UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

Alterações ósseas e edição gênica intra-articular com o sistema CRISPR/Cas9 no modelo murino da mucopolissacaridose tipo I

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Hallana Souza Santos

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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LISTA DE ABREVIATURAS

BHE: Barreira hematoencefálica

BRA: Bloqueador do receptor de angiotensina

Cas9: do inglês CRISPR Associated protein 9

CRISPR: do inglês clustered regularly interspaced palindromic repeats

CS: Condroitin Sulfato

DLs: Doenças Lisossômicas

DNA: Ácido Desoxirribonucleico

DS: Dermatan Sulfato

EIM: Inatos do metabolismo

FLS: Sinoviócitos semelhantes a fibroblastos

GAGs: Glicosaminoglicanos

GAGs: Glicosaminoglicanos

gRNA: RNA guia

HS: Heparan Sulfato

IDUA: Alfa-L-iduronidase

KS: Keratan Sulfato

M6F: Manose-6-fosfato

ML: Mucolipidoses

MPS I: Mucopolissacaridose tipo I

MPS II: Mucopolissacaridose tipo II

MPS III A: Mucopolissacaridose tipo III A

MPS III B: Mucopolissacaridose tipo III B

MPS III C: Mucopolissacaridose tipo III C

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MPS IX: Mucopolissacaridose tipo IX

MPS VI: Mucopolissacaridose tipo V I

MPS VII: Mucopolissacaridose tipo VII

MPS: Mucopolissacaridose

mRNA: RNA mensageiro

OMIM: do inglês Online Mendelian Inheritance in Man

OPG: Osteoprotegerin

PAM: do inglês protospacer adjacent motifs

RANK: Receptor activator of nuclear factor Kappa-Beta

RANKL: Receptor activator of nuclear factor-kappa beta ligand

RAS: Sistema renina angiotensina

RE: Reticulo endoplasmático

RNA: Ácido ribonucleico

RNA: Ácido ribonucléico

RNP: Complexo de ribonucleoproteína

SUS: Sistema Único de Saúde

TCTH: Transplante de Células Tronco Hematopoiéticas

TGF-Beta: Fator de transformação do crescimento beta

TNF-Alfa: Fatores de Necrose Tumoral Alfa

TRE: Terapia de Reposição Enzimática

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RESUMO

A mucopolissacaridose tipo I (MPS I) é causada pela deficiência da atividade da enzima alfa-L-iduronidase (IDUA), uma enzima lisossomal responsável pela degradação dos glicosaminoglicanos (GAGs) dermatan sulfato (DS) e heparan sulfato (HS). GAGs são importantes componentes da matriz extracelular. O acúmulo de GAGS progride ao longo da vida e causa manifestações clínicas altamente variáveis, incluindo comprometimento ósseo e articular, que causam um grande impacto na saúde e qualidade de vida dos pacientes. A fisiopatologia osteoarticular da MPS I ainda não é totalmente compreendida. Adicionalmente, os ossos e articulações são regiões de difícil acesso para fornecimento dos produtos terapêuticos atualmente disponíveis para MPS I, a terapia de reposição enzimática (TRE) e o transplante de células-tronco hematopoiéticas (TCTH). Assim, o objetivo deste trabalho foi, a partir de um modelo murino de MPS I, elucidar as alterações presentes no tecido ósseo e desenvolver um protocolo de edição gênica com o sistema CRISPR/Cas9, como terapia *in situ*, buscando corrigir as alterações articulares. Demonstramos informações importantes sobre características morfológicas e biomecânicas ósseas que são diferentes entre os camundongos selvagens e MPS I, aos 6 meses de idade. Avaliamos se a edição gênica com o sistema CRISPR/Cas9 intravenoso e os tratamentos farmacológicos com inibidor de catepsina B, losartana ou propranolol, que anteriormente apresentaram efeitos promissores e relevantes em tecidos de difícil acesso, como aorta, válvulas cardíacas e ossos, eram capazes de melhorar características morfológicas e biomecânicas do tecido ósseo. Demonstramos que esses tratamentos não foram capazes de normalizar completamente as alterações ósseas presentes no modelo animal. Comprovamos que os lipossomas catiônicos carregando plasmídeos com o sistema CRISPR-Cas9 aplicado por via intra-articular levaram a um aumento localizado na atividade da IDUA e redução no acúmulo de GAGS nesse tecido. A partir dos resultados gerais, pudemos constatar que existem parâmetros que podem ser mensurados para avaliar alterações ósseas na MPS I, e que podem ser usados para testar futuras abordagens terapêuticas. Além disso, a edição gênica intra-articular, utilizando vetores não virais para a entrega do sistema CRISPR-Cas9, pode ser utilizada como alternativa para tratar os problemas articulares da MPS I, bem como outras doenças articulares.

