer to the nervous system: A clinical reality. Neuron 2019. http://dx.doi.org/10.1016/j.neuron.2019.02.017

[30] Ikezu T. The use of viral vectors to enhance cognition. Cogn Enhanc Pharmacol Environ Genet Factors 2015. http://dx.doi.org/10.1016/B978-0-12-417042-1.00006-1

[31] Gray SJ, Woodard KT, Samulski RJ. Viral vectors and delivery strategies for CNS gene therapy. Ther Deliv 2010; 1(4): 517-34. http://dx.doi.org/10.4155/tde.10.50 PMID: 22833965

[32] Hardcastle N, Boulis NM, Federici T. AAV gene delivery to the spinal cord: Sserotypes, methods, candidate diseases, and clinical trials. Expert Opin Biol Ther 2018; 18(3): 293-307. http://dx.doi.org/10.1080/14712598.2018.1416089 PMID: 29249183

[33] Hocquemiller M, Giersch L, Audrain M, Parker S, Cartier N. Adeno-associated virus-based gene therapy for CNS diseases. Hum Gene Ther 2016; 27(7): 478-96. http://dx.doi.org/10.1089/hum.2016.087 PMID: 27267688

[34] Ojala DS, Amara DP, Schaffer DV. Adeno-associated virus vectors and neurological gene therapy. Neuroscientist 2015; 21(1): 84-98. http://dx.doi.org/10.1177/1073858414521870 PMID: 24557878

[35] Pardridge WM. Blood-brain barrier and delivery of protein and gene therapeutics to brain. Front Aging Neurosci 2020; 11: 373. http://dx.doi.org/10.3389/fnagi.2019.00373 PMID: 31998120

[36] Sudhakar V, Richardson RM. Gene therapy for neurodegenerative diseases. Neurotherapeutics 2019; 16(1): 166-75. http://dx.doi.org/10.1007/s13311-018-00694-0 PMID: 30542906

[37] Salegio EA, Samaranch L, Kells AP, *et al.* Axonal transport of adeno-associated viral vectors is serotype-dependent. Gene Ther 2013; 20(3): 348-52. http://dx.doi.org/10.1038/gt.2012.27 PMID: 22418061

[38] Pietersz KL, Martier RM, Baatje MS, *et al.* Transduction patterns in the CNS following various routes of AAV-5-mediated gene delivery. Gene Ther 2020. http://dx.doi.org/10.1038/s41434-020-0178-0 PMID: 32801344

[39] Spronck EA, Brouwers CC, Vallès A, *et al.* AAV5-miHTT gene therapy demonstrates sustained huntingtin lowering and functional improvement in huntington disease mouse models. Mol Ther Methods Clin Dev 2019; 13: 334-43. http://dx.doi.org/10.1016/j.omtm.2019.03.002 PMID: 30984798

[40] Green F, Samaranch L, Zhang HS, *et al.* Axonal transport of AAV9 in nonhuman primate brain. Gene Ther 2016; 23(6): 520-6. http://dx.doi.org/10.1038/gt.2016.24 PMID: 26953486

[41] Sands MS, Haskins ME. CNS-directed gene therapy for lysosomal storage diseases. Acta Paediatr Int J Paediatr 2008.

[42] Hocquemiller M, Hemsley KM, Douglass ML, *et al.* AAVrh10 vector corrects disease pathology in MPS IIIA mice and achieves widespread distribution of SGSH in large animal brains. Mol Ther Methods Clin Dev 2020. http://dx.doi.org/10.1016/j.omtm.2019.12.001 PMID: 31909089

[43] Rosenberg JB, Sondhi D, Rubin DG, *et al.* Comparative efficacy and safety of multiple routes of direct CNS administration of adeno-associated virus gene transfer vector serotype rh.10 expressing the human arylsulfatase A cDNA to nonhuman primates. Hum Gene Ther Clin Dev 2014; 25(3): 164-77. http://dx.doi.org/10.1089/humc.2013.239 PMID: 25144894

[44] Taghian T, Marosfoi MG, Puri AS, *et al.* A safe and reliable technique for central nervous system delivery of AAV vectors in the cisterna magna. Mol Ther 2020; 5: 411-21. http://dx.doi.org/10.1016/j.ymthe.2019.11.012 PMID: 31813800

[45] Vera LNP, Baldo G. The potential of gene therapy for mucopolysaccharidosis type I. Expert Opin Orphan Drugs 2020; 8: 33-41. http://dx.doi.org/10.1080/21678707.2020.1715208

[46] Vázquez-Domínguez I, Garanto A, Collin RWJ. Molecular therapies for inherited retinal diseases-current standing, opportunities and challenges. Genes (Basel) 2019; 10(9): 654. http://dx.doi.org/10.3390/genes10090654 PMID: 31466352

[47] Geary RS, Henry SP, Grillone LR. Fomivirsen: Clinical pharmacology and potential drug interactions. Clin Pharmacokinet 2002; 41(4): 255-60. http://dx.doi.org/10.2165/00003088-200241040-00002 PMID: 11978144

[48] Kourlas H, Schiller DS. Pegaptanib sodium for the treatment of neovascular age-related macular degeneration: A review. Clin Ther 2006; 28(1): 36-44. http://dx.doi.org/10.1016/j.clinthera.2006.01.009 PMID: 16490578

[49] Lloyd A, Piglowska N, Ciulla T, *et al.* Estimation of impact of *RPE65*-mediated inherited retinal disease on quality of life and the potential benefits of gene therapy. Br J Ophthalmol 2019; 103(11): 1610-4. http://dx.doi.org/10.1136/bjophthalmol-2018-313089 PMID: 30658988

[50] Zorzi GK, Schuh RS, de Campos AM, *et al.* Biomateriais para formulações de base nanotecnológica visando terapia genética ocular. In: scielo 2017; 40.

[51] Ahmad R. The challenges of ophthalmic drug delivery: A review. Obesity and vitamin D deficiency view project 2011.

[52] MacLaren REA. A 2020 vision of ocular gene therapy. Gene Ther 2020. PMID: 32601358

[53] Bennett J, Maguire AM. Gene therapy for ocular disease. Mol Ther 2000; 1(6): 501-5. http://dx.doi.org/10.1006/mthe.2000.0080 PMID: 10933974

[54] Hou AY, Kiss S. An update on ocular gene therapy for monogenic and multifactorial retinal diseases. J Vitreoretin Dis 2019; 3: 366-77. http://dx.doi.org/10.1177/2474126419876750

[55] Bucher K, Rodríguez-Bocanegra E, Dauletbekov D, Fischer MD. Immune responses to retinal gene therapy using adeno-associated viral vectors -implications for treatment success and safety. Prog Retin Eye Res 2020; 100915. http://dx.doi.org/10.1016/j.preteyeres.2020.100915 PMID: 33069860

[56] Dalkara D, Kolstad KD, Caporale N, *et al.* Inner limiting membrane barriers to AAV-mediated retinal transduction from the vitreous. Mol Ther 2009; 17(12): 2096-102. http://dx.doi.org/10.1038/mt.2009.181 PMID: 19672248

[57] Stein L, Roy K, Lei L, Kaushal S. Clinical gene therapy for the treatment of RPE65-associated Leber congenital amaurosis. Expert Opin Biol Ther 2011; 11(3): 429-39. http://dx.doi.org/10.1517/14712598.2011.557358 PMID: 21299439

[58] Meyer CH, Krohne TU, Issa PC, *et al.* CHAPTER 10 - Routes for drug delivery to the eye and retina: intravitreal injections and vitrectomy.Retin Pharmacother. Edinburgh: W.B. Saunders 2010; pp. 67-73. http://dx.doi.org/10.1016/B978-1-4377-0603-1.00015-6

[59] Alhalafi AM. Applications of polymers in intraocular drug delivery systems. Oman J Ophthalmol 2017; 10(1): 3-8. http://dx.doi.org/10.4103/0974-620X.200692 PMID: 28298856

[60] Tsai C-H, Wang P-Y, Lin I-C, Huang H, Liu GS, Tseng CL. Ocular drug delivery: Role of degradable polymeric nanocarriers for ophthalmic application. Int J Mol Sci 2018; 19(9): 19. http://dx.doi.org/10.3390/ijms19092830 PMID: 30235809

[61] Bigot K, Gondouin P, Bénard R, *et al.* Transferrin non-viral gene therapy for treatment of retinal degeneration. Pharmaceutics 2020; 12(9): 836. http://dx.doi.org/10.3390/pharmaceutics12090836 PMID: 32882879

[62] Wójcik-Gryciuk A, Gajewska-Woźniak O, Kordecka K, Boguszewski PM, Waleszczyk W, Skup M. Neuroprotection of retinal ganglion cells with AAV2-BDNF pretreatment restoring normal TrkB receptor protein levels in glaucoma. Int J Mol Sci 2020; 21(17): 21. http://dx.doi.org/10.3390/ijms21176262 PMID: 32872441

[63] Osborne A, Khatib TZ, Songra L, *et al.* Neuroprotection of retinal ganglion cells by a novel gene therapy construct that achieves sustained enhancement of brain-derived neurotrophic factor/tropomyosin-related kinase receptor-B signaling. Cell Death Dis 2018; 9(10): 1007. http://dx.doi.org/10.1038/s41419-018-1041-8 PMID: 30258047

[64] Reid CA, Nettesheim ER, Connor TB, Lipinski DM. Development of an inducible anti-VEGF rAAV gene therapy strategy for the treatment of wet AMD. Sci Rep 2018; 8(1): 11763. http://dx.doi.org/10.1038/s41598-018-29726-7 PMID: 30082848

[65] Yang F, Ma H, Boye SL, Hauswirth WW, Ding XQ. Overexpression of type 3 iodothyronine deiodinase reduces cone death in the leber congenital amaurosis model mice. Adv Exp Med Biol 2018; 1074: 125-31. http://dx.doi.org/10.1007/978-3-319-75402-4_16 PMID: 29721936

[66] Askou AL, Benckendorff JNE, Holmgaard A, *et al.* Suppression of choroidal neovascularization in mice by subretinal delivery of multigenic lentiviral vectors encoding anti-angiogenic microRNAs. Hum Gene Ther Methods 2017; 28(4): 222-33. http://dx.doi.org/10.1089/hgtb.2017.079 PMID: 28817343

[67] Krishnan A, Fei F, Jones A, *et al.* Overexpression of soluble fas ligand following adeno-associated virus gene therapy prevents retinal ganglion cell death in chronic and acute murine models of glaucoma. J Immunol 2016; 197(12): 4626-38. http://dx.doi.org/10.4049/jimmunol.1601488 PMID: 27849168

[68] Jacobson SG, Cideciyan A V, Ratnakaram R, *et al.* Gene therapy for leber congenital amaurosis caused by RPE65 mutations: Safety and efficacy in 15 children and adults followed up to 3 years. Arch Ophthalmol 2012; 9-24. http://dx.doi.org/10.1001/archophthalmol.2011.298

[69] Testa F, Maguire AM, Rossi S, *et al.* Three-year follow-up after unilateral subretinal delivery of adeno-associated virus in patients with Leber congenital Amaurosis type 2. Ophthalmology 2013; 120(6): 1283-91. http://dx.doi.org/10.1016/j.ophtha.2012.11.048 PMID: 23474247

[70] Bainbridge JWB, Mehat MS, Sundaram V, *et al.* Long-term effect of gene therapy on Leber's congenital amaurosis. N Engl J Med 2015; 372(20): 1887-97. http://dx.doi.org/10.1056/NEJMoa1414221 PMID: 25938638

[71] Russell S, Bennett J, Wellman JA, *et al.* Efficacy and safety of voretigene neparvovec (AAV2-hRPE65v2) in patients with RPE65-mediated inherited retinal dystrophy: A randomised, controlled, open-label, phase 3 trial. Lancet 2017; 390(10097): 849-60. http://dx.doi.org/10.1016/S0140-6736(17)31868-8 PMID: 28712537

[72] FDA approves hereditary blindness gene therapy. Nat Biotechnol 2018; 36(1): 6. http://dx.doi.org/10.1038/nbt0118-6a PMID: 29319697

[73] Evans CH, Ghivizzani SC, Robbins PD. Gene delivery to joints by intra-articular injection. Hum Gene Ther 2018; 29(1): 2-14. http://dx.doi.org/10.1089/hum.2017.181 PMID: 29160173

[74] Jiménez G, Cobo-Molinos J, Antich C, López-Ruiz E. Osteoarthritis: Trauma *vs* disease. Adv Exp Med Biol 2018; 1059: 63-83. http://dx.doi.org/10.1007/978-3-319-76735-2_3 PMID: 29736569

[75] Moss KL, Jiang Z, Dodson ME, *et al.* Sustained interleukin-10 transgene expression following intra-articular AAV5-IL-10 administration to horses. Hum Gene Ther 2020; 31(1-2): 110-8. http://dx.doi.org/10.1089/hum.2019.195 PMID: 31773987

[76] Sotobayashi D, Kawahata H, Anada N, Ogihara T, Morishita R, Aoki M. Therapeutic effect of intra-articular injection of ribbon-type decoy oligonucleotides for hypoxia inducible factor-1 on joint contracture in an immobilized knee animal model. J Gene Med 2016; 18(8): 180-92. http://dx.doi.org/10.1002/jgm.2891 PMID: 27352194

[77] van Vulpen LFD, Popov-Celeketic J, van Meegeren MER, *et al.* A fusion protein of interleukin-4 and interleukin-10 protects against blood-induced cartilage damage *in vitro* and *in vivo*. J Thromb Haemost 2017; 15(9): 1788-98. http://dx.doi.org/10.1111/jth.13778 PMID: 28696534

[78] Ohmori T, Mizukami H, Katakai Y, *et al.* Safety of intra-articular transplantation of lentivirally transduced mesenchymal stromal cells for haemophilic arthropathy in a non-human primate. Int J Hematol 2018; 108(3): 239-45. http://dx.doi.org/10.1007/s12185-018-2465-8 PMID: 29737459

[79] Zhang F, Yan X, Li M, *et al.* Exploring the potential feasibility of intra-articular adeno-associated virus-mediated gene therapy for hemophilia arthropathy. Hum Gene Ther 2020; 31(7-8): 448-58.

http://dx.doi.org/10.1089/hum.2019.355 PMID: 32079420

[80] Bevaart L, Aalbers CJ, Vierboom MPM, *et al.* Safety, biodistribution, and efficacy of an AAV-5 vector encoding human interferon-beta (ART-I02) delivered *via* intra-articular injection in rhesus monkeys with collagen-induced arthritis. Hum Gene Ther Clin Dev 2015; 26(2): 103-12.
http://dx.doi.org/10.1089/humc.2015.009 PMID: 26086763

[81] Chen S-Y, Shiau A-L, Wu C-L, Wang CR. Intraarticular overexpression of Smad7 ameliorates experimental arthritis. Sci Rep 2016; 6: 35163.
http://dx.doi.org/10.1038/srep35163 PMID: 27731365

[82] Denadai-Souza A, Ribeiro CM, Rolland C, *et al.* Effect of tryptase inhibition on joint inflammation: A pharmacological and lentivirus-mediated gene transfer study. Arthritis Res Ther 2017; 19(1): 124.
http://dx.doi.org/10.1186/s13075-017-1326-9 PMID: 28587618

[83] Liu S, Kiyoi T, Takemasa E, Maeyama K. Intra-articular lentivirus-mediated gene therapy targeting CRACM1 for the treatment of collagen-induced arthritis. J Pharmacol Sci 2017; 133(3): 130-8.
http://dx.doi.org/10.1016/j.jphs.2017.02.001 PMID: 28258822

[84] Hong S-S, Marotte H, Courbon G, Firestein GS, Boulanger P, Miossec P. PUMA gene delivery to synoviocytes reduces inflammation and degeneration of arthritic joints. Nat Commun 2017; 8(1): 146.
http://dx.doi.org/10.1038/s41467-017-00142-1 PMID: 28747638

[85] Kanazawa T, Tamano K, Sogabe K, *et al.* Intra-articular Retention and Anti-arthritic Effects in Collagen-Induced Arthritis Model Mice by Injectable Small Interfering RNA Containing Hydrogel. Biol Pharm Bull 2017; 40(11): 1929-33.
http://dx.doi.org/10.1248/bpb.b17-00481 PMID: 29093340