ABSTRACT

Mucopolysaccharidosis type I (MPS I) is caused by a deficiency in the activity of the enzyme alpha-L-iduronidase (IDUA), a lysosomal enzyme responsible for the degradation of glycosaminoglycans (GAGs) dermatan sulfate (DS) and heparan sulfate (HS). GAGs are important components of the extracellular matrix. The accumulation of GAGs progresses throughout life and causes highly variable clinical manifestations, including bone and joint involvement, which have a major impact on patients' health and quality of life. The osteoarticular pathophysiology of MPS I is still not fully understood. Additionally, the bones and joints are regions of difficult access to therapeutic products currently available for MPS I, such as enzyme replacement therapy (ERT) and hematopoietic stem cell transplantation (HSCT). Thus, the objective of this work was to elucidate the alterations present in the bone tissue in MPS I mice and to develop a gene editing protocol with the CRISPR/Cas9 system, as an in-situ therapy, seeking to correct the joint alterations. We demonstrated important information regarding morphological and biomechanical characteristics of MPS I bones, that are different between wild-type and MPS I mice at 6 months of age. We also evaluated whether gene editing with intravenous CRISPR/Cas9 system and pharmacological treatments with cathepsin B inhibitor, losartan, or propranolol, which previously showed promising and relevant effects in difficult-to-access tissues, such as aorta, heart valves and bones, were able to improve morphological and biomechanical characteristics of bone tissue. We showed that none of these treatments were able to completely normalize the bone changes present in the animal model. We proved that intra-articular administration of cationic liposomes carrying plasmids with the CRISPR-Cas9 system led to a localized increase in IDUA activity and a reduction in the accumulation of GAGS in this tissue. From these results, we could observe that there are parameters that can be measured to assess bone changes in MPS I, and that can be used to test future therapeutic approaches. In addition, intra-articular gene editing, using non-viral vectors for the delivery of the CRISPR-Cas9 system, can be used as an alternative to treat the joint problems of MPS I, as well as other joint diseases.

1. INTRODUÇÃO

1.1 **Erros inatos do metabolismo**

Os erros inatos do metabolismo (EIM) são um grupo heterogêneo de doenças causadas por mutações em genes, que codificam proteínas que atuam no metabolismo (Figura 1). Podem ser herdados ou podem ocorrer como resultado de mutações espontâneas (de novo). A maioria deles são autossômicos recessivos e raramente eles podem ser autossômicos dominantes ou ligados ao X (Rice and Steiner 2016).

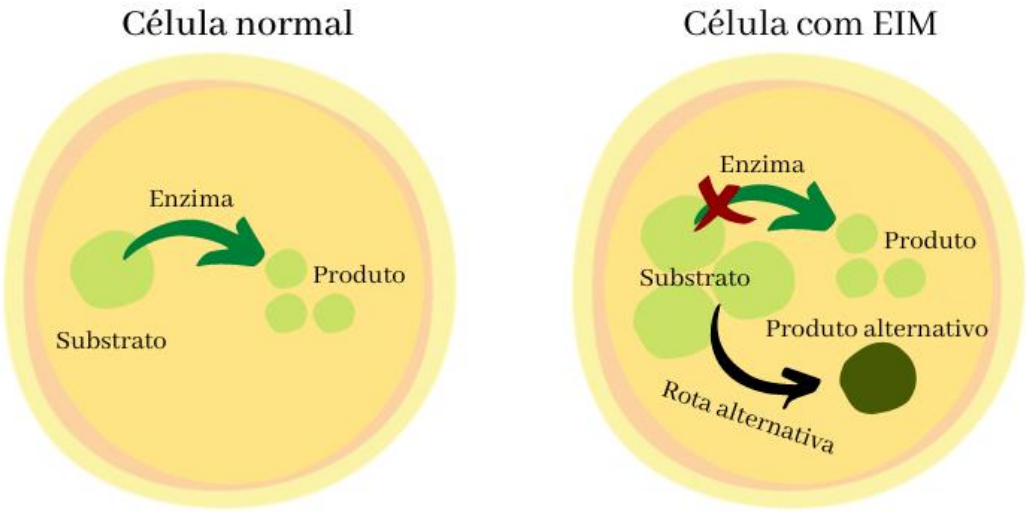

Figura 1: Representação esquemática de um erro inato do metabolismo (EIM): Cada etapa de uma via metabólica é controlada por uma enzima, a sua ausência, resulta em interrupção da via. Em consequência, substâncias precursoras (substratos) podem se acumular no organismo, enquanto os compostos subsequentes (produtos) deixam de ser sintetizados em quantidade suficientes. Ainda, podem ser produzidos produtos alternativos, devido ao não-funcionamento da enzima.

Os EIM são individualmente raros, mas coletivamente numerosos. A maioria envolve defeitos em enzimas e transporte de proteínas. Podem ser classificados em duas grandes categorias clínicas (Figura 2). A categoria 1 inclui os EIM que envolvem apenas um sistema funcional ou afetam apenas um órgão ou sistema anatômico. Os sintomas apresentados são uniformes e o diagnóstico correto geralmente é mais fácil de ser realizado. A categoria 2

inclui EIM que afetam uma via metabólica comum a muitas células ou órgãos. Os EIM nesta categoria têm uma grande diversidade de sintomas de apresentação e são subdivididos em 3 grupos: o grupo 1 inclui alterações do metabolismo intermediário que afetam pequenas moléculas, o grupo 2 inclui alterações que envolvem principalmente o metabolismo energético e o grupo 3 inclui alterações que envolvem moléculas complexas (Saudubray and Garcia-Cazorla 2018).

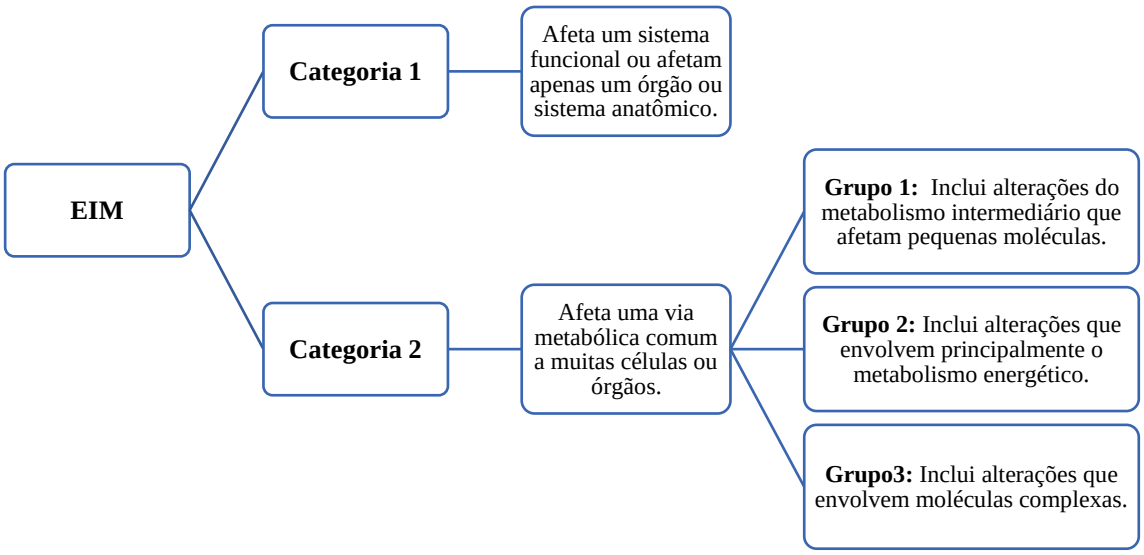

Figura 2: Classificação dos EIM segundo The Metabolic and Molecular Bases of Inherited Disease, 1995 edition, by Saudubray &Charpentier (Saudubray and Garcia-Cazorla 2018).

Atualmente, mais de 750 EIM já foram descritos. Embora não tenham cura, alguns podem ser tratados se ocorrer uma intervenção precoce, que previnem o aparecimento de danos permanentes. A maioria pode ser diagnosticada a partir de testes metabólicos plasmáticos/urinários, realizados em laboratórios especializados em bioquímica, ou por análise molecular (Saudubray and Garcia-Cazorla 2018).

1.2 **Doenças lisossômicas**

Doenças Lisossômicas (DLs) fazem parte da categoria 2 do grupo dos EIM. As DLs também são um grupo amplo de doenças, que inclui mais de 70 distúrbios. Eles são causados pelo mau funcionamento de componentes do lisossomo, como enzimas lisossômicas,

proteínas de membrana, transportadores, modificadores, entre outros (Saudubray and Garcia-Cazorla 2018).

Os lisossomos são importantes organelas celulares responsáveis pela digestão de componentes na célula. Sendo assim, são nessas organelas que ocorrem processos como catabolismo ou degradação de moléculas complexas, como os glicosaminoglicanos (GAGs), esfingolipídios e oligossacarídeos. Dessa forma, a disfunção lisossomal pode resultar em armazenamento de moléculas complexas e esse armazenamento afeta a homeostase celular, pois compromete processos celulares importantes como a endocitose e a autofagia (Figura 3), causando danos gerais nos tecidos e órgão, resultando em disfunção orgânica generalizada(Vera and Baldo 2020).