[86] Luo X, Chen Y, Lv G, *et al.* Adenovirus-mediated small interfering RNA targeting TAK1 ameliorates joint inflammation with collagen-induced arthritis in mice. Inflammation 2017; 40(3): 894-903.
http://dx.doi.org/10.1007/s10753-017-0534-4 PMID: 28220341

[87] Jansen MAA, Klausen LH, Thanki K, *et al.* Lipidoid-polymer hybrid nanoparticles loaded with TNF siRNA suppress inflammation after intra-articular administration in a murine experimental arthritis model. Eur J Pharm Biopharm 2019; 142: 38-48.
http://dx.doi.org/10.1016/j.ejpb.2019.06.009 PMID: 31199978

[88] Xia Q, Zhu S, Wu Y, *et al.* Intra-articular transplantation of atsttrin-transduced mesenchymal stem cells ameliorate osteoarthritis development. Stem Cells Transl Med 2015; 4(5): 523-31.
http://dx.doi.org/10.5966/sctm.2014-0200 PMID: 25824140

[89] Ortved K, Wagner B, Calcedo R, Wilson J, Schaefer D, Nixon A. Humoral and cell-mediated immune response, and growth factor synthesis after direct intraarticular injection of rAAV2-IGF-I and rAAV5-IGF-I in the equine middle carpal joint. Hum Gene Ther 2015; 26(3): 161-71.
http://dx.doi.org/10.1089/hum.2014.050 PMID: 25705927

[90] Pi Y, Zhang X, Shao Z, Zhao F, Hu X, Ao Y. Intra-articular delivery of anti-Hif-2α siRNA by chondrocyte-homing nanoparticles to prevent cartilage degeneration in arthritic mice. Gene Ther 2015; 22(6): 439-48.
http://dx.doi.org/10.1038/gt.2015.16 PMID: 25876463

[91] Zhang X, Crawford R, Xiao Y. Inhibition of vascular endothelial growth factor with shRNA in chondrocytes ameliorates osteoarthritis. J Mol Med (Berl) 2016; 94(7): 787-98.
http://dx.doi.org/10.1007/s00109-016-1425-0 PMID: 27164955

[92] Aini H, Itaka K, Fujisawa A, *et al.* Messenger RNA delivery of a cartilage-anabolic transcription factor as a disease-modifying strategy for osteoarthritis treatment. Sci Rep 2016; 6: 18743.
http://dx.doi.org/10.1038/srep18743 PMID: 26728350

[93] Wang G, Evans CH, Benson JM, *et al.* Safety and biodistribution assessment of sc-rAAV2.5IL-1Ra administered *via* intra-articular injection in a mono-iodoacetate-induced osteoarthritis rat model. Mol Ther Methods Clin Dev 2016; 3: 15052.
http://dx.doi.org/10.1038/mtm.2015.52 PMID: 26817025

[94] Watson Levings RS, Broome TA, Smith AD, *et al.* Gene therapy for osteoarthritis: Pharmacokinetics of intra-articular self-complementary adeno-associated virus interleukin-1 receptor antagonist delivery in an equine model. Hum Gene Ther Clin Dev 2018; 29(2): 90-100.
http://dx.doi.org/10.1089/humc.2017.142 PMID: 29869540

[95] Watson Levings RS, Smith AD, Broome TA, *et al.* Self-complementary adeno-associated virus-mediated interleukin-1 receptor antagonist gene delivery for the treatment of osteoarthritis: Test of efficacy in an equine model. Hum Gene Ther Clin Dev 2018; 29(2): 101-12.
http://dx.doi.org/10.1089/humc.2017.143 PMID: 29869535

[96] Nakagawa R, Akagi R, Yamaguchi S, *et al.* Single *vs*. repeated matrix metalloproteinase-13 knockdown with intra-articular short interfering RNA administration in a murine osteoarthritis model. Connect Tissue Res 2019; 60(4): 335-43.
http://dx.doi.org/10.1080/03008207.2018.1539082 PMID: 30345823

[97] Hu G, Zhang N, Li J, *et al.* Tumor necrosis factor receptor associated factor 3 modulates cartilage degradation through suppression of interleukin 17 signaling. Am J Pathol 2020; 190(8): 1701-12.
http://dx.doi.org/10.1016/j.ajpath.2020.04.016 PMID: 32416098

[98] Madry H, Gao L, Rey-Rico A, *et al.* Thermosensitive hydrogel based on PEO-PPO-PEO poloxamers for a controlled *in situ* release of recombinant adeno-associated viral vectors for effective gene therapy of cartilage defects. Adv Mater 2020; 32(2): e1906508.
http://dx.doi.org/10.1002/adma.201906508 PMID: 31763733

[99] Watkins LR, Chavez RA, Landry R, *et al.* Targeted interleukin-10 plasmid DNA therapy in the treatment of osteoarthritis: Toxicology and pain efficacy assessments. Brain Behav Immun 2020; 90: 155-66.
http://dx.doi.org/10.1016/j.bbi.2020.08.005 PMID: 32800926

[100] Song SY, Hong J, Go S, *et al.* Interleukin-4 gene transfection and spheroid formation potentiate therapeutic efficacy of mesenchymal stem cells for osteoarthritis. Adv Healthc Mater 2020; 9(5): e1901612.
http://dx.doi.org/10.1002/adhm.201901612 PMID: 31977158

[101] Zhao L, Huang J, Fan Y, *et al.* Exploration of CRISPR/Cas9-based gene editing as therapy for osteoarthritis. Ann Rheum Dis 2019; 78(5): 676-82.
http://dx.doi.org/10.1136/annrheumdis-2018-214724 PMID: 30842121

[102] Stone A, Grol MW, Ruan MZC, *et al.* Combinatorial Prg4 and Il-1ra gene therapy protects against hyperalgesia and cartilage degeneration in post-traumatic osteoarthritis. Hum Gene Ther 2019; 30(2): 225-35.
http://dx.doi.org/10.1089/hum.2018.106 PMID: 30070147

[103] Kyostio-Moore S, Berthelette P, Cornell CS, Nambiar B, Figueiredo MD. Hyaluronic acid synthase-2 gene transfer into the joints of Beagles by use of recombinant adeno-associated viral vectors. Am J Vet Res 2018; 79(5): 505-17.
http://dx.doi.org/10.2460/ajvr.79.5.505 PMID: 29688780

[104] Si H-B, Zeng Y, Liu S-Y, *et al.* Intra-articular injection of microRNA-140 (miRNA-140) alleviates osteoarthritis (OA) progression by modulating extracellular matrix (ECM) homeostasis in rats. Osteoarthritis Cartilage 2017; 25(10): 1698-707.
http://dx.doi.org/10.1016/j.joca.2017.06.002 PMID: 28647469

[105] Xiang X, Tang Y, Leng Q, Zhang L, Qiu L. Targeted gene delivery to the synovial pannus in antigen-induced arthritis by ultrasound-targeted microbubble destruction *in vivo*. Ultrasonics 2016; 65: 304-14.
http://dx.doi.org/10.1016/j.ultras.2015.09.011 PMID: 26433434

[106] Sieker JT, Kunz M, Weißenberger M, *et al.* Direct bone morphogenetic protein 2 and Indian hedgehog gene transfer for articular cartilage repair using bone marrow coagulates. Osteoarthritis Cartilage 2015; 23(3): 433-42.
http://dx.doi.org/10.1016/j.joca.2014.11.008 PMID: 25463442

[107] Ha C-W, Noh MJ, Choi KB, Lee KH. Initial phase I safety of retrovirally transduced human chondrocytes expressing transforming growth factor-beta-1 in degenerative arthritis patients. Cytotherapy 2012; 14(2): 247-56.
http://dx.doi.org/10.3109/14653249.2011.629645 PMID: 22242865

[108] Ha C-W, Cho JJ, Elmallah RK, *et al.* A multicenter, single-blind, phase IIa clinical trial to evaluate the efficacy and safety of a cell-mediated gene therapy in degenerative knee arthritis patients. Hum Gene Ther Clin Dev 2015; 26(2): 125-30.
http://dx.doi.org/10.1089/humc.2014.145 PMID: 25760423

[109] Kim M-K, Ha C-W, In Y, *et al.* A multicenter, double-blind, phase III clinical trial to evaluate the efficacy and safety of a cell and gene therapy in knee osteoarthritis patients. Hum Gene Ther Clin Dev 2018; 29(1): 48-59.
http://dx.doi.org/10.1089/humc.2017.249 PMID: 29641281

[110] Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000; 100(1): 57-70. http://dx.doi.org/10.1016/S0092-8674(00)81683-9 PMID: 10647931
[111] World Health Organization nd
[112] Fouad YA, Aanei C. Revisiting the hallmarks of cancer. Am J Cancer Res 2017; 7(5): 1016-36. PMID: 28560055
[113] Vessoni AT, Filippi-Chiela EC, Lenz G, Batista LFZ. Tumor propagating cells: Drivers of tumor plasticity, heterogeneity, and recurrence. Oncogene 2020; 39(10): 2055-68. http://dx.doi.org/10.1038/s41388-019-1128-4 PMID: 31801972
[114] Gatenby RA, Brown JS. Integrating evolutionary dynamics into cancer therapy. Nat Rev Clin Oncol 2020; 17(11): 675-86. http://dx.doi.org/10.1038/s41571-020-0411-1 PMID: 32699310
[115] Fojo T, Mailankody S, Lo A. Unintended consequences of expensive cancer therapeutics—the pursuit of marginal indications and a me-too mentality that stifles innovation and creativity: the John Conley Lecture. JAMA Otolaryngol Head Neck Surg 2014; 140(12): 1225-36. http://dx.doi.org/10.1001/jamaoto.2014.1570 PMID: 25068501
[116] Hanahan D, Coussens LM. Accessories to the crime: Functions of cells recruited to the tumor microenvironment. Cancer Cell 2012; 21(3): 309-22. http://dx.doi.org/10.1016/j.ccr.2012.02.022 PMID: 22439926
[117] Baliaka A, Zarogoulidis P, Domvri K, *et al.* Intratumoral gene therapy *versus* intravenous gene therapy for distant metastasis control with 2-diethylaminoethyl-dextran methyl methacrylate copolymer non-viral vector-p53. Gene Ther 2014; 21(2): 158-67. http://dx.doi.org/10.1038/gt.2013.68 PMID: 24285215
[118] Xiao W, Zhang W, Huang H, *et al.* Cancer targeted gene therapy for inhibition of melanoma lung metastasis with eIF3i shRNA loaded liposomes. Mol Pharm 2020; 17(1): 229-38. http://dx.doi.org/10.1021/acs.molpharmaceut.9b00943 PMID: 31765158
[119] Senapati D, Patra BC, Kar A, *et al.* Promising approaches of small interfering RNAs (siRNAs) mediated cancer gene therapy. Gene 2019; 719: 144071. http://dx.doi.org/10.1016/j.gene.2019.144071 PMID: 31454539
[120] Vinhas R, Fernandes AR, Baptista PV. Gold nanoparticles for BCR-ABL1 gene silencing: Improving tyrosine kinase inhibitor efficacy in chronic myeloid leukemia. Mol Ther Nucleic Acids 2017; 7: 408-16. http://dx.doi.org/10.1016/j.omtn.2017.05.003 PMID: 28624216
[121] Li J, Chen J, Wang S, *et al.* Blockage of transferred exosome-shuttled miR-494 inhibits melanoma growth and metastasis. J Cell Physiol 2019; 234: 15763-74. http://dx.doi.org/10.1002/jcp.28234 PMID: 30723916
[122] Huang K, Yang C, Wang Q-X, *et al.* The CRISPR/Cas9 system targeting EGFR exon 17 abrogates NF-κB activation *via* epigenetic modulation of UBXN1 in EGFRwt/vIII glioma cells. Cancer Lett 2017; 388: 269-80. http://dx.doi.org/10.1016/j.canlet.2016.12.011 PMID: 27998759
[123] Gupta A, Christensen RG, Rayla AL, Lakshmanan A, Stormo GD, Wolfe SA. An optimized two-finger archive for ZFN-mediated gene targeting. Nat Methods 2012; 9(6): 588-90. http://dx.doi.org/10.1038/nmeth.1994 PMID: 22543349
[124] Kim Y, Kweon J, Kim A, *et al.* A library of TAL effector nucleases spanning the human genome. Nat Biotechnol 2013; 31(3): 251-8. http://dx.doi.org/10.1038/nbt.2517 PMID: 23417094
[125] Koo T, Yoon A-R, Cho H-Y, Bae S, Yun CO, Kim JS. Selective disruption of an oncogenic mutant allele by CRISPR/Cas9 induces efficient tumor regression. Nucleic Acids Res 2017; 45(13): 7897-908. http://dx.doi.org/10.1093/nar/gkx490 PMID: 28575452
[126] Kim YI, Ahn BC, Ronald JA, *et al.* Intratumoral *versus* intravenous gene therapy using a transcriptionally targeted viral vector in an orthotopic hepatocellular carcinoma rat model. J Vasc Interv Radiol 2012; 23(5): 704-11. http://dx.doi.org/10.1016/j.jvir.2012.01.053 PMID: 22387029
[127] Kauczor HU, Schuler M, Heussel CP, *et al.* CT-guided intratumoral gene therapy in non-small-cell lung cancer. Eur Radiol 1999; 9(2): 292-6. http://dx.doi.org/10.1007/s003300050670 PMID: 10101653
[128] Li S. Electroporation gene therapy: New developments *in vivo* and *in vitro*. Curr Gene Ther 2004; 4(3): 309-16. http://dx.doi.org/10.2174/1566523043346336 PMID: 15384944
[129] Formenti SC, Demaria S. Systemic effects of local radiotherapy. Lancet Oncol 2009; 10(7): 718-26. http://dx.doi.org/10.1016/S1470-2045(09)70082-8 PMID: 19573801
[130] Tetzlaff MT, Teh BS, Timme TL, *et al.* Expanding the therapeutic index of radiation therapy by combining *in situ* gene therapy in the treatment of prostate cancer. Technol Cancer Res Treat 2006; 5(1): 23-36. http://dx.doi.org/10.1177/153303460600500104 PMID: 16417399
[131] Tamura RE, Lana MG, Costanzi-Strauss E, Strauss BE. Combination of cabazitaxel and p53 gene therapy abolishes prostate carcinoma tumor growth. Gene Ther 2020; 27(1-2): 15-26. http://dx.doi.org/10.1038/s41434-019-0071-x PMID: 30926960
[132] Hillman GG, Xu M, Wang Y, *et al.* Radiation improves intratumoral gene therapy for induction of cancer vaccine in murine prostate carcinoma. Hum Gene Ther 2003; 14(8): 763-75. http://dx.doi.org/10.1089/104303403765255156 PMID: 12804139
[133] Field AK, Davies ME, DeWitt C, *et al.* 9-([2-hydroxy-1-(hydroxymethyl)ethoxy]methyl)guanine: a selective inhibitor of herpes group virus replication. Proc Natl Acad Sci 1983; 139 LP-4143.
[134] Xiong F, Zhang F, Jin Y, *et al.* Orthotopic hepatic cancer: Radiofrequency hyperthermia-enhanced intratumoral herpes simplex virus-thymidine kinase gene therapy. Oncotarget 2017; 9(18): 14099-108. http://dx.doi.org/10.18632/oncotarget.23586 PMID: 29581830
[135] Düzgüneş N. Origins of suicide gene therapy. Methods Mol Biol 2019; 1895: 1-9. http://dx.doi.org/10.1007/978-1-4939-8922-5_1 PMID: 30539525
[136] Tian D, Sun Y, Yang Y, Lei M, Ding N, Han R. Human telomerase reverse-transcriptase promoter-controlled and herpes simplex virus thymidine kinase-armed adenoviruses for renal cell carcinoma treatment. OncoTargets Ther 2013; 6: 419-26. PMID: 23723709
[137] Miles BJ, Shalev M, Aguilar-Cordova E, *et al.* Prostate-specific antigen response and systemic T cell activation after *in situ* gene therapy in prostate cancer patients failing radiotherapy. Hum Gene Ther 2001; 12(16): 1955-67. http://dx.doi.org/10.1089/104303401753204535 PMID: 11686937
[138] Hillman GG, Slos P, Wang Y, *et al.* Tumor irradiation followed by intratumoral cytokine gene therapy for murine renal adenocarcinoma. Cancer Gene Ther 2004; 11(1): 61-72. http://dx.doi.org/10.1038/sj.cgt.7700656 PMID: 14681727
[139] Mukhopadhyay A, Wright J, Shirley S, *et al.* Characterization of abscopal effects of intratumoral electroporation-mediated IL-12 gene therapy. Gene Ther 2019; 26(1-2): 1-15. http://dx.doi.org/10.1038/s41434-018-0044-5 PMID: 30323352
[140] Daud A, Algazi AP, Ashworth MT, *et al.* Systemic antitumor effect and clinical response in a phase 2 trial of intratumoral electroporation of plasmid interleukin-12 in patients with advanced melanoma. J Clin Oncol 2014; 32: 9025. http://dx.doi.org/10.1200/jco.2014.32.15_suppl.9025
[141] Tamura T, Nishi T, Goto T, *et al.* Intratumoral delivery of interleukin 12 expression plasmids with *in vivo* electroporation is effective for colon and renal cancer. Hum Gene Ther 2001; 12(10): 1265-76. http://dx.doi.org/10.1089/104303401750270922 PMID: 11440620
[142] Lucas ML, Heller L, Coppola D, Heller R. IL-12 plasmid delivery by *in vivo* electroporation for the successful treatment of established subcutaneous B16.F10 melanoma. Mol Ther 2002; 5(6): 668-75. http://dx.doi.org/10.1006/mthe.2002.0601 PMID: 12027550
[143] Goto T, Nishi T, Kobayashi O, *et al.* Combination electro-gene therapy using herpes virus thymidine kinase and interleukin-12 expression plasmids is highly efficient against murine carcinomas *in vivo*. Mol Ther 2004; 10(5): 929-37. http://dx.doi.org/10.1016/j.ymthe.2004.07.028 PMID: 15509510
[144] Burkart C, Mukhopadhyay A, Shirley SA, *et al.* Improving therapeutic efficacy of IL-12 intratumoral gene electrotransfer through novel plasmid design and modified parameters. Gene Ther 2018; 25(2): 93-103. http://dx.doi.org/10.1038/s41434-018-0006-y PMID: 29523878