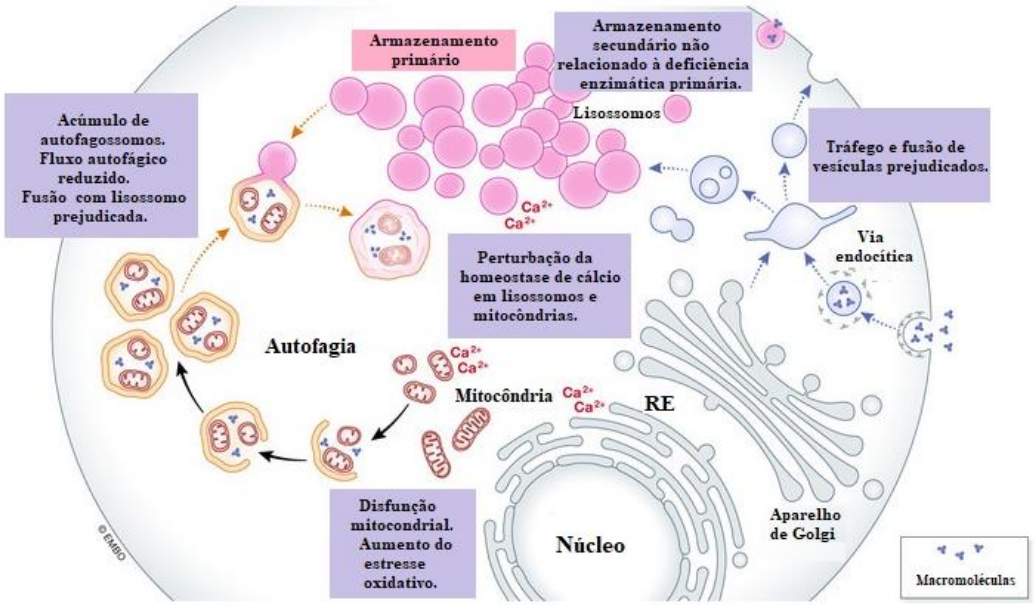

Figura 3: Alguns dos mecanismos envolvidos na fisiopatologia das DLs. O acúmulo de substratos não degradados desencadeia múltiplos eventos como o armazenamento de substratos secundários não relacionados à enzima defeituosa; composição anormal de membranas e fusão e tráfego anormal de vesículas intracelulares; comprometimento da autofagia; perturbação da homeostase de cálcio; e disfunção mitocondrial e estresse oxidativo. Reticulo endoplasmático (RE). Adaptada de (Parenti et al. 2021).

O tecido conjuntivo é um dos mais afetados nas DLs, por conta da sua constituição rica em moléculas complexas que precisam da ação de hidrolases lisossomais para serem degradadas. Assim, as DLs afetam vários órgãos desse tecido, incluindo o esqueleto,

causando morbidade significativa. As manifestações clínicas envolvem baixa estatura, disostose múltipla, deformidades da coluna e articulações, e até osteopenia. A fisiopatologia da doença esquelética nas DLs é compreendida parcialmente e envolve diferentes aspectos, incluindo armazenamento direto de substrato, inflamação, alterações epigenéticas e outras alterações complexas do metabolismo da cartilagem e do osso (Clarke and Hollak 2015).

Um exemplo de DLs são as Mucolipidoses (ML). As MLs são causadas por mutações no complexo enzimático N-acetilglucosamina-1-fosfotransferase (GlcNAc-fosfotransferase; EC 2.7.8.17), responsável por realizar modificações pós-traducionais em hidrolases lisossômicas. Essas modificações são essenciais para que essas enzimas sejam reconhecidas pelos lisossomos. Assim, quando ocorrem falhas nesse complexo, as hidrolases lisossômicas são secretadas para fora das células, resultando em uma escassez de múltiplas enzimas lisossômicas dentro dos lisossomos. Por consequência, ocorre o acúmulo de colesterol, fosfolipídios, glicosaminoglicanos (GAGs) e outros substratos não degradados nos lisossomos(Khan and Tomatsu 2020).

Outro grupo maior de DLs são as mucopolissacaridoses (MPS), que serão abordadas em detalhes na próxima seção e são o foco do presente trabalho.

1.3 **Mucopolissacaridoses**

As MPS são uma família de DLs causadas por deficiências na atividade de enzimas lisossômicas responsáveis pela degradação de glicosaminoglicanos (GAGs)(Giugliani 2012; Josahkian et al. 2021)Os GAGs, são carboidratos complexos, componentes da matriz extracelular que participam da sinalização celular e modulam vários processos bioquímicos, como a regulação do crescimento e proliferação celular, promoção da adesão celular, retenção de água, controle de fluxos de íons, sinalização neuronal e reparo de lesões (Sodhi and Panitch 2021). Por isso, seu acúmulo causa danos multissistêmicos. Na literatura, estão descritos sete tipos de MPS que são causadas por onze deficiências enzimáticas. Todas têm herança autossômica recessiva, com exceção da MPS II, que está ligada ao cromossomo X (Neufeld and Muenzer 2001; Santos et al. 2021a)

A incidência das MPS varia amplamente de acordo com o tipo e a localização geográfica e/ou origem étnica, havendo assim, lugares com incidências maiores para MPS específicas (Celik et al. 2021).No Brasil, uma pesquisa mais atual, realizada pelo Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre, com base em dados epidemiológicos de 1982 a 2019, mostrou que a prevalência de MPS nesse período foi de 1,57 por 100.000 nascidos vivos. Segundo esse estudo, a MPS II foi a mais comum com uma prevalência de 0,48 por 100.000 nascidos vivos. Em relação ao número de casos por região, a MPS VI foi a mais comum no Nordeste, enquanto que a MPS I e MPS II foram os tipos mais comuns no Sul. Essas diferenças observadas nas regiões brasileiras provavelmente estão ligadas ao efeito fundador, endogamia e consanguinidade, entre outros fatores (Josahkian et al. 2021). Contudo, apesar de constante atualização de dados relacionados à prevalência de MPS, os dados epidemiológicos sobre os tipos de MPS estão disponíveis para poucos países e regiões e sua prevalência ao nascimento pode ser subestimada devido à heterogeneidade clínica desse grupo de doenças e das dificuldades para sua investigação laboratorial (Celik et al. 2021; Josahkian et al. 2021).

O diagnóstico de MPS pode ser realizado por uma triagem inicial, pela análise de GAGs na urina e no sangue, por meio de eletroforese em gel de poliacrilamida ou por espectrometria de massas em tandem. O diagnóstico é confirmado por ensaio enzimático para determinar a atividade das hidrolases lisossômicas em leucócitos, fibroblastos ou plasma. Ainda, pode-se complementar a análise por técnicas moleculares, como sequenciamento do DNA para a determinação da variante patogênica (Zhou et al. 2020).Para obter o máximo benefício das terapias disponíveis, a detecção e a intervenção precoces são críticas, por isso a triagem neonatal é extremamente importante. Um exemplo de triagem neonatal é o teste do pezinho, que consiste na coleta de gotas de sangue dos pés de recém-nascidos (Giugliani 2012).

No Brasil, o teste do pezinho oferecido pelo Sistema Único de Saúde (SUS) é obrigatório em todo o território nacional. O exame oferecido pelo SUS era capaz de detectar apenas seis doenças, entre elas a fenilcetonúria, que é um EIM. A partir de 26/05/2021, com a nova lei número 14.154, o exame passou a abranger quatorze grupos de doenças e ampliou para cinquenta o número de doenças a serem detectadas. Entre elas, está prevista a inclusão das DLs, grupo do qual a MPS faz parte. A implementação será feita em etapas e as mudanças entram em vigor 365 dias após a publicação da lei.

As MPS apresentam um amplo espectro de características clínicas que estão relacionadas com a via de degradação do GAG que é afetada (Tabela 1). Então, para cada tipo de MPS, diferentes órgãos e tecidos são afetados, existindo, porém, um conjunto de características comuns, como as alterações faciais grosseiras e a hepatoesplenomegalia. Além disso, diversas MPS apresentam problemas neurológicos, como deficiência intelectual, cardiorrespiratórios, ósseos e articulares(Santos et al. 2021a)

Tabela 2: Características gerais e principais achados clínicos das mucopolissacaridoses (Adaptada de (Santos et al. 2021a)).

Tipo de MPS	OMIM	Epônimo	Enzima envolvida	Gene	GAG acumulado	Principais órgãos afetados		
						Cérebro	Ossos/ Articulações	Vísceras (Fígado e Baço)
MPS I	#607014	Hurler / Scheie	Alfa-L-iduronidase	*IDUA*	HS, DS	+++/++	++	+++
	#607016					+/-	++	++
MPS II	#309900	Hunter	Iduronato sulfatase.	*IDS*	HS, DS	+++/ -	++	++/+++
MPS III A	#252900	Sanfilippo A	Heparan N-sulfatase (sulfamidase)	*SGSH*	HS	+++	+	+
MPS III B	#252920	Sanfilippo B	Alfa-N-acetil-glucosaminidase	*NAGLU*	HS	+++	+	+
MPS III C	#252930	Sanfilippo C	Acetil-CoA: alfa-glucosamina acetiltransferase	*HGSNAT*	HS	+++	+	+
MPS III D	#252940	Sanfilippo D	N-acetilglucosamina- 6-sulfatase	*GNS*	HS	+++	+	+
MPS IV A	#253000	Morquio A	Galactose 6-sulfatase	*GALNS*	KS, CS	-	+++	++
MPS IV B	#253010	Morquio B	Beta-Galactosidase	*GLB1*	KC	-	+++	++
MPS VI	#253200	Maroteaux-Lamy	N-acetil-galactosamina- 4-sulfatase	*ARSB*	DS, CS	-	+++	+++
MPS VII	#253220	Sly	Beta-glucuronidase	*GUSB*	HS, DS, CS	++	++	+++
MPS IX	#601492	Natowicz	Hialuronidase	*HYAL1*	HA	-	++	-

CS: Sulfato de condroitina; DS: Sulfato de dermatano; HA: ácido hialurônico; HS: Sulfato de heparano; KS: Sulfato de queratano; MPS: Mucopolissacaridose; GAG: Glicosaminoglicanos. -: Sem envolvimento, +: Leve, ++: Moderado, +++: Grave.

1.4 **Mucopolissacaridose tipo I**

A Mucopolissacaridose tipo I (MPS I) é uma DL causada por mutações no gene *IDUA* (4q16.3). Esse gene codifica a enzima lisossomal alfa-L-iduronidase (IDUA, EC 3.2.1.76), responsável pela hidrólise dos resíduos dos GAGs dermatan sulfato (DS) e heparan sulfato (HS) (Neufeld and Muenzer 2001). Na MPS I, as manifestações clínicas osteoarticulares estão associadas principalmente ao acúmulo do DS, enquanto a patologia do sistema nervoso central está associada ao acúmulo de HS (Opoka-Winiarska et al. 2013). As variantes genéticas patogênicas levam à diminuição na atividade da enzima e ao amplo espectro de manifestações clínicas observada. Os pacientes com a forma mais grave da doença são historicamente classificados como tendo a síndrome de Hurler (OMIM #67014). Nestes casos, hepatoesplenomegalia, opacidade de córnea e alterações cardiorrespiratórias aparecem precocemente e estes pacientes apresentam sintomas neurológicos como deficiência intelectual. Pacientes com as formas menos graves, classificados historicamente como possuindo a Síndrome de Scheie (OMIM#67015), não possuem deficiência intelectual e as demais alterações são mais tardias, comparados à história natural dos pacientes com síndrome de Hurler (Kubaski et al. 2020). Na realidade, os pacientes situam-se num espectro contínuo de gravidade da doença, que nas suas formas mais graves pode levar ao óbito já na primeira década de vida, se não tratados.