[145] Chen J, Guo Z, Tian H, Chen X. Production and clinical development of nanoparticles for gene delivery. Mol Ther Methods Clin Dev 2016; 3: 16023. http://dx.doi.org/10.1038/mtm.2016.23 PMID: 27088105

[146] Yoon A-R, Jung B-K, Choi E, *et al.* CRISPR-Cas12a with an oAd induces precise and cancer-specific genomic reprogramming of EGFR and efficient tumor regression. Mol Ther 2020; 28(10): 2286-96. http://dx.doi.org/10.1016/j.ymthe.2020.07.003 PMID: 32682455

[147] Yin H, Kanasty RL, Eltoukhy AA, Vegas AJ, Dorkin JR, Anderson DG. Non-viral vectors for gene-based therapy. Nat Rev Genet 2014; 15(8): 541-55. http://dx.doi.org/10.1038/nrg3763 PMID: 25022906

[148] Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J Control Release 2000; 65(1-2): 271-84. http://dx.doi.org/10.1016/S0168-3659(99)00248-5 PMID: 10699287

[149] Amer MH. Gene therapy for cancer: Present status and future perspective. Mol Cell Ther 2014; 2: 27. http://dx.doi.org/10.1186/2052-8426-2-27 PMID: 26056594

[150] Zylberberg C, Gaskill K, Pasley S, Matosevic S. Engineering liposomal nanoparticles for targeted gene therapy. Gene Ther 2017; 24(8): 441-52. http://dx.doi.org/10.1038/gt.2017.41 PMID: 28504657

[151] Chen Z, Liu F, Chen Y, *et al.* Targeted delivery of CRISPR/Cas9-mediated cancer gene therapy *via* liposome-templated hydrogel nanoparticles. Adv Funct Mater 2017; 27(46): 1703036. http://dx.doi.org/10.1002/adfm.201703036 PMID: 29755309

[152] Kim S-S, Rait A, Kim E, Pirollo KF, Chang EH. A tumor-targeting p53 nanodelivery system limits chemoresistance to temozolomide prolonging survival in a mouse model of glioblastoma multiforme. Nanomedicine (Lond) 2015; 11(2): 301-11. http://dx.doi.org/10.1016/j.nano.2014.09.005 PMID: 25240597

[153] Roma-Rodrigues C, Rivas-García L, Baptista PV, Fernandes AR. Gene therapy in cancer treatment: Why go nano? Pharmaceutics 2020; 12(3): 233. http://dx.doi.org/10.3390/pharmaceutics12030233 PMID: 32151052

[154] Brown MS, Diallo OT, Hu M, *et al.* CK2 modulation of NF-kappaB, TP53, and the malignant phenotype in head and neck cancer by anti-CK2 oligonucleotides *in vitro* or *in vivo via* sub-50-nm nanocapsules. Clin Cancer Res 2010; 16(8): 2295-307. http://dx.doi.org/10.1158/1078-0432.CCR-09-3200 PMID: 20371694

[155] Smith TT, Stephan SB, Moffett HF, *et al. In situ* programming of leukaemia-specific T cells using synthetic DNA nanocarriers. Nat Nanotechnol 2017; 12(8): 813-20. http://dx.doi.org/10.1038/nnano.2017.57 PMID: 28416815

[156] Maier-Hauff K, Ulrich F, Nestler D, *et al.* Efficacy and safety of intratumoral thermotherapy using magnetic iron-oxide nanoparticles combined with external beam radiotherapy on patients with recurrent glioblastoma multiforme. J Neurooncol 2011; 103(2): 317-24. http://dx.doi.org/10.1007/s11060-010-0389-0 PMID: 20845061

[157] Mamot C, Ritschard R, Wicki A, *et al.* Tolerability, safety, pharmacokinetics, and efficacy of doxorubicin-loaded anti-EGFR immunoliposomes in advanced solid tumours: A phase 1 dose-escalation study. Lancet Oncol 2012; 13(12): 1234-41. http://dx.doi.org/10.1016/S1470-2045(12)70476-X PMID: 23153506

[158] Wang K, Kievit FM, Jeon M, Silber JR, Ellenbogen RG, Zhang M. Nanoparticle-mediated target delivery of TRAIL as gene therapy for glioblastoma. Adv Healthc Mater 2015; 4(17): 2719-26. http://dx.doi.org/10.1002/adhm.201500563 PMID: 26498165

[159] Naoum GE, Tawadros F, Farooqi AA, *et al.* Role of nanotechnology and gene delivery systems in TRAIL-based therapies. E-cancermedicalscience 2016; 10: 660. http://dx.doi.org/10.3332/ecancer.2016.660 PMID: 27594905

[160] Luo C, Miao L, Zhao Y, *et al.* A novel cationic lipid with intrinsic antitumor activity to facilitate gene therapy of TRAIL DNA. Biomaterials 2016; 102: 239-48. http://dx.doi.org/10.1016/j.biomaterials.2016.06.030 PMID: 27344367

[161] Balkwill FR, Capasso M, Hagemann T. The tumor microenvironment at a glance. J Cell Sci 2012; 5591 LP-6.

[162] Abdalla AME, Xiao L, Ullah MW, Yu M, Ouyang C, Yang G. Current challenges of cancer anti-angiogenic therapy and the promise of nanotherapeutics. Theranostics 2018; 8(2): 533-48. http://dx.doi.org/10.7150/thno.21674 PMID: 29290825

[163] Hegde PS, Chen DS. Top 10 challenges in cancer immunotherapy. Immunity 2020; 52(1): 17-35. http://dx.doi.org/10.1016/j.immuni.2019.12.011 PMID: 31940268

[164] Marabelle A, Tselikas L, de Baere T, Houot R. Intratumoral immunotherapy: Using the tumor as the remedy. Ann Oncol 2017; 28(suppl_12): xii33-43. http://dx.doi.org/10.1093/annonc/mdx683 PMID: 29253115

[165] Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. Cell 2011; 144(5): 646-74. http://dx.doi.org/10.1016/j.cell.2011.02.013 PMID: 21376230

[166] Cesca M, Morosi L, Berndt A, *et al.* Bevacizumab-induced inhibition of angiogenesis promotes a more homogeneous intratumoral distribution of paclitaxel, improving the antitumor response. Mol Cancer Ther 2016; 125 LP-35.

[167] Yuan Z, Pastoriza J, Quinn T, *et al.* Chapter 2 - Targeting tumor vasculature using adeno-associated virus phage vectors coding tumor necrosis factor-α.Lattime EC, Gerson SLBT-GT of C. San Diego: Academic Press 2014; pp. 19-33. http://dx.doi.org/10.1016/B978-0-12-394295-1.00002-0

[168] Kim SH, Jeong JH, Lee SH, Kim SW, Park TG. Local and systemic delivery of VEGF siRNA using polyelectrolyte complex micelles for effective treatment of cancer. J Control Release 2008; 129(2): 107-16. http://dx.doi.org/10.1016/j.jconrel.2008.03.008 PMID: 18486981

[169] Qin H, Valentino J, Manna S, *et al.* Gene therapy for head and neck cancer using vaccinia virus expressing IL-2 in a murine model, with evidence of immune suppression. Mol Ther 2001; 4(6): 551-8. http://dx.doi.org/10.1006/mthe.2001.0493 PMID: 11735339

[170] Tsang KY, Palena C, Yokokawa J, *et al.* Analyses of Recombinant Vaccinia and Fowlpox Vaccine Vectors Expressing Transgenes for Two Human Tumor Antigens and Three Human Costimulatory Molecules. Clin Cancer Res 2005; 1597 LP-607. http://dx.doi.org/10.1158/1078-0432.CCR-04-1609

[171] Merino MJ, Pinto PA, Moreno V, Gil S, Schlom J, Gulley JL. Morphological changes induced by intraprostatic PSA-based vaccine in prostate cancer biopsies (phase I clinical trial). Hum Pathol 2018; 78: 72-8. http://dx.doi.org/10.1016/j.humpath.2018.04.013 PMID: 29709495

[172] Zamarin D, Holmgaard RB, Ricca J, *et al.* Intratumoral modulation of the inducible co-stimulator ICOS by recombinant oncolytic virus promotes systemic anti-tumour immunity. Nat Commun 2017; 8: 14340. http://dx.doi.org/10.1038/ncomms14340 PMID: 28194010

[173] Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. Nat Immunol 2002; 3(11): 999-1005. http://dx.doi.org/10.1038/ni1102-999 PMID: 12407407

[174] Wang G, Chow RD, Bai Z, *et al.* Multiplexed activation of endogenous genes by CRISPRa elicits potent antitumor immunity. Nat Immunol 2019; 20(11): 1494-505. http://dx.doi.org/10.1038/s41590-019-0500-4 PMID: 31611701

[175] Johnson ML, Spira A, Carbone DP, *et al.* 93O -first results of phase I/II studies evaluating viral vector-based heterologous prime/boost immunotherapy against predicted HLA class I neoantigens demonstrate CD8 T cell responses in patients with advanced cancers. Ann Oncol 2019; 30: xi34. http://dx.doi.org/10.1093/annonc/mdz451.002

[176] Prieto-Vila M, Takahashi R-U, Usuba W, Kohama I, Ochiya T. Drug resistance driven by cancer stem cells and their niche. Int J Mol Sci 2017; 18(12): 2574. http://dx.doi.org/10.3390/ijms18122574 PMID: 29194401

[177] Subramaniam V, Vincent IR, Gilakjan M, Jothy S. Suppression of human colon cancer tumors in nude mice by siRNA CD44 gene therapy. Exp Mol Pathol 2007; 83(3): 332-40. http://dx.doi.org/10.1016/j.yexmp.2007.08.013 PMID: 17945212

[178] Fujita T, Teh BS, Timme TL, *et al.* Sustained long-term immune responses after *in situ* gene therapy combined with radiotherapy

and hormonal therapy in prostate cancer patients. Int J Radiat Oncol Biol Phys 2006; 65(1): 84-90.
http://dx.doi.org/10.1016/j.ijrobp.2005.11.009 PMID: 16472937

[179] Boulay J-L, Perruchoud AP, Reuter J, Bolliger C, Herrmann R, Rochlitz C. P21 gene expression as an indicator for the activity of adenovirus-p53 gene therapy in non-small cell lung cancer patients. Cancer Gene Ther 2000; 7(9): 1215-9.
http://dx.doi.org/10.1038/sj.cgt.7700227 PMID: 11023193

[180] Goldberg EP, Hadba AR, Almond BA, Marotta JS. Intratumoral cancer chemotherapy and immunotherapy: Opportunities for nonsystemic preoperative drug delivery. J Pharm Pharmacol 2002; 54(2): 159-80.
http://dx.doi.org/10.1211/0022357021778268 PMID: 11848280

[181] Huang A, Pressnall MM, Lu R, *et al.* Human intratumoral therapy: Linking drug properties and tumor transport of drugs in clinical trials. J Control Release 2020; 326: 203-21.
http://dx.doi.org/10.1016/j.jconrel.2020.06.029 PMID: 32673633

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SHORT COMMUNICATION

Evidence for inflammasome activation in the brain of mucopolysaccharidosis type II mice

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Abstract

Hunter syndrome or mucopolysaccharidosis type II (MPS II) is an X-linked recessive disease caused by the deficiency of iduronate 2-sulfatase (IDS), leading to storage of undegraded heparan and dermatan sulfate. Patients with the severe form present neurological abnormalities, but the mechanisms of such alterations are unknown. Here, we hypothesized that the undegraded substances found in this disease could be recognized as damage-associated molecular patterns (DAMPS), leading to activation of the inflammasome. Brains from 2 and 5 months normal and MPS II mice were studied. We observed an increase in cathepsin B activity in the brain tissue and leakage of this enzyme from the lysosome to the cytoplasm in a MPS II neuronal cell line, which is a known activator of the inflammasome. Furthermore, Caspase-1 activity and IL-1-beta levels were elevated at 5 months, confirming that this pathway is indeed altered. Our results suggest that undegraded GAG activate the inflammasome pathway in MPS II and future studies could focus on blocking such pathway to better understand the role of this process to the pathogenesis of MPS II.

Keywords Mucopolysaccharidosis type II · Hunter syndrome · Inflammasome · NRLP3 · Caspase-1 · Interleukin-1-beta · CRISPR-Cas9

Introduction

Hunter syndrome or mucopolysaccharidosis type II (MPS II, OMIM 309900) is an X-linked recessive disease caused by the deficiency of the lysosomal hydrolase iduronate 2-sulfatase (IDS, EC3.1.6.13) resulting in accumulation of two glycosaminoglycans (GAG), heparan- and dermatan-sulfate (Brusius-Facchin et al. 2014). The disease is characterized by multisystemic abnormalities, including hepatosplenomegaly, joint contractures, dysostosis multiplex, cardiac and respiratory abnormalities. In the severe form, it also affects the central nervous system (CNS). Severe MPS II patients have impairment of cognitive skills and regression of mental development (Giugliani et al. 2018).

The mechanisms by which MPS II patients develop such brain abnormalities are not well-established. Storage of material in the lysosomes can be observed in neurons and glial cells. It has been shown in animal models that due to the storage, processes such as neuroinflammation, secondary accumulation of molecules as gangliosides and lysosome permeabilization occur and may have a role in the pathogenesis of the disease (Parker and Bigger 2019).

As an example, GAG fragments have been recently identified as a potential endogenous danger-associated molecular pattern (DAMPs), being able to activate innate immune response pathways such as the ones mediated by Toll-like receptor 4 (TLR4) and the NLR Family Pyrin Domain Containing 3 (NLRP3) or other proteins involved in the inflammasome (Latz 2010; Simonaro 2016; Parker and Bigger 2019). The activation of the NLRP3 is caused by cathepsin B (CtsB) release to the cytoplasm, which stimulates secretion of cytokines such as interleukin-1-beta (IL 1-β) and activates caspase-1 (Casp-1), recruiting immune cells and leading to a cell death process called pyroptosis (Tschopp and Schroder 2010).

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We and others have shown that the MPS II mouse model develops a progressive brain disease. Behavioral abnormalities can be detected from 4 to 6 months of age, and include impaired memory, motor and neuropsychological alterations (Gleitz et al. 2017; Azambuja et al. 2018). We have also shown that CtsB is released from the lysosome in MPS I patients' cells in vitro (Gonzalez et al. 2018), and since both MPS I and II have storage of the same GAGs, we hypothesized that this could be true for both diseases.