Os tratamentos atualmente disponíveis para MPS I são a terapia de reposição enzimática (TRE) e transplante de células-tronco hematopoiéticas (TCTH). A TRE consiste na administração semanal da forma recombinante da enzima ao paciente, enquanto o TCTH consiste em fornecer ao paciente células progenitoras do sistema hematopoiético para que essas células transplantadas produzam e distribuam a enzima aos diferentes sistemas de órgãos (Poletto et al. 2020; Vera and Baldo 2020).Essas duas abordagens são baseadas em uma propriedade conhecida como correção cruzada, que é a capacidade de uma célula restaurada fornecer enzima para células vizinhas. Isso ocorre, pois, enzimas lisossomais passam por mudanças pós-traducionais para adição de resíduos de manose-6-fosfato (M6F). Esses resíduos M6F permitem que essas enzimas sejam levadas até o lisossomo e sejam capturadas por células vizinhas (Vera and Baldo 2020). Os tratamentos, idealmente, devem ser realizados precocemente, antes dos aparecimentos dos sintomas da doença, pois eles não possuem a capacidade de restaurar certos danos já estabelecidos.

No entanto, esses tratamentos possuem limitações. Com relação à TRE, ela é bem tolerada e não possui efeitos adversos graves, porém tem limitações por não cruzar a barreira hematoencefálica (BHE), não tendo efeito sobre a doença neurológica. Além disso, há a necessidade de administrações ao longo da vida e é uma terapia de alto custo, de mais de US$ 200.000,00 por ano por paciente. O TCTH, quando bem-sucedido, é uma abordagem eficaz para o tratamento da MPS I. No entanto, apresenta alta morbidade e mortalidade relacionadas ao procedimento, precisa empregar quimioterapia preparativa e tem potencial para falha do enxerto. Além dos problemas com esses tratamentos individualmente, nenhum dos dois previne totalmente as manifestações clínicas da doença (Poletto et al. 2020). Essas opções não são capazes de entregar a enzima para articulações e ossos em quantidades significativas(Bidone et al. 2018) e os pacientes ainda podem precisar de tratamentos complementares (Poletto et al. 2020).

1.4.1 **Alterações osteoarticulares na MPS I**

A MPS I é caracterizada por possuir envolvimento osteoarticular (Figura 4), que muitas vezes é uma característica precoce e proeminente da doença e geralmente resulta em déficits de crescimento e na necessidade de muitas cirurgias ortopédicas (Bartok and Firestein 2010). Embora achados clínicos demonstrem o comprometimento osteoarticular na MPS I, sua fisiopatologia não é totalmente compreendida. Até o momento mais de 300 variantes patogênicas do gene *IDUA* já foram descritas (Voskoboeva et al. 2022) No entanto, a correlação genótipo/fenótipo da MPS I ainda não está bem estabelecida (Bartok and Firestein 2010). Isso, aliado a estudos, principalmente em modelos animais, indicam que existem eventos secundários à alteração enzimática e ao acúmulo de GAGs envolvidos neste aspecto da patogênese da doença.

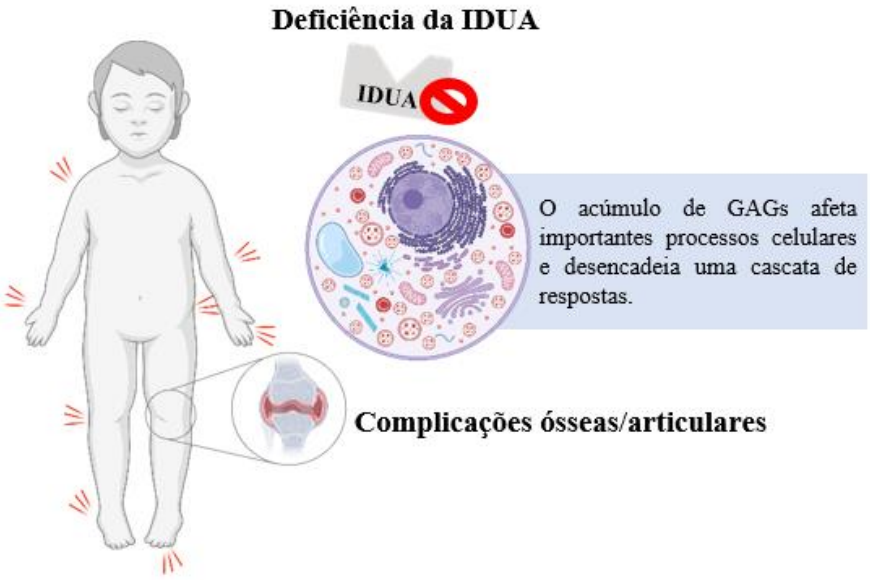

Figura 4: A mucopolissacaridose tipo I (MPS I) é causada pela deficiência da enzima alfa-L-iduronidase (IDUA) e acúmulo lisossômico de glicosaminoglicanos (GAGs) que causa, entre outras alterações, complicações ósseas e articulares.