Therefore, we decided to test if leakage of CtsB from the lysosome occurs in the brains of MPS II mice and if it can lead to activation of the inflammasome pathway in this animal model, with activation of capase-1 and secretion of interleukins, thus contributing to the disease process.

Methods

Animals and study design

Male wild-type (WT) and MPS II mice were used ($n = 4$–8 animals per group, depending on the assay). All animal studies were approved by the authors' institutional review board and MPS II mice on a C57BL/6 background (The Jackson Lab, USA, strain B6N.Cg-Idstm1Muen/J) were used. The MPS II mice carry a disrupted version of the Ids gene, detected by PCR after DNA extraction from ear tissue at 21 days as previously described (Azambuja et al. 2018). Ids^{-} mice (referred as "MPS II" group) and their normal littermate controls (Ids+, referred as wild type, "WT" group) were the subjects for the experiments. At 3 weeks of age, offspring were separated from the dam, genotyped and housed (2–5 per cage) by gender. Animals were maintained to conventional housing under a 12 h light/12 h dark cycle with controlled temperature (19 ± 1 °C) and humidity (50 ± 10%). To study brain alterations animals were anesthetized with isoflurane and sacrificed at either 2 months of age (60 ± 7 days) or at 5 months (150 ± 30 days). Brains were removed and total brain cortex was collected and separated in 2 portions. One was flash frozen in -80oC and the other one was put in buffered formalin for 48 h, and then embedded in paraffin for histological analyses.

Assessment of neuroinflammation

Neuroinflamation was evaluated using immunohistochemistry for GFAP (Glial fibrillary acidic protein) staining. The protocol was described in detail previously (Baldo et al. 2012). Briefly, the mouse cortex was embedded in paraffin and thin sections (6 μm) were incubated with anti-GFAP antibody (Abcam, dilution 1:5000) and a secondary antibody anti-rabbit IgG, HRP-linked (Cell Signaling, USA #7074). Five high-power fields (400X Magnification) from each slide were analyzed by a pathologist, blinded to the groups. The number of positive cells was recorded for each field and the average from the five fields was used.

Cathepsin B activity and localization

Cathepsin B (CtsB) activity was assessed using the specific fluorimetric substrate Z-Arg-Arg-AMC (Enzo Life Sciences, USA) at pH 7.0. As previously described (Gonzalez et al. 2018). Results were expressed as nmol/h/mg protein.

To assess CtsB localization in the cells, we used CRISPR-Cas9 system to create a cell line knockout for Ids. For that, we cloned into the PrecisionXTM CRISPR-Cas9 SmartNuclease plasmid (System Biology, USA) the sequence for the gRNA 5'GAGGAAAGAAACGCGGCTCG-3′, aiming to disrupt the exon 3 of the Ids gene. We transfected a neuroblastoma cell line (SHSY-5Y, ATCC® CRL-2266®) with the Lipofectamine3000 (Thermo, USA) according to fabricant instructions and selected positive clones using flow cytometry. Each clone was sequenced by Sanger sequencing using the primers forward 5' GCGATGCTTACCTCTGCTTC 3′ e reverse 5' GCTGGATTCAGACACCACAA 3′. In clones with alterations in the DNA sequence, IDS activity was assessed to confirm gene knockout, using fluorimetric assay with 4-MU-alpha-L-iduronide-2-sulphate substrate (Carbosynth, USA). For immunofluorescence analyses of CtsB and Lamp-1 (lysosomal membrane-associated protein 1), cells were grown in coverslips, fixed and permeabilized with cold methanol. After, cells were washed with PBS and incubated with blocking solution (PBS, 2% bovine serum albumin) for 30 min. The coverslips were incubated with primary antibodies, with a 1:100 dilution for Mouse anti-Cathepsin B antibody (Abcam #ab58802) and a 1:500 dilution of Goat anti-Lamp-1 antibody (Santacruz, #sc-8098) at 4 °C overnight. After washing, secondary detection was performed separately. First an incubation with a 1:1000 dilution of donkey anti-goat Alexa 555 (Abcam #150130) for 1 h at room temperature for Lamp-1 and then the second antibody with a 1: 800 dilution of a Goat Anti-mouse FITC (Millipore #92590) for CtsB. Samples were observed under a confocal laser-scanning microscope. Co-localization was assessed using the ImageJ's pluggin co-localization threshold and values of Manders' coefficients and scatter plot used for the discussion (Dunn et al. 2011).

Western blot for NLRP3

Mouse cortex samples were homogenized in 150 mM NaCl, 50 mM Tris, 1% IGEPAL (CA-630), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 1 mM of protease inhibitor phenylmethylsulfonyl fluoride (PMSF) and immediately centrifuged. The supernatants were collected and protein concentrations were determined by the Lowry method. Samples were diluted in 0.1 M Tris, pH 6.8, to

achieve final protein concentrations of 4 μg/μL in Laemmli buffer (250 mM Tris, 8% SDS, 40% glycerol, 0.008% bromophenol blue, pH 6.8 and 20% β-mercaptoethanol). They were then heated at 70 °C for 10 min and 50 μg of total protein were loaded on SDS-PAGE (8%) gels and run for 60 min, first at 50 V, and then for 90 min at 120 V. The proteins in the gel were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corporation Inc., USA) using a transfer buffer (48 mM Tris, pH 9–9.3, 39 mM glycine, 20% methanol) on a wet system (Bio-Rad Laboratories Inc., USA) at 100 V. The membranes were stained with Coomassie Blue and the molecular weights of the proteins were determined by comparison to standard molecular weights (Bio-Rad Laboratories Inc., USA - #1610374).

The membranes were blocked for 2 h followed by overnight incubation at 4 °C with the primary antibody anti-NLRP3 (1:1000, Thermo, USA) diluted in TTBS buffer with 5% non-fat milk. Afterwards, the membranes were washed and incubated with anti-rabbit (IgG) secondary antibody conjugated to horseradish peroxidase (1:1000) (Cell Signaling Technology, USA - #7074 s). Chemiluminescent detection was performed using the Immobilon Western kit (Millipore Corporation Inc., USA – #WBKLS0050) and the membranes were then exposed to a digital image acquisition system. Images were analyzed in ImageJ, NLRP3 results were normalized by Coomassie blue and shown as fold-change from WT.

Caspase-1 activity and production of IL 1-beta

Caspase-1 activity was assessed using a fluorogenic assay. Samples were homogenized in acetate buffer and incubated with the Ac-YVAD-AMC substrate (Enzo Life Sciences, USA) at a final concentration of 25 μM. Fluorescence was measured using Spectramax M3 every 5 min for 60 min at an excitation of 355 nm and an emission 460 nm using kinetic reading and comparison with 7-amino-4-methulcourmarin (AMC) standards. Results are expressed as nmol/h/mg protein.

Interleukin-1-beta (IL 1-beta) levels were assessed using a commercial ELISA Kit from eBioscience (Catalog # 88–7019). Briefly, tissues were homogenized in 100 μL of a 100 mM Tris-HCl buffer, EDTA 1 mM, PMSF 0,1 mM and Triton X-100 1%, pH 7,4 and submitted to the ELISA protocol, according to manufacturer. Samples were read with a Spectramax M3 at 450 nm and results are shown in pg/mL.

Ethics and statistics

The protocol was approved by our local Ethics committee (project #160442) and all experiments followed the Brazilian legislation (Lei 11,794-CONCEA) and the Guide for the Care and Use of Laboratory Animals (2008) published by the National Research Council (Washington, DC, USA).

The Levene's test was performed to assess the equality of variances. Differences between normal and MPS II mice within each time point were compared by student's t test. Other comparisons are described in the text. A $p < 0.05$ was considered statistically significant.

Results

MPS II mice had a number of GFAP positive-cells almost twice as high as WT mice as early as 2-months old. This number was even higher at later time points ($p < 0.05$ comparing MPS II at 2 and 5 months, two way ANOVA), reaching statistical significance (Fig. 1a) at 5 months compared to WT.

We hypothesized that the GAG storage could lead to lysosome leakage with increase in CtsB activity in the cytoplasm, activating the inflammasome and leading to the neuroinflammation observed. We performed CtsB activity assay and it followed the same pattern observed in GFAP staining. CtsB activity was almost twice as high in MPS II mice compared to WT mice at 2 months ($p = 0.149$), and almost 3-times higher at 5 months of age ($p = 0.019$, Fig. 1b). The increase from 2 to 5 months was significant in MPS II ($p < 0.05$, two-way ANOVA).

To confirm lysosomal membrane permeabilization and leakage of CtsB, we created a MPS II neuron-like cell line using CRISPR-Cas9. The clone obtained was homozygous for a 1pb insertion in the exon 3 of the IDS gene (c.259dupA) and had an enzyme activity of 0.008 nmol/h/mg prot (versus 4.76 nmol/h/mg in wild type cells). Using immunofluorescence, we observed that a fraction of the CtsB did not colocalize with Lamp-1. Mander's coefficient (an indicator of colocalization, of 2 channels, where a value of 1 indicates perfect co-localization) for Wild type cells was 0.99 ± 0.01 in both channels (red or green), while it was 0.87 ± 0.02 (for red channel) and 0.93 ± 0.01 (for green channel, $p < 0.05$ in both cases), suggesting that part of the enzyme escapes the lysosome (Fig. 1c).

Since CtsB is known to activate NLRP3, we decided to assess the presence of this protein in the brains by western blot. Results show that the protein is present in similar amount at 2 months ($p = 0.65$). At 5 months a 20% increase in the expression of NLRP3 was observed, without statistical difference ($p = 0.13$, Fig. 1d). Despite not finding differences between the groups, the presence of the NLRP3 confirmed by western blot made us hypothesize that activation of Casp-1 and secretion of interleukins, especially IL-1β, could be happening. We performed Casp-1 activity assay, and it was already highly elevated in 2-month-old animals (more than 10-fold normal values, $p = 0.025$, Fig. 2a). The enzyme activity was still elevated at 5 months (5-fold normal, $p = 0.036$) although lower than at 2 months of age ($p < 0.05$, two-way ANOVA). Despite high Casp-1 activity at 2-months, IL-1β levels were not altered at this time point ($p = 0.97$). However,

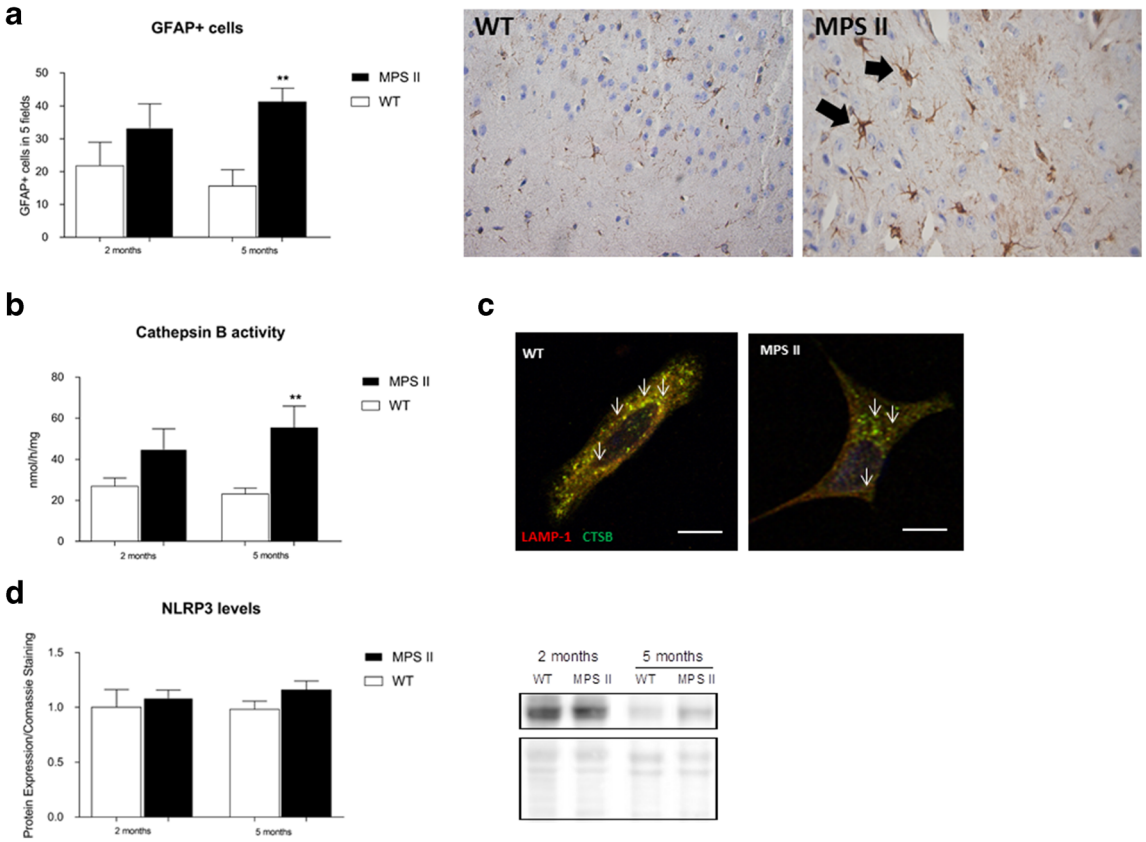

Fig. 1 Neuroinflammation and lysosomal leakage of cathepsin B in MPS II. **a** Representative section of a WT and MPS II mouse brain after GFAP staining. On the right, quantification in 5-high-power fields (400X magnification) evidencing neuroinflammation. $N = 4$ per group at 2 months and 8 per group at 5 months. **b** Cathepsin B activity in tissue homogenates. N = 4–8 per group. **c** Cathepsin B localization in a SHSY-5Y normal cell line (WT) or knockout for IDS (MPS II). Note that arrows in the WT cell show co-localization of LAMP-1 and CTSB (yellow) while in the MPS II neuron green dots (CTSB) can be observed (arrows), suggesting leakage of CTSB from the lysosome (see quantification method in the text). **d** Western blot for NLRP3 protein (upper lane) and coomassie blue (lower lane). On the right, results from quantification of the western blot images ($n = 4$–7 per group). *$p < 0.05$ compared to WT mice, Student's t test. WT- wild type; MPS - mucopolysaccharidosis type II; GFAP- glial fibrillary acidic protein; NLRP3- NLR Family Pyrin Domain Containing 3

they were significantly elevated at 5-months ($p = 0.023$, Fig. 2b) compared no WT mice.

Discussion

The mechanisms leading to neuronal dysfunction in MPS II are unknown. MPS II mice present behavior abnormalities from 4 to 6 months onwards, suggesting problems in the central nervous system (Azambuja et al. 2018), however, at least in animal models, a high degree of apoptosis was never observed. Indeed, in the current study, we assessed Caspase-3 activity, and found no difference between the 2 groups (data not shown). These initial results led us to hypothesize that other mechanisms could be responsible for the brain damage observed.

The lysosomal enzymes whose activity is reduced/abolished in the MPS are responsible for degradation of glycosaminoglycans. Therefore, as a consequence, undegraded or partially degraded molecules of heparan and dermatan

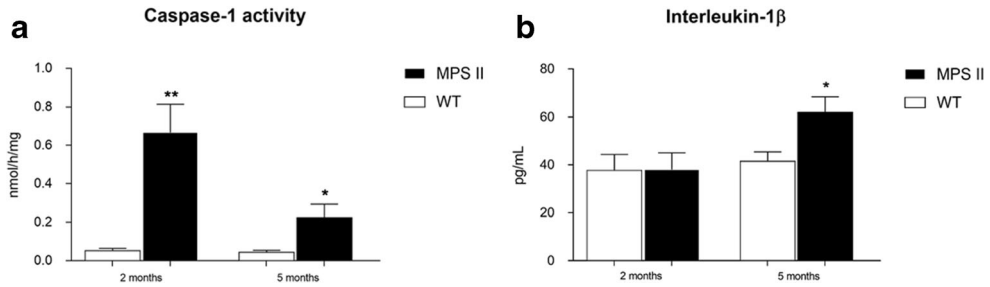

Fig. 2 Downstream effects of inflammasome activation. **a** Caspase-1 activity. **b** Secretion of IL-1β. N = 4–8/group *p < 0.05 compared to WT mice, Student's t test. WT- wild type; MPS - mucopolysaccharidosis type II; IL-1β - Interleukin1-beta

sulphate accumulate both within the lysosomes as well as in the extracellular space. A few years ago, some studies were published suggesting that these molecules could activate innate immune pathways, such as the TLR-4 pathway (DiRosario et al. 2009). Furthermore, blocking the activation of these pathways led to improvements in some aspects of the diseases (Simonaro et al. 2010).

It is well- known that, in the brains of both MPS patients and animal models, a neuroinflammatory process occurs (Zalfa et al. 2016). However, the events that lead to the activation of such process are not well understood. In the present work, we hypothesize that the undegraded GAG could be recognized as damage-associated molecular patterns (DAMPs), leading to a neuroinflammatory process due to activation of the inflammasome. Interestingly, it was found recently (Burkovetskaya et al. 2019) that in other lysosomal storage disorder with neuroinflammation (Batten disease) the caspase-1 pathway is activated as well, which suggest that other accumulated molecules could also be recognized as DAMPs, and this could be a common mechanism of disease in lysosomal disorders. To strengthen this hypothesis, it was also recently shown the role of the inflammasome activation in the pathogenesis in MPS IIIA, which also accumulates heparan sulphate GAG as in MPS II (Parker et al. 2020).

We observed increased cathepsin B activity levels in the brain cortex, and leakage of a fraction of this enzyme to the cytoplasm. In the cytoplasm, CtsB participates in the NLRP3 inflammasome assembly and activation (Campden and Zhang 2019). This then activates caspase-1, which finally cleaves pro-IL-1beta into IL-1beta whose potent proinflammatory activity directs host responses to injury, including recruitment of immune cells such as macrophages (Schroder and Tschopp 2010). The early increase in Casp-1 activity observed without immediate secretion of IL-1-beta suggests that other processes may be needed to cleave IL-1-beta. Very interestingly, it has been shown that autophagy controls IL-1beta secretion by targeting pro-IL-1beta for degradation (Harris et al. 2011) and it has been extensively proven that there is a progressive block of autophagy in lysosomal storage disorders, including in MPS (Settembre et al. 2008). Therefore, the 2 processes may act together in the secretion of IL-1-beta. Furthermore, the Il-1-beta secretion has been proven to occur in MPS II patients, and an elevation in this cytokine was found in serum of MPS II patients, even under enzyme replacement (Jacques et al. 2016).

The neuroinflammation that occurs as a consequence of activation of such pathway was visualized by increase number of cells positive for GFAP stain. Proliferation of glial cells has been reported in several neurodegenerative diseases and it is related to impaired neuronal function. The pro-inflammatory cytokines released can disrupt nerve terminals activity causing dysfunction of synapses, which correlates with cognitive decline in neurodegenerative diseases (Kawashita et al. 2009; Arranz and De Strooper 2019). Interestingly, cognitive alterations in the MPS II mouse model were only reported later in life (around 4 to 6 months of age), while at 2 months no alterations were observed in a time-dependent fashion similar to the abnormalities found in the present study (Azambuja et al. 2018). It is possible that the chronic pro-inflammatory state causes progressive dysfunction of synapses, causing the behavior abnormalities seen later in this animal model (Gleitz et al. 2017; Azambuja et al. 2018).

Altogether, our results suggest that alterations in the lysosomal membrane (possibly caused by GAG storage or other unknown alterations) leads to cathepsin B leakage from the organelle. The high interleukin-1-beta levels observed suggest activation of the inflammasome, but since we did not see an increase in NLRP3 levels, we suggest it may be mediated either by other inflammasome proteins (such as NRLP1) or even via other pathways. Future studies could focus on blocking the inflammasome to observe the role of this process to the pathogenesis of MPS II brain disease. Since current treatments such as enzyme replacement therapy do not address the brain abnormalities, the results from the current study could lead to creation of new and more effective therapies for the disease.

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References

Arranz AM, De Strooper B (2019) The role of astroglia in Alzheimer's disease: pathophysiology and clinical implications. Lancet Neurol 18(4):406–414. https://doi.org/10.1016/S1474-4422(18)30490-3

Azambuja AS, Correa L, Gabiatti BP, Martins GR, de Oliveira FÁ, Ribeiro MFM, Baldo G (2018) Versive and non-aversive memory impairment in the mucopolysaccharidosis II mouse model. Metab Brain Dis 33(1):343–345. https://doi.org/10.1007/s11011-017-0110-5

Baldo G, Mayer FQ, Martinelli B, Dilda A, Meyer F, Ponder KP, Giugliani R, Matte U (2012) Evidence of a progressive motor dysfunction in Mucopolysaccharidosis type I mice. Behav Brain Res 233(1):169–175. https://doi.org/10.1016/j.bbr.2012.04.051

Brusius-Facchin AC, Schwartz IV, Zimmer C, Ribeiro MG, Acosta AX, Horovitz D, Monlleó IL, Fontes MI, Fett-Conte A, Sobrinho RP, Duarte AR, Boy R, Mabe P, Ascurra M, de Michelena M, Tylee KL, Besley GT, Garreton MC, Giugliani R, Leistner-Segal S (2014) Mucopolysaccharidosis type II: identification of 30 novel mutations among Latin American patients. Mol Genet Metab 111(2):133–138

Burkovetskaya M, Bosch ME, Karpuk N, Fallet R, Kielian T (2019) Caspase 1 activity influences juvenile Batten disease (CLN3) pathogenesis. J Neurochem 148(5):652–668

Campden RI, Zhang Y (2019) The role of lysosomal cysteine cathepsins in NLRP3 inflammasome activation. Arch Biochem Biophys. https://doi.org/10.1016/j.abb.2019.02.015

DiRosario J, Divers E, Wang C, Etter J, Charrier A, Jukkola P, Auer H, Best V, Newsom DL, McCarty DM, Fu H (2009) Innate and

adaptive immune activation in the brain of MPS IIIB mouse model. J Neurosci Res 87(4):978–990

Dunn KW, Kamocka MM, McDonald JH (2011) A practical guide to evaluating colocalization in biological microscopy. Am J Physiol Cell Physiol 300(4):C723–C742

Giugliani R, Vairo F, Kubaski F, Poswar F, Riegel M, Baldo G, Saute JÁ (2018) Neurological manifestations of lysosomal disorders and emerging therapies targeting the CNS. Lancet Child Adolesc Health 2(1):56–68. https://doi.org/10.1016/S2352-4642(17)30087-1

Gleitz HF, O'Leary C, Holley RJ, Bigger BW (2017) Identification of age-dependent motor and neuropsychological behavioural abnormalities in a mouse model of Mucopolysaccharidosis Type II. PLoS One 12(2):e0172435. https://doi.org/10.1371/journal.pone.0172435

Gonzalez EA, Martins GR, Tavares AMV, Viegas M, Poletto E, Giugliani R, Matte U, Baldo G (2018) Cathepsin B inhibition attenuates cardiovascular pathology in mucopolysaccharidosis I mice. Life Sci 196:102–109. https://doi.org/10.1016/j.lfs.2018.01.020

Harris J, Hartman M, Roche C, Zeng SG, O'Shea A, Sharp FA, Lambe EM, Creagh EM, Golenbock DT, Tschopp J, Kornfeld H, Fitzgerald KA, Lavelle EC (2011) Autophagy controls IL-1beta secretion by targeting pro-IL-1beta for degradation. J Biol Chem 286(11):9587–9597

Jacques CE, Donida B, Mescka CP, Rodrigues DG, Marchetti DP, Bitencourt FH, Burin MG, de Souza CF, Giugliani R, Vargas CR (2016) Oxidative and nitrative stress and pro-inflammatory cytokines in Mucopolysaccharidosis type II patients: effect of long-term enzyme replacement therapy and relation with glycosaminoglycan accumulation. Biochim Biophys Acta 1862(9):1608–1616

Kawashita E, Tsuji D, Kawashima N, Nakayama K, Matsuno H, Itoh K (2009) Abnormal production of macrophage inflammatory protein-1alpha by microglial cell lines derived from neonatal brains of Sandhoff disease model mice. J Neurochem 109(5):1215–1224. https://doi.org/10.1111/j.1471-4159.2009.06041.x

Latz E (2010) The inflammasomes: mechanisms of activation and function. Curr Opin Immunol 22(1):28–33

Parker H, Bigger BW (2019) The role of innate immunity in mucopolysaccharide diseases. J Neurochem 148(5):639–651

Parker H, Ellison SM, Holley RJ, O'Leary C, Liao A, Asadi J, Glover E, Ghosh A, Jones S, Wilkinson FL, Brough D, Pinteaux E, Boutin H, Bigger BW (2020) Haematopoietic stem cell gene therapy with IL-1Ra rescues cognitive loss in mucopolysaccharidosis IIIA. EMBO Mol Med 12(3):e11185

Schroder K, Tschopp J (2010 Mar 19) The inflammasomes. Cell. 140(6):821–832

Settembre C, Fraldi A, Jahreiss L, Spampanato C, Venturi C, Medina D, de Pablo R, Tacchetti C, Rubinsztein DC, Ballabio A (2008) A block of autophagy in lysosomal storage disorders. Hum Mol Genet 17(1):119–129

Simonaro CM (2016) Lysosomes, Lysosomal storage diseases, and inflammation. Journal of Inborn Errors of Metabolism 4:1–8. https://doi.org/10.1177/2326409816650465

Simonaro CM, Ge Y, Eliyahu E, He X, Jepsen KJ, Schuchman EH (2010) Involvement of the Toll-like receptor 4 pathway and use of TNF-alpha antagonists for treatment of the mucopolysaccharidoses. Proc Natl Acad Sci U S A 107(1):222–227. https://doi.org/10.1073/pnas.0912937107

Tschopp J, Schroder K (2010) NLRP3 Inflammasome activation: the convergence of multiple signalling pathways on ROS production? Nat Rev Immunol 10(3):210–5. https://doi.org/10.1038/nri2725

Zalfa C, Verpelli C, D'Avanzo F, Tomanin R, Vicidomini C, Cajola L, Manara R, Sala C, Scarpa M, Vescovi AL, De Filippis L (2016) Glial degeneration with oxidative damage drives neuronal demise in MPSII disease. Cell Death Dis 7(8):e2331. https://doi.org/10.1038/cddis.2016.231

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ARTICLE

Neonatal nonviral gene editing with the CRISPR/Cas9 system improves some cardiovascular, respiratory, and bone disease features of the mucopolysaccharidosis I phenotype in mice

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Abstract

Mucopolysaccharidosis type I (MPS I) is caused by deficiency of alpha-L-iduronidase (IDUA), leading to multisystemic accumulation of glycosaminoglycans (GAG). Untreated MPS I patients may die in the first decades of life, mostly due to cardiovascular and respiratory complications. We previously reported that the treatment of newborn MPS I mice with intravenous administration of lipossomal CRISPR/Cas9 complexes carrying the murine *Idua* gene aiming at the *ROSA26* locus resulted in long-lasting IDUA activity and GAG reduction in various tissues. Following this, the present study reports the effects of gene editing in cardiovascular, respiratory, bone, and neurologic functions in MPS I mice. Bone morphology, specifically the width of zygomatic and femoral bones, showed partial improvement. Although heart valves were still thickened, cardiac mass and aortic elastin breaks were reduced, with normalization of aortic diameter. Pulmonary resistance was normalized, suggesting improvement in respiratory function. In contrast, behavioral abnormalities and neuroinflammation still persisted, suggesting deterioration of the neurological functions. The set of results shows that gene editing performed in newborn animals improved some manifestations of the MPS I disorder in bone, respiratory, and cardiovascular systems. However, further studies will be imperative to find better delivery strategies to reach "hard-to-treat" tissues to ensure better systemic and neurological effects.

Introduction

Mucopolysaccharidosis type I (MPS I) is an autosomal recessive disease caused by deficiency of the lysosomal enzyme alpha-L-iduronidase (IDUA, EC 3.2.1.76), which is involved in the catabolism of the glycosaminoglycans (GAGs) heparan and dermatan sulfate (DS). Enzyme replacement therapy (ERT) and hematopoietic stem cell transplantation (HSCT) are the two treatments currently available for MPS I. However, these therapies are not completely effective, as ERT is not capable of crossing the blood–brain barrier (BBB) and reach the brain, joints, heart valves, or bones, while HSCT shows to be mostly effective if performed before cognitive decline [1–3].

MPS I clinical spectrum varies from the severe Hurler syndrome (OMIM #67014) to the attenuated Scheie syndrome (OMIM #67016), with intermediate disease phenotypes classified as Hurler–Scheie syndrome (OMIM#67015) [1, 4]. Multisystemic manifestations as organomegaly, corneal clouding, heart and valve diseases, pulmonary

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hypertension, and joint stiffness are commonly present in all forms of MPS I, although Hurler patients also present severe neurocognitive decline [4–9]. Short and thick limbs and swollen joints may cause movement impairment, flattened facial bone and cartilage, and narrowing of the trachea can contribute to upper airway disease [8, 10]. MPS I hearts are often dilated, congestive heart failure is frequent in patients with Hurler syndrome, and both Hurler and Scheie phenotypes usually undergo valve replacement [11]. In addition, restrictive lung disease is common in MPS [12] and aortic dilatation and increased breaks in the elastin structure of the aorta may occur [13, 14].

The MPS I mouse model used in this study was established in 2003 by the disruption of the *Idua* gene with the neomycin resistance gene and has proven to be a useful model for studying the pathogenesis of the disease as well as for the development of treatment options. This animal model develops multiple progressive symptoms also found in patients, including visceral and brain disease [13, 15–21].

As MPS I is a progressive disorder, intervention in the neonatal period allows the effects of treatments to be potentially prophylactic, before lysosomal GAG accumulation and related pathology occur, as treatments performed after GAG accumulation lead to irreversible organ damage [20]. In addition, treatment implemented at a later age would need to clear accumulated GAGs in addition to preventing additional deleterious effects, considering that clearance of preexisting storage may be a slow process [3, 20].

We have previously reported that neonatal hydrodynamic injection of liposomal CRISPR/Cas9 complexes containing the murine *Idua cDNA* resulted in long-lasting detection of the enzyme mainly in the serum, lung, and heart of MPS I mice. Furthermore, secreted IDUA was taken up from blood by other organs, except for the brain, and the therapy led to reduced GAG storage in these organs, correcting the biochemical defects and improving heart contractility [22]. To have a better insight of these results, in the present study we expanded these analyses and report the effects of this gene editing therapy on bone, cardiovascular, respiratory, and brain abnormalities in liposome-treated MPS I mice.

Materials and methods

Animal procedures

MPS I mice were genotyped by PCR reaction as previously described [23] and maintained under standard conditions. Newborn MPS I C57BL/6 mice (2–3 days old) (*Idua*-KO, kindly donated by Dr Elizabeth Neufeld, UCLA, USA) were treated. Briefly, the treatment consisted in one hydrodynamic injection (volume corresponding to 10% of body weight) of liposome complexes (liposome + plasmid containing the sequence encoding for the *Cas9* gene and a guide sequence to the *ROSA26* locus + sequence of the murine *IDUA* cDNA for homologous recombination at *ROSA26* locus) in the superficial temporal vein of newborn MPS I mice, as previously described [22].

Two control groups were used, untreated MPS I mice and normal 6-month old mice ($n =$ up to 14/group, depending on the test). At 6 months, behavioral and echocardiographic tests were performed. After that, mice were weighed, anesthetized, and plethysmography was performed. Then, blood was collected and mice were euthanized by cervical dislocation under anesthesia. Diameter of the ascending aorta was measured using a digital pachymeter (MTX, BRZ) and liver, lungs, kidneys, heart, testicles, aorta, and brain cortex were isolated and systematically divided in two pieces. One was flash frozen in liquid nitrogen for biochemical analysis and the other portion was fixed in buffered formalin. Thin cross sections were submitted to routine histologic processing, stained with hematoxylin–eosin/alcian blue or Verhoeff-van Gieson Stain (in the case of aortas), and then analyzed.