As principais características ortopédicas da MPS I incluem contraturas articulares e disostose múltipla. A disostose múltipla é a anormalidade radiográfica classicamente observada na MPS, resultantes do crescimento endocondral e membranoso defeituoso em todo o corpo (Bartok and Firestein 2010). A contratura articular parece ser causada pelo depósito de GAGs nas articulações e possui um processo inflamatório associado. Também se observa desaceleração no crescimento em estatura logo nos primeiros estágios da infância (Borgo et al. 2018).

Esses problemas podem ser explicados, ao menos parcialmente, por estudos de osteoimunologia. Esse campo de estudo explica as complexas interações entre o sistema imunológico e o osso. Esses sistemas possuem moléculas reguladoras em comum como citocinas, receptores, moléculas sinalizadoras e fatores de transcrição. O acúmulo de GAGs desencadeia uma cascata de respostas metabólicas, inflamatórias e imunológicas inter-relacionadas e provoca uma inflamação secundária, que é muito semelhante ao processo inflamatório de doenças reumáticas, principalmente a artrite reumatoide. Há evidências que nas MPS o sistema imunológico do corpo acaba se ativando e leva à inflamação, provocando a degradação da matriz extracelular da cartilagem e dos ossos e resultam na destruição dessas estruturas (Opoka-Winiarska et al. 2013).

Estudos demonstram que na MPS I ocorre uma inflamação sinovial. Ela ocorre em consequência do aumento dos níveis de citocinas pró-inflamatórias, como o fator de necrose tumoral alfa (TNF- alfa) e interleucina 1. O aumento dessas moléculas no ambiente articular faz com que as células sinoviais se proliferem mais rapidamente, resultando no sinal clínico de hiperplasia da membrana sinovial. Devido às alterações nos processos proliferativos, ocorre o aumento na quantidade de células como sinoviócitos semelhantes a fibroblastos (FLS) e macrófagos. Os FLS expressam várias moléculas importantes para a homeostase articular e os macrófagos são células do sistema imune, responsáveis por fagocitose. A proliferação dessas células sinaliza inflamação para outras células do sistema imune, que irão tentar combatê-la. Além disso, o aumento dessas moléculas no ambiente articular provoca a apoptose dos condrócitos, que são células da cartilagem. O comprometimento dos condrócitos leva a uma degradação da matriz da cartilagem, devido à superexpressão de várias proteases (que são enzimas destrutivas) como as metaloproteinases da matriz e catepsinas (Simonaro et al. 2010). Portanto, ao conhecer estes mecanismos, abre-se a possibilidade do desenvolvimento de novos tratamentos, como uso de inibidores destas catepsinas, ou a modulação de outras moléculas, como as citocinas TGF-beta (fator de transformação do crescimento beta) ou do TNF-alfa, envolvidas nas alterações osteoarticulares na MPS I.

Estudos histológicos demonstram que na MPS I a cartilagem de crescimento do osso está desorganizada (Simonaro et al. 2001). Isso desequilibra o processo de crescimento e compromete a formação de fibras elásticas. O aumento dos níveis de citocinas pró-inflamatórias impede o amadurecimento dos condrócitos e a transformação deles em células osteoprogenitoras, que posteriormente serão convertidas em osteoblastos, que são os responsáveis por secretar a matriz óssea. Isso aumenta a quantidade de cartilagem e diminui a formação óssea. Além disso, os GAGs acumulados nos ossos causam ruptura de fibras elásticas, que são essenciais para a manutenção das formas adequadas do osso. (Opoka-Winiarska et al. 2013).

A inflamação metabólica que começa no início da vida e, no curso natural, progride ao longo da vida e causa a destruição do osso. Estudos sugerem que na MPS I ocorre uma desregulação na via RANK (receptor activator of nuclear factor Kappa-Beta) -RANKL (receptor activator of nuclear factor-kappa beta ligand) -OPG (osteoprotegerin) que é uma via de diferenciação e ativação dos osteoclastos, que são responsáveis pela reabsorção e remodelagem do tecido ósseo (Opoka-Winiarska et al. 2013). A desregulação nessa via

altera a homeostase de moléculas importantes, como o cálcio e, consequentemente, também pode alterar propriedades biomecânicas e morfológicas do tecido ósseo. O estudo das propriedades biomecânicas nas MPS ainda é muito escasso, mas estudos preliminares em camundongos MPS I demonstraram alterações aos 3 meses que tornam os ossos desses animais menos flexíveis e, portanto, mais suscetíveis a fraturas, enquanto aos 6 meses de idade, demonstram alterações que concederam os ossos desses animais maior resistência a forças exercidas, indicando que eles se tornam mais resistentes com o aumento da idade (Ferreira et al. 2021). Estas propriedades biomecânicas dos ossos são obtidas a partir da combinação das seguintes variáveis: máxima tensão, máxima força, elasticidade, tenacidade e resiliência. Juntas, essas variáveis fornecem informações sobre rigidez, força e a qualidade da matriz óssea. Individualmente, referem-se a aspectos específicos do tecido ósseo. A variável máxima tensão refere-se a máxima tensão suportada antes que ocorra a fratura. A variável máxima força refere-se a carga necessária para causar fratura. A variável elasticidade refere-se à capacidade de resistir à deformação e retornar a sua forma original quando a força exercida é interrompida. A variável tenacidade refere-se ao maior estresse suportado. A resiliência é a maior quantidade de energia absorvida até o limite elástico sem que haja deformação permanente. (Ferreira et al. 2021).

Os ossos e articulações são considerados regiões de difícil acesso para fornecimento de produtos terapêuticos na MPS I (Baldo et al. 2014; Rai and Pham 2018) .O que acontece regularmente é que os pacientes com MPS I que fazem a TRE ou TCTH, não melhoram as condições osteoarticulares e são frequentemente submetidos a tratamentos e cirurgias paliativas na tentativa de melhorar a qualidade de vida (Borgo et al. 2018).

1.4.2 **Modelo animal da MPS I**

Modelos animais caninos, felinos e murinos têm sido utilizados na pesquisa para estudar a MPS I. O modelo de camundongo MPS I, foi desenvolvido na década de 90, mimetiza vários sintomas progressivos que também são encontrados em pacientes com MPS I, incluindo as características faciais e o comprometimento osteoarticular (Clarke et al. 1997) (Figura 5). Os camundongos que foram utilizados neste estudo são C57BL/6 MPS I (Idua-KO) e camundongos C57BL/6 não afetados. Esse modelo foi criado em 2003 pela ruptura do gene *Idua* com o gene de resistência à neomicina e provou ser um modelo

útil para o estudo da patogenia da doença e para o desenvolvimento de novas opções de tratamento. Os animais nocaute produzem atividade negligenciável da enzima, acumulam GAG nos tecidos e apresentam múltiplas anormalidades, incluindo cardíacas, cerebrais ósseas e articulares, entre outras (Schuh et al. 2020).

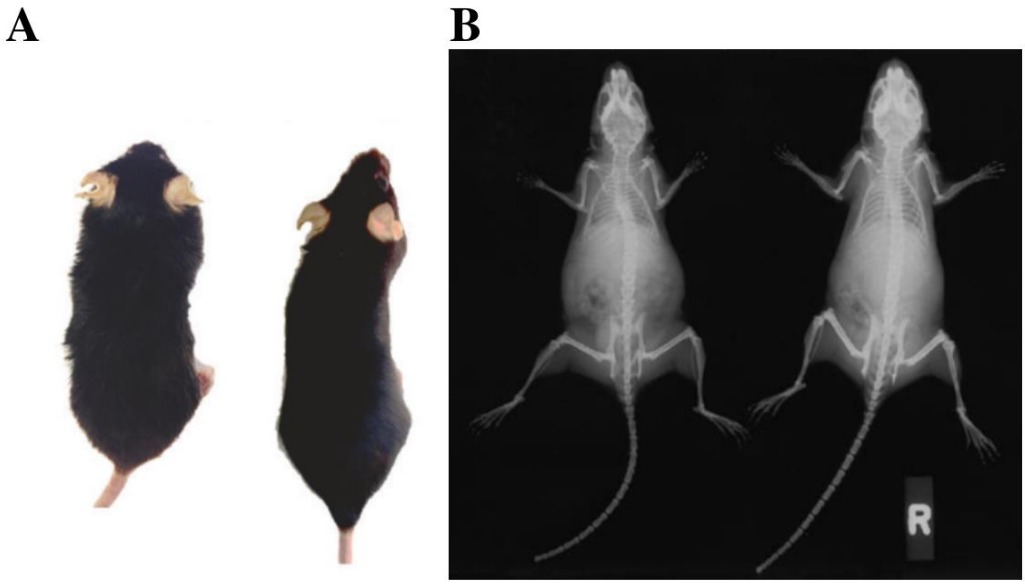

Figura 5: Camundongos MPS I e normal. **(A)** Morfologia facial e corporal de camundongos MPSI (esquerda) e normal (direta) com 6 meses de idade adaptado de (Schuh et al. 2020). **(B)** Radiografia de camundongos MPS I (direita) e normal (esquerda) com 15 semanas de idade. Comprometimento osteoarticular do camundongos MPS I: Costelas alargadas com acentuação anterior, ossos faciais grosseiros com espessura dos arcos zigomáticos e espessamento geral dos ossos longos adaptado de (Clarke et al. 1997).

1.5 **Terapia gênica**

A terapia gênica é uma abordagem na qual células modificadas (endógenas ou exógenas) produzem persistentemente fatores terapêuticos *in vivo* após a manipulação genética, através do uso de vetores, que são partículas utilizadas para direcionar o material genético ao interior das células. A terapia gênica pode