GAG levels

GAG levels were measured by tandem mass spectrometry. GAGs were extracted from tissues after acetone precipitation. DS, heparan sulfate with O- or N-sulfation (HS–OS and HS–NS), and mono- and di-keratan sulfate (KS) disaccharides were obtained through digestion with chondroitinase B, heparitinase, and keratanase II followed by quantification through liquid chromatography tandem mass spectrometry (LC/MS/MS) as previously described [24]. In summary, 10 µL of extracted tissues were added to omega 10 K filter plates (Pall Co, MI, USA) with 90 µL of 50 mM Tris HCL (pH 7) and centrifuged by 15 min. After centrifugation, samples were incubated with 60 µL of 50 mM Tris HCL, 10 µl of 5 µg/ml of internal standard (chondrosine), 10 µl of 0.6 mU chondroitinase B (in BSA 1%), 10 µl of 1 mU heparitinase (in BSA 1%), and 10 µl of 1 mU keratanase II (in BSA 1%) (enzymes and IS were provided by Seikagaku Co, Tokyo, JPN) [24]. Samples were incubated in a shaker overnight at 37 °C followed by centrifugation and injection into the 6460 triple quad mass spectrometer (Agilent Techonologies, USA) operated in the negative ion mode with electrospray ionization. The mobile phase was a gradient elution of 148 mM ammonia (solution A) to 100% acetonitrile (solution B). Specific precursor ion and product ion were used to detect and quantify each disaccharide (354.3, 193.1 IS; 462, 97 mono-KS; 542, 462 di-KS; 416, 138 HS-NS; 378.3, 175.1 HS-OS, DS). Peak areas for all components were integrated automatically using QQQ Quantitative Analysis software (Agilent

Technologies, USA). The concentration of each disaccharide was calculated using QQQ Quantitative Analysis software [24].

Echocardiographic assessment

Six-month-old mice were anesthetized with isofluorane and placed in left lateral decubitus position to obtain cardiac images. An EnVisor HD System, Philips Medical (Andover, Mass, USA), with a 12–4-MHz transducer was used, at 2-cm depth with fundamental and harmonic imaging. Images were captured by a trained operator with experience in echocardiography of small animals. Cardiac mass parameter was analyzed. All details of echocardiographic assessment were previously described [25].

Behavioral tests

Open field test

Locomotor and exploratory activities were assessed using an open field test. The test consisted of a square arena ($52 \times 52\ cm^2$) with 60-cm high walls. The floor was divided in 16 squares by parallel and intersecting lines, obtaining 4 centered squares and 12 periphery squares. Mice were placed in one of the corners of the open field and (a) ambulation (number of times a mouse crossed with 4 paws one of the lines in the floor), and (b) exploratory behavior (rearings) were observed during 5 min.

Inhibitory avoidance

The inhibitory avoidance test in rodents is a widely used animal model of aversively motivated learning and memory. The inhibitory avoidance training box was a $50 \times 25 \times 25$-cm acrylic box whose floor consisted of parallel stainless steel bars (1 mm diameter) spaced 1 cm apart (Insight, Sao Paulo, Brazil). We adapted a small platform ($1 \times 3 \times 2$ cm) in the center of the apparatus. The animal was gently placed in the platform and their latency to step-down on the grid with all four paws was recorded. In the training session, immediately after stepping down on the grid, animals were given a 0.5 mA foot shock for 2 s. In retention test session, carried out 24 h after training (to evaluate long-term memory retention), no foot shocks were given and a maximum of 300 s was imposed in the step-down latency. The time when the animal stepped down was recorded and used for analysis.

Plethysmography

At the age of 6 months, all mice were anesthetized with intraperitoneal administration of ketamine/xylazine at 100 and 10 mg/kg, respectively. The trachea was cannulated, and the mice were mechanically ventilated at 150 strokes/min with a 150 µl stroke volume. Mice were placed in a forced pulmonary maneuver system where pulmonary resistance was recorded using a FinePointe™ RC System (Buxco Research Systems, USA). Results are shown in cm H_2O.s/mL [26].

Radiographs

Mouse limbs were isolated, cleaned of soft tissue, and radiographed as described [27]. Bone width in mice was measured on radiographs and were evaluated and reported as width in mm.

Histological analyses

The tissues were fixed with 10% buffered formalin, embedded in paraffin and submitted to semi-thin sections for the assembly of the histological slides. Hematoxylin–eosin (H&E) and alcian-blue (1%) staining were used to investigate the GAG accumulation. At least two different slides from each animal were analyzed. Analyses were performed by our pathologist, blinded to groups. At least five-high-power fields (×400 magnification) were observed.

Wall thickness of the ascending aorta was measured after Verhoeff-van Gieson staining by obtaining the average of at least five wall measurements in different points of the cut, as previously described [24]. Heart valve thickness was measured at ten different points and the mean result was considered. Glial fibrillary acid protein (GFAP) was evaluated by immunohistochemistry in five-high power (×400 magnification) fields as previously reported [28]. The microscope slides were analyzed under a microscope (Olympus BX51TF, JPN) (×200 for overview images, or ×400 for closer images).

Ethics statement and statistics

This study was approved by the authors' institutional ethics committee on animal experimentation (Comissão de Ética no Uso de Animais do Hospital de Clínicas de Porto Alegre - permit number #150416) and all experiments with animals were monitored by a veterinarian. IBM SPSS Statistics version 20 was used for statistical analysis.

All injections were performed by our vet, who was blinded to treatment groups. Additional control groups (mice injected with only the donor sequence- without Cas9-, or mice injected with plasmids but without the liposome) were also performed, but since they did not differ from untreated mice in biochemical parameters [22], functional tests were not performed and their results are not shown in

the present study. Possible gender effects were analyzed in all tests, and no significant differences were found between males and females if not specified. Results were compared using ANOVA and Tukey or Kruskal–Wallis and Mann–Whitney, as indicated. A Pearson test was used for correlation analysis. *P* values lower than 0.05 were considered statistically significant. GraphPad Prism 7 software was used to graphic design.

Results

Serum IDUA and tissue histology

MPS I mice received a hydrodynamic injection at 2–3 days after birth with a liposomal complex containing a CRISPR/Cas9 plasmid and a plasmid containing the mouse *Idua* cDNA. IDUA activity in serum was maintained at stable levels ranging from 5 to 7% of normal levels in mice for 6 months. Also, at 6 months, we observed a substantial reduction in GAG staining in most organs analyzed but the brain, as previously reported and summarized in Table 1. In addition to the data previously reported, here we describe an inverse correlation between tissue GAG levels at 6 months and IDUA activity in heart and lungs as well as serum IDUA levels versus urine GAG excretion levels (Supplementary Fig. 1).

Table 1 Effect of neonatal gene therapy on serum IDUA levels, urinary GAG and average tissue pathology findings.

Parameters	Normal ($n = 6$)	Untreated ($n = 6$)	Treated ($n = 6$)
Serum IDUA (nmol/h/mL)[a]	6.14 ± 0.11*	0.03 ± 0.014	0.40 ± 0.050*
Urinary GAGs[b] (μg/mg creatinine)	185.5 ± 27.90*	510.5 ± 45.90	356.2 ± 17.40*
Brain cortex	0	++	++
Heart	0	++++	+
Kidney	0	++++	++
Liver	0	++++	++
Lung	0	++++	+

Zero represents histology that is indistinguishable from normal; + represents animals in which storage was absent in some fields and present in others; ++ represents animals in which storage was present in all fields, but at low levels; +++ represents animals in which storage was present in all fields at moderate levels; and ++++ represents animals with large amounts of storage material in all fields

*$p < 0.05$, when compared to untreated MPS I mice (ANOVA and Tukey post hoc)

[a]The average serum Idua activity in nmol/h/mL for normal (wild type mice), untreated MPS I mice, and liposome-treated MPS I mice, are shown. The severity of pathological evidence of lysosomal storage disease at 6 months after birth was evaluated in the indicated tissues of normal, untreated MPS I, or liposome-treated MPS I mice. Pathology in brain cortex, heart, kidney, liver, and lung tissues were evaluated

[b]Urinary GAGs shown here were measured using the DMB assay and do not distinguish Dermatan sulfate, heparan sulfate or other GAGs

GAG levels

GAGs were also quantified according to the composition of mono and disaccharides present in the samples using tandem mass spectrometry, as can be observed in Fig. 1. Serum DS, HS–NS, and HS–OS were statistically decreased in treated animals, while only heparan sulfate saccharides were reduced in the urine of the same group. There was no difference in mono- or di-KS in serum or any other samples. Regarding tissue accumulation in treated animals, there was a significantly reduction in DS, HS–NS, and HS–OS in the kidney, lung, and spleen. In the meanwhile, there was HS-OS decrease in the heart and DS in the liver. The correlation between serum and urinary DS, HS–OS, HS–NS, mono-KS, and di-KS levels and serum IDUA activity was investigated, as can be seen in Suplementary Fig. 2. All graphs which correlated DS, HS–OS or HS–NS (A, B, C, F, G, H) showed an inverse significant correlation with IDUA activity found in treated MPS I mice ($P < 0.05$, Pearson test), except for (D) serum mono-KS versus serum IDUA and (E) serum di-KS versus serum IDUA.

Mouse facial morphology and body weight

Figure 2a shows that an untreated MPS I mouse has a short, broad face at 6 months after birth. In contrast, the facial morphology of a treated mouse was in between of an untreated MPS I and a normal mouse. The average body weight of the treated MPS I male mice at 6 months was 32.6 ± 0.8 g [standard error of the mean (SEM)], as shown in Fig. 2b. This was similar to that in age-matched normal male mice (31.1 ± 1.6 g) and was markedly lower than untreated MPS I males (34.7 ± 0.9 g; $p < 0.001$ for treated vs. untreated MPS I mice). Similarly, the average body weight of the treated MPS I female mice (28.8 ± 0.6 g) was lower than the average weight of 29.4 ± 0.8 g in the untreated MPS I females, although this difference was not statistically significant ($p > 0.05$). The weights of the normal females were significantly lower than in the other groups (24.9 ± 0.2 g).

Bone pathology

Radiographs of untreated MPS I mice (column 2 in Fig. 3), demonstrated that the long femoral bone (panel 3B) and the zygomatic facial bone (panel 3 A) were thick. These features were improved (but not normalized) in the treated MPS I mice (column 3). The width of the zygomatic bone

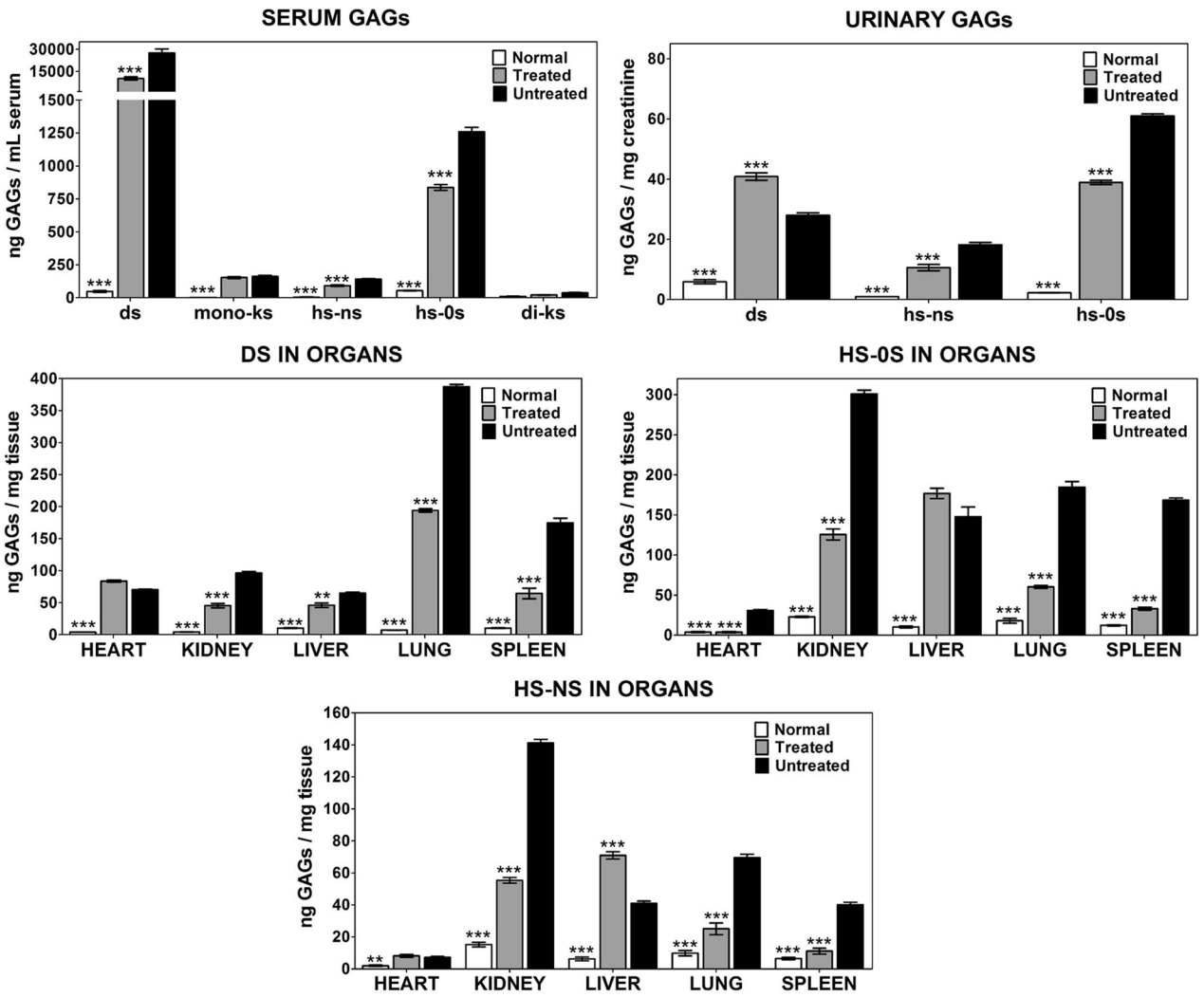

Fig. 1 GAG quantification using Tandem Mass Spectrometry. Dermatan sulfate (DS), heparan sulfate (HS-NS and HS-OS), disaccharides were determined in serum, urine, and tissues from normal (wild type mice, $n = 6$, white bars), untreated (MPS I mice, $n = 6$, black bars), and treated MPS I mice ($n = 6$, gray bars). KS levels in organs and urine were under the lower limits of detection of the method, thus they were not shown. Normal and untreated MPS I mice did not receive any injections prior to euthanasia. Liposome-treated MPS I mice were injected once with the liposome associated to a CRISPR/Cas9 plasmid and a donor plasmid of *Idua* cDNA in a volume corresponding to 10% of body weight of at 2–3 days after birth. ANOVA and Tukey post hoc, where $*p < 0.05$ and $**p < 0.005$, and $***p < 0.001$ versus untreated. Averages for all animals in each group ± the standard error of the mean (SEM) are shown. MPS I, mucopolysaccharidosis type I. Liposome, liposomal carrier associated to the CRISPR/Cas9 plasmid and *Idua* donor plasmid.

was 0.3 mm for treated mice (Fig. 3c), but there was significant difference when compared with values from both untreated MPS I and normal mice ($p < 0.05$). The width of the femoral bone evaluated was significantly reduced in the treated MPS I mice (Fig. 3c) compared to the MPS I group, and not different from normal mice.

Cardiovascular disease

Echocardiographic analysis was performed in 6-month-old mice to assess heart function. We have previously shown that heart contraction was improved with the treatment. We expanded these results here, showing that left ventricular mass is increased in MPS I mice, and treatment leads to normalization of this parameter (Fig. 4). We also measured the left ventricular anterior and posterior wall thickness in systole and diastole, and found no difference among the 3 groups (data not shown).

Pathology in the aorta and heart valves

GAG storage in the MPS I mouse heart valves could be visualized in histological sections from 6-month-old animals (Fig. 5a).

Cells presenting GAG storage were vacuolated interstitial cells. Heart valves were stained with H–E and

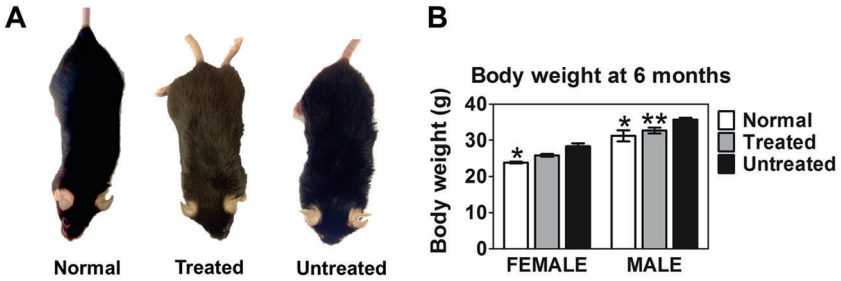

Fig. 2 Facial morphology and body weight. Normal and untreated MPS I mice did not receive any injections prior to euthanasia. Liposome-treated MPS I mice were injected once with the liposome associated to a CRISPR/Cas9 plasmid and a donor plasmid of *Idua* cDNA in a volume corresponding to 10% of body weight of at 2–3 days after birth. **a** Facial morphology. Normal, liposome-treated, and untreated MPS I mice were photographed at 6 months of age. **b** Body weight. The weights for males (four normal, three liposome-treated MPS I, and four untreated MPS I mice were evaluated) and females (four normal, two liposome-treated MPS I, and four untreated MPS I mice) were determined at 6 months of age, and averages for all animals in each group ± the standard error of the mean (SEM) are shown. Anova and Tukey post hoc, where $*p < 0.05$ and $**p < 0.005$, versus untreated. MPS I, mucopolysaccharidosis type I. Liposome, liposomal carrier associated to the CRISPR/Cas9 plasmid and *Idua* donor plasmid.

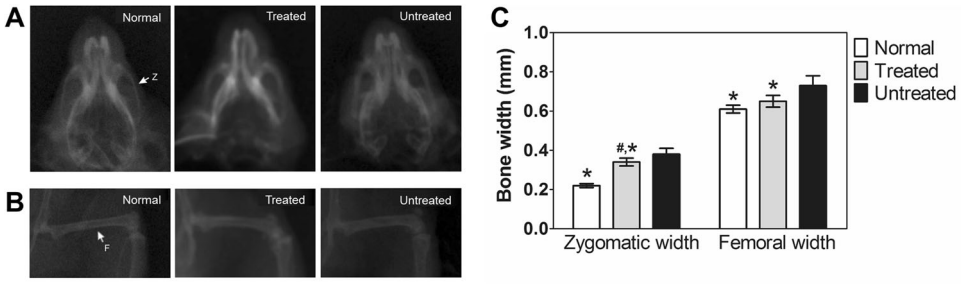

Fig. 3 Effect of gene editing on bone abnormalities. Normal (wild type mice, $n = 14$, white bars), liposome-treated ($n = 6$, gray bars), and untreated MPS I ($n = 14$, black bars) mice radiographs at 6 months of age. **a** Zygomatic bone (indicated as "Z"). **b** Femoral bone (indicated as "F"). **c** Bone width comparison (mm). Averages for all animals in each group ± the standard error of the mean (SEM) are shown. Anova and Tukey post hoc, where $*p < 0.05$, (*) when significantly different from untreated and (#) from normal group. MPS I mucopolysaccharidosis type I.

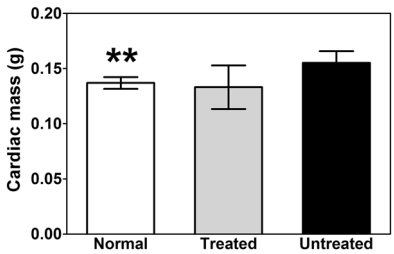

Fig. 4 Left ventricular mass assessed by echocardiography. Normal ($n = 14$, white bars), liposome-treated ($n = 6$, gray bars), and untreated MPS I ($n = 14$, black bars) mice were submitted to echocardiography at 6 months of age. Averages for all animals in each group ± the standard error of the mean (SEM) are shown. ANOVA and Tukey post hoc, where $*p < 0.05$, $**p < 0.005$, and $***p < 0.0005$, compared to untreated group. MPS I mucopolysaccharidosis type I. Liposome, liposomal carrier associated to the CRISPR/Cas9 plasmid and *Idua* donor plasmid.

alcian blue for GAG analysis since they were too small to quantify with a biochemical assay (Fig. 5a). In addition, measuring the valve thickness at ten different points revealed that the heart valves were thickened in the MPS I mice (Fig. 5b), and the treated group was not different from the untreated.

Analyzes of the aorta revealed that untreated MPS I mice presented increased aortic diameter and wall thickness (Fig. 6a), with numerous white vacuoles in the tissue, which correspond to GAG storage (Fig. 6c). Treated mice presented decreased aortic diameter compared with untreated MPS I mice, although it was not normalized, which suggests a mild effect of treatment in this organ (Fig. 6a).

When analyzing elastin breaks, one can observe that there was no difference between treated mice and normal or MPS I mice, demonstrating that the treated group was in between the two control groups (Fig. 6b).

Respiratory disease

Lung resistance obtained during plethysmography was used as a measure of obstructive airway disease [26]. In Fig. 7

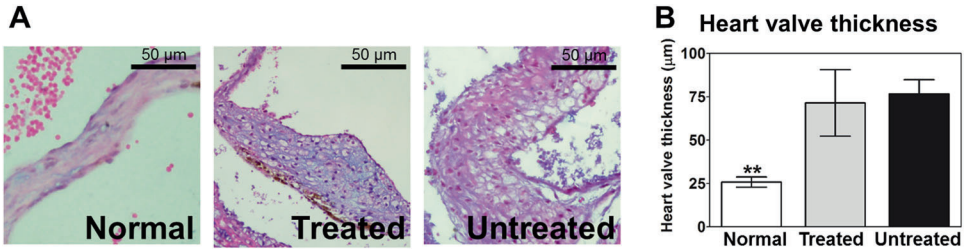

Fig. 5 Heart valve thickening and GAG storage. a An example of heart valve from the left ventricle of normal, untreated, and treated 6-month old MPS I mice. **b** Heart valve thickness at 6 months. Normal (wild type mice, white bar, $n = 10$), treated MPS I (gray bar, $n = 6$), and untreated MPS I mice (black bar, $n = 10$). Thickening of the heart valves was measured in ten different points, and the average value was recorded. Averages for all animals in each group ± the standard error of the mean (SEM) are shown. ANOVA and Tukey post hoc, where $*p < 0.05$, $**p < 0.005$, significantly different from untreated MPS I group. Magnification: ×200. MPS I mucopolysaccharidosis type I. Liposome, liposomal carrier associated to the CRISPR/Cas9 plasmid and *Idua* donor plasmid.

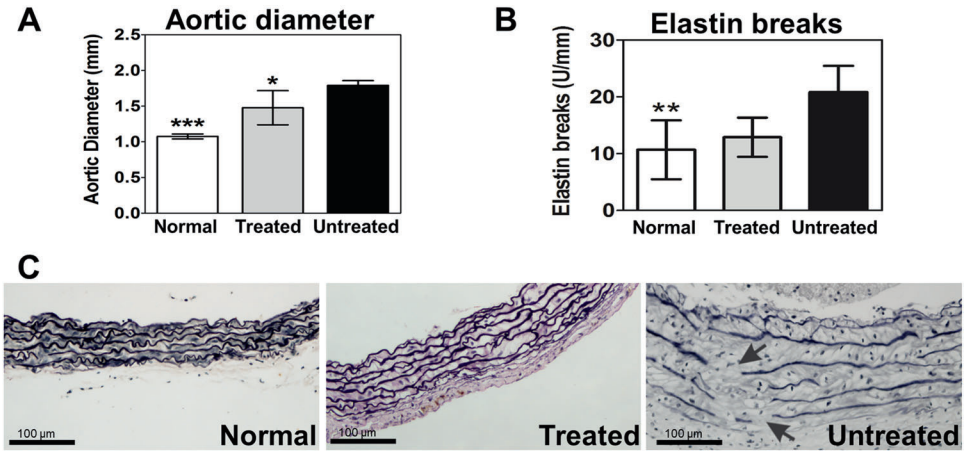

Fig. 6 Pathology in the aorta. a Diameter of the ascending aorta at 6 months measured at the moment of tissue collection. **b** Quantification of elastin breaks at 6 months. **c** Aortic disease: Verhoeff–Van Gieson stain for elastic fiber content. Representative sections of an aorta from a normal (wild type mice, $n = 10$, white bars), a treated MPS I ($n = 6$, gray bars) and untreated MPS I mice ($n = 10$, black bars). Black arrows indicate GAG storage and elastin breaks in the tissue. Averages for all animals in each group ± the standard error of the mean (SEM) are shown. ANOVA and Tukey post hoc, where $*p < 0.05$, $**p < 0.005$, and $***p < 0.0005$, (*) when significantly different from untreated MPS I group. MPS I mucopolysaccharidosis type I. Liposome, liposomal carrier associated to the CRISPR/Cas9 plasmid and *Idua* donor plasmid.

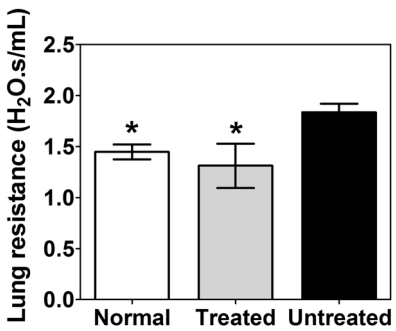

Fig. 7 Respiratory disease. Lung resistance obtained from plethysmography analysis from normal (wild type, white bar, $n = 5$), treated MPS I (gray bar, $n = 3$), and untreated MPS I mice (black bar, $n = 5$). Averages for all animals in each group ± the standard error of the mean (SEM) are shown. ANOVA and Tukey post-hoc, $*p < 0.05$ versus untreated MPS I group. MPS I mucopolysaccharidosis type I.

one can observe that MPS I mice presented higher lung resistance values, while treated mice were similar to normal mice, and both were significantly different from the untreated group.

Behavior analysis and brain histology

As a measure of locomotor activity and exploratory behavior, animals were submitted to the open field test. MPS I mice presented reduced activity in both parameters, although only results from crossings were statistically significant (Fig. 8a). Treatment failed to prevent the abnormalities in this test, as treated mice also had reduced locomotor activity (Fig. 8a).

In the inhibitory avoidance test, latency to step down the platform during the training session in the inhibitory avoidance apparatus was not different among groups, as

expected. However, 24 h after training, MPS I mice stepped down from the platform significantly faster than normal mice, suggesting memory deficits. Treated mice also failed to remember the aversive stimulus and performed similarly to untreated MPS I mice (Fig. 8c).

GFAP positive cells were increased in MPS I mice and also in the treated group, which evidences that treatment was not able to reduce neuroinflammation (Fig. 9).

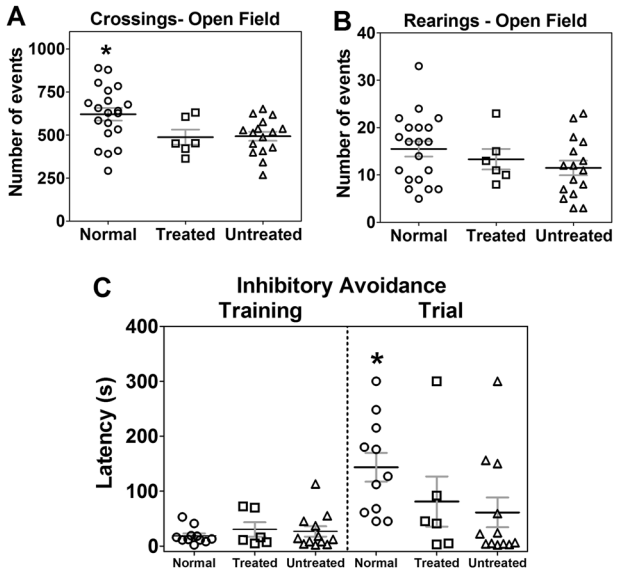

Fig. 8 Behavior analysis: open field and inhibitory avoidance test. **a** Open field test: mice were analyzed at 6 months and locomotor activity of normal (wild type, circles, $n = 20$), treated MPS I (squares, $n = 6$), and untreated MPS I mice (triangles, $n = 16$) and **b** exploratory behavior were compared among groups, considering number of crossings and number of rearings of normal (wild type, circles, $n = 20$), treated MPS I (squares, $n = 6$), and untreated MPS I mice (triangles, $n = 16$). **c** Results from the inhibitory avoidance test were based on the time to step down the platform during training session and time to step down the platform during trial session, 24 h after training of normal (wild type, circles, $n = 12$), treated MPS I (squares, $n = 6$), and untreated MPS I mice (triangles, $n = 12$). Dots represent individual mice and trace indicated average and standard deviation. Kruskal–Wallis and Dunns post hoc, $^{*}p < 0.05$, difference from untreated MPS I group. MPS I mucopolysaccharidosis type I.

Discussion

We have previously shown that the treatment of newborn MPS I mice with the CRISPR-Cas9 system enabled constant production of IDUA by tissues and its secretion. Furthermore, it significantly reduced GAGs present in urineand tissues [22]. The increased production of IDUA and reduced GAG storage in tissues as lungs and heart (where gene editing and enzyme production was most efficient) led to the expectation of finding functional improvements in these systems, as reported here in. However, some other organs could also benefit from treatment despite low frequency of gene editing, since a fraction of the enzyme can be secreted to the bloodstream from edited cells, and could be taken up by cells in other organs via the classical M6P receptor, in a process that would resemble ERT [29–31].

Firstly, it should be pointed out that hydrodynamic delivery was performed as a proof-of-concept, as this form of injection may damage cells and tissues and could not be easily extrapolated to human clinic yet. In this sense, future studies will focus on alternative forms of administration.

We deeply analyzed serum and tissue IDUA and GAG levels, which were previously reported in our latest study [22], aiming to look further at possible correlations. We consider particularly important the strong correlation found between the reduction in urinary GAGs and the increase in serum IDUA activity observed here, as urine GAG levels is the main biomarker used for monitoring therapeutic efficacy in clinical practice [32]. In addition, we performed GAG analysis by tandem mass spectrometry. It was interesting to notice that total urinary GAGs were reduced when considering the DMB assay results, but in fact only the HS was actually lower. Also, heart DS levels were not different from untreated mice, while HS were reduced. This is particularly interesting because heart function in these mice was normalized after treatment, and these results could suggest a bigger role for HS in heart disease progression than previously anticipated.

Looking at phenotype correction, treated male mice showed improved results in body weight while female mice

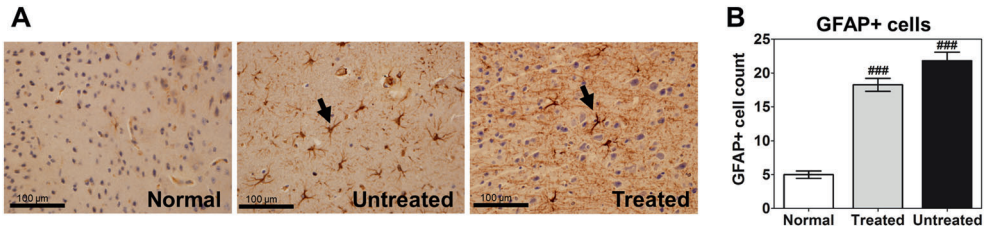

Fig. 9 Neuroinflammation. **a** Glial fibrillary acidic protein (black arrows) was detected in the cortex of normal (wild type mice, $n = 6$), untreated MPS I ($n = 6$), and treated ($n = 6$) MPS I mice. **b** Quantification of GFAP positive cells per field evaluated in 10 high-power fields of normal (wild type mice, $n = 6$, white bar), untreated MPS I ($n = 6$, black bar), and treated ($n = 6$, gray bar) MPS I mice. Mean ± the standard error of the mean (SEM) are shown. ANOVA and Tukey post hoc, where $^{\#}p < 0.05$, $^{\#\#}p < 0.005$, and $^{\#\#\#}p < 0.0005$ *versus* normal, as both treated and untreated were not significantly different. Magnification: ×200. MPS I, mucopolysaccharidosis type I.

did not reach statistically significant values, probably due to small sample size. Also, we could observe that facial morphology of treated mice was in between normal and untreated groups. This led us to perform X-ray analysis. The zygomatic bones showed lower widths in comparison to untreated mice, although not completely normalized. The femoral long bone width differed from MPS I as well, but not from the normal group. These surprising results suggest that the treatment was able to reach the bone tissue in some extent, although unfortunately not sufficiently to normalize bone width. It is important to point out that treatments such as ERT and even HSCT have limited effect on bone disease, since the enzyme does not reach bone tissue [33, 34]. In fact, HSCT may provide substantial impact on bone lesions for MPS I mice, as demonstrated by Pievani et al. [35] and Azario et al. [36], which described GAG (DS, HS, and KS) normalization in blood and marked improvements in bone pathology and skull after neonatal bone marrow transplantation and umbilical cord blood transplantation in MPS I mice. It is reasonable that HSCT provides a better clinical outcome since the secreted enzyme activity is much higher than our current report. In this sense, our future studies will look at distribution and affinity of this specific formulation for the bone and evaluation of enzyme and tissue GAG levels in this tissue as well, as it is clinically relevant [34, 37].

MPS I patients frequently develop heart disease, therefore we aimed to look at aspects of cardiac function [11]. The parameters of contractility of the left ventricle as well as heart dimensions in systole and diastole previously reported suggest that the treatment is able to prevent both left ventricle dysfunction and heart enlargement [13, 17]. Here, we show that LV mass was also normalized, which suggest that the ventricular hypertrophy frequently found in patients could be improved with our gene therapy.

The thickness of the heart valves showed no improvement in treated mice, which suggest that the treatment may not be efficient in preventing valve dysfunction. The heart valves are poorly vascularized, being composed mainly of collagen fibrils [38]. This characteristic may be likely responsible for a poor distribution of the vector and the enzyme throughout this tissue, and it could explain why our gene therapy approach failed in correcting this aspect of the disease. Previous work has shown that even very high enzyme levels achieved with gene therapy are still only partially effective in correcting heart valves [39].

The ascending aorta is another tissue considered as "difficult-to-treat" by current therapies [20, 40]. Therefore, it was unexpected that our treated mice presented intermediate results of wall thickness and aortic diameter, as well as lower count of elastin breaks. Although GAG storage could still be observed in treated mice and IDUA activity levels were not measured due to insufficient sample, these results highlight that the therapy could be a potential alternative to treat some of the most affected organs in MPS I.

This is the first time that lung function is measured in MPS I mice to date, and this parameter is important because upper and lower airway obstruction and restrictive pulmonary disease are very common in both children and adults with the disease, being one of the causes of MPS patients mortality [41, 42]. The pulmonary function results of the treated group were similar to normal, and reduced compared to MPS I, in agreement with higher IDUA levels (10% of normal values) and lower GAG levels found in the lungs of treated mice previously reported [20].

It is a consensus that IDUA does not cross the blood–brain barrier in significant amount in human patients, although some researchers have shown that, in mice, a small fraction of the enzyme is able to reach the brain, when found in high levels in serum [25, 43]. We had previously shown that IDUA activity in the brain of treated-mice was indeed undetectable, and GAG levels were not reduced [22]. We confirmed those findings with behavior analysis and GFAP content (as a marker of neuroinflammation, which has been previously shown to be elevated in MPS I mice) [15, 19, 28, 44, 45], which showed no improvement. These results confirm that the treatment is not able to reach the brain when applied intravenously.

This study shows promising findings in the frontier of knowledge, being the first one to report these outcomes. In contrast, enzyme levels found did not reach normal levels and were not able to normalize GAG levels. However cardiac and respiratory findings may suggest that supraphysiological levels of enzyme may not be strictly necessary, since a combination with other therapies could be viable. In this sense, finding a treatment that is effective in the central nervous system which could be used in combination with ERT, for example, is imperative to prevent neurological symptoms [3, 46]. Other studies have shown that gene/enzyme targeting to specific tissues is possible [47], and we are aware that, to achieve better results, it is necessary to produce enzyme at higher levels or to address specific tissues. New routes and formulations are being tested to address the brain as nose-to-brain delivery [48], as well as other difficult-to-treat tissues as joints with intra-articular delivery [49].

Taken together, our set of results suggest deterioration of function in some organs (particularly heart valves and brain) while other tissues, such as the cardiovascular system, the aorta, the bones, and the lungs, seem to have their function at least partially improved after the neonatal treatment. It is important to notice that despite the lack of complete normalization of tissues, this treatment could be combined with other approaches such as AAV delivery, HSCT, or ERT to prevent some important deterioration found in patients treated by currently available therapies. In addition,

increasing gene editing efficiency with improved vector design, other routes of administration, or multiple injections could lead to even better outcomes, and we are currently focusing on that.

Conclusion

This study demonstrates that neonatal gene therapy can result in improvements in bone, heart, and lung pathologies. Although low levels of IDUA activity were detected in serum (6% of normal IDUA levels), improved facial dysmorphism, bone width, heart, and lung function, and aortic dilatation were observed. To reach even better results, identification of new effective ways to improve the delivery of IDUA to difficult-to-treat tissues through modifications of the protein or by increasing blood enzyme levels should be pursued.

The long-term risks of any gene therapy approach need to be considered. However, we have not seen any evidence of tumors in treated mice, and the administration of liposome to transgenic mice or man did not result in insertional oncogenesis. Further studies will be necessary to evaluate this potential risk prior to implementation of this approach in patients and studies by our group as well as other are ongoing to verify the long-term safety of this strategy. We are currently searching for new alternative approaches as new delivery routes and investigating combined approaches to gene editing, expecting to achieve supra or normalization of the enzyme activity, hoping to find a more feasible and effective treatment to translate to clinical practice. In conclusion, the set of results show for the first time benefits of gene editing as a gene therapy approach in multiple aspects of a lysosomal disorder. However, for a complete correction of these multisystemic disorders, delivery alternatives should be tested to increase enzyme activity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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References

1. Giugliani R, Federhen A, Rojas MVM, Vieira T, Artigalás O, Pinto LL, et al. Mucopolysaccharidosis I, II, and VI: Brief review and guidelines for treatment. Genet Mol Biol. 2010;33:589–604.
2. Hollak CEM, Wijburg FA. Treatment of lysosomal storage disorders: successes and challenges. J Inherit Metab Dis. 2014;37:587–98.
3. Patel P, Suzuki Y, Tanaka A, Yabe H, Kato S, Shimada T, et al. Impact of enzyme replacement therapy and hematopoietic stem cell therapy on growth in patients with Hunter syndrome. Mol Genet Metab Rep. 2014;1:184–96.
4. Muenzer J, Wraith JE, Clarke LA. Mucopolysaccharidosis I: management and treatment guidelines. Pediatrics. 2009;123. https://doi.org/10.1542/peds.2008-0416.
5. Giugliani R. Mucopolysacccharidoses: from understanding to treatment, a century of discoveries. Genet Mol Biol. 2012;35:924–31.
6. Hirth A, Berg A, Greve G. Successful treatment of severe heart failure in an infant with Hurler syndrome. J Inherit Metab Dis. 2007;30:820.
7. Soliman OII, Timmermans RGM, Nemes A, Vletter WB, Wilson JHP, ten Cate FJ, et al. Cardiac abnormalities in adults with the attenuated form of mucopolysaccharidosis type I. J Inherit Metab Dis. 2007;30:750–7.
8. Chan MO, Sen ES, Hardy E, Hensman P, Wraith E, Jones S, et al. Assessment of musculoskeletal abnormalities in children with mucopolysaccharidoses using pGALS. Pediatr Rheumatol Online J. 2014;12:32.
9. Chan D, Li A, Yam M, Li C, Fok T. Hurler's syndrome with cor pulmonale secondary to obstructive sleep apnoea treated by continuous positive airway pressure. J Paediatr Child Health. 2003;39:558–9.
10. Taylor C, Brady P, O'Meara A, Moore D, Dowling F, Fogarty E. Mobility in Hurler syndrome. J Pediatr Orthop. 2008;28:163–8.
11. Braunlin EA, Harmatz PR, Scarpa M, Furlanetto B, Kampmann C, Loehr JP, et al. Cardiac disease in patients with mucopolysaccharidosis: presentation, diagnosis and management. J Inherit Metab Dis. 2011;34:1183–97.
12. Lin S-P, Shih S-C, Chuang C-K, Lee K-S, Chen M-R, Niu D-M, et al. Characterization of pulmonary function impairments in patients with mucopolysaccharidoses-changes with age and treatment. Pediatr Pulmonol. 2014;49:277–84.
13. Baldo G, Tavares AMV, Gonzalez E, Poletto E, Mayer FQ, Matte U, da S, et al. Progressive heart disease in mucopolysaccharidosis type I mice may be mediated by increased cathepsin B activity. Cardiovasc Pathol. 2017;27:45–50.
14. Poswar F de O, de Souza CFM, Giugliani R, Baldo G. Aortic root dilatation in patients with mucopolysaccharidoses and the impact of enzyme replacement therapy. Heart Vessels. 2018;34:290–5.
15. Ohmi K, Greenberg DS, Rajavel KS, Ryazantsev S, Li HH, Neufeld EF. Activated microglia in cortex of mouse models of mucopolysaccharidoses I and IIIB. Proc Natl Acad Sci USA. 2003;100:1902–7.
16. de Oliveira PG, Baldo G, Mayer FQ, Martinelli B, Meurer L, Giugliani R, et al. Characterization of joint disease in mucopolysaccharidosis type I mice. Int J Exp Pathol. 2013;94:305–11.
17. Braunlin E, Mackey-Bojack S, Panoskaltsis-Mortari A, Berry J, Mcelmurry R, Riddle M, et al. Cardiac functional and histopathologic findings in humans and mice with mucopolysaccharidosis type I: implications for assessment of therapeutic interventions in Hurler syndrome. Pediatr Res. 2006;59:27–32.
18. Garcia AR, Pan J, Lamsa JC, Muenzer J. The characterization of a murine model of mucopolysaccharidosis II (Hunter syndrome). J Inherit Metab Dis. 2007;30:924–34.

19. Baldo G, Mayer FQ, Martinelli B, Dilda A, Meyer F, Ponder KP, et al. Evidence of a progressive motor dysfunction in mucopolysaccharidosis type I mice. Behav Brain Res. 2012;233:169–75.
20. Schneider AP, Matte U, Pasqualim G, Tavares AMV, Mayer FQ, Martinelli B, et al. Deleterious effects of interruption followed by reintroduction of enzyme replacement therapy on a lysosomal storage disorder. Transl Res. 2016;176:29–37.e1.
21. Schuh R, Baldo G, Teixeira H. Nanotechnology applied to treatment of mucopolysaccharidoses. Expert Opin Drug Deliv. 2016;13:1709–18.
22. Schuh RS, Poletto E, Pasqualim G, Tavares AMV, Meyer FS, Gonzalez EA, et al. In vivo genome editing of mucopolysaccharidosis I mice using the CRISPR/Cas9 system. J Control Release. 2018;288:23–33.
23. Baldo G, Quoos Mayer F, Burin M, Carrillo-Farga J, Matte U, Giugliani R. Recombinant encapsulated cells overexpressing alpha-L-iduronidase correct enzyme deficiency in human mucopolysaccharidosis type I cells. Cells Tissues Organs. 2012;195:323–9.
24. Kubaski F, Mason RW, Nakatomi A, Shintaku H, Xie L, van Vlies NN, et al. Newborn screening for mucopolysaccharidoses: a pilot study of measurement of glycosaminoglycans by tandem mass spectrometry. J Inherit Metab Dis. 2017;40:151–8.
25. Baldo G, Mayer FQ, Martinelli BZ, de Carvalho TG, Meyer FS, de Oliveira PG, et al. Enzyme replacement therapy started at birth improves outcome in difficult-to-treat organs in mucopolysaccharidosis I mice. Mol Genet Metab. 2016;109:33–40.
26. Criée CP, Sorichter S, Smith HJ, Kardos P, Merget R, Heise D, et al. Body plethysmography—Its principles and clinical use. Respir Med. 2011;105:959–71.
27. Sands MS, Barker JE, Vogler C, Levy B, Gwynn B, Galvin N, et al. Treatment of murine mucopolysaccharidosis type VII by syngeneic bone marrow transplantation in neonates. Lab Investig. 1993;68:676–86.
28. Baldo G, Lorenzini DM, Santos DS, Mayer FQ, Vitry S, Bigou S, et al. Shotgun proteomics reveals possible mechanisms for cognitive impairment in Mucopolysaccharidosis I mice. Mol Genet Metab. 2015;114:138–45.
29. Hartung SD, Reddy RG, Whitley CB, McIvor RS. Enzymatic correction and cross-correction of mucopolysaccharidosis type I fibroblasts by adeno-associated virus-mediated transduction of the alpha-L-iduronidase gene. Hum Gene Ther. 1999;10:2163–72.
30. Kakkis ED, Muenzer J, Tiller GE, Waber L, Belmont J, Passage M, et al. Enzyme-replacement therapy in mucopolysaccharidosis I. N Engl J Med. 2001;344:182–8.
31. Neufeld ES, Muenzer J. The online metabolic and molecular basis of inherited disease. In: Valle D, Beaudet AL, Vogelstein B, editors. New York: MacGrow Hill; 2007.
32. Tomatsu S, Fujii T, Fukushi M, Oguma T, Shimada T, Maeda M, et al. Newborn screening and diagnosis of mucopolysaccharidoses. Mol Genet Metab. 2013;110:42–53.
33. Parini R, Deodato F, Di Rocco M, Lanino E, Locatelli F, Messina C, et al. Open issues in Mucopolysaccharidosis type I-Hurler. Orphanet J Rare Dis. 2017;12:112.
34. Tomatsu S, Alméciga-Díaz CJ, Montaño AM, Yabe H, Tanaka A, Dung VC, et al. Therapies for the bone in mucopolysaccharidoses. Mol Genet Metab. 2015;114:94–109.
35. Pievani A, Azario I, Antolini L, Shimada T, Patel P, Remoli C. Neonatal bone marrow transplantation prevents bone pathology in a mouse model of mucopolysaccharidosis type I. Blood. 2015;125:1662–71.
36. Azario I, Pievani A, Del Priore F, Antolini L, Santi L, Corsi A, et al. Neonatal umbilical cord blood transplantation halts skeletal disease progression in the murine model of MPS-I. Sci Rep. 2017;7:9473.
37. Rowan DJ, Tomatsu S, Grubb JH, Haupt B, Montaño AM, Oikawa H, et al. Long circulating enzyme replacement therapy rescues bone pathology in mucopolysaccharidosis VII murine model. Mol Genet Metab. 2012;107:161–72.
38. Hinton RB, Yutzey KE. Heart valve structure and function in development and disease. Annu Rev Physiol. 2011;73:29–46.
39. Bigg PW, Baldo G, Sleeper MM, O'Donnell PA, Bai H, Rokkam VRP, et al. Pathogenesis of mitral valve disease in mucopolysaccharidosis VII dogs. Mol Genet Metab. 2013;110:319–28.
40. Poletto E, Pasqualim G, Giugliani R, Matte U & Baldo G. Effects of gene therapy on cardiovascular symptoms of lysosomal storage diseases. Genet Mol Biol. 2019;42:261–85.
41. Berger KI, Fagondes SC, Giugliani R, Hardy KA, Lee KS, McArdle C, et al. Respiratory and sleep disorders in mucopolysaccharidosis. J Inherit Metab Dis. 2013;36:201–10.
42. Muhlebach MS, Wooten W, Muenzer J. Respiratory manifestations in mucopolysaccharidoses. Paediatr Respir Rev. 2011;12:133–8.
43. Ou L, Herzog T, Koniar BL, Gunther R, Whitley CB. High-dose enzyme replacement therapy in murine Hurler syndrome. Mol Genet Metab. 2014;111:116–22.
44. O'Callaghan JP, Sriram K. Glial fibrillary acidic protein and related glial proteins as biomarkers of neurotoxicity. Expert Opin Drug Saf. 2005;4:433–42.
45. Siemionow K, Klimczak A, Brzezicki G, Siemionow M, McLain RF. The effects of inflammation on glial fibrillary acidic protein expression in satellite cells of the dorsal root ganglion. Spine. 2009;34:1631–7.
46. Lund TC. Hematopoietic stem cell transplant for lysosomal storage diseases. Pediatr Endocrinol Rev. 2013;11:91–8. Suppl 1.
47. Almeciga-Diaz CJ, Montano AM, Barrera LA, Tomatsu S. Tailoring the AAV2 capsid vector for bone-targeting. Pediatr Res. 2018;84:545–51.
48. Schuh RS, Bidone J, Poletto E, Pinheiro CV, Pasqualim G, de Carvalho TG, et al. Nasal Administration of cationic nanoemulsions as nucleic acids delivery systems aiming at mucopolysaccharidosis type I gene therapy. Pharm Res. 2018;35:221.
49. Bidone J, Schuh RS, Farinon M, Poletto É, Pasqualim G, de Oliveira PG, et al. Intra-articular nonviral gene therapy in mucopolysaccharidosis I mice. Int J Pharm. 2018;548:151–8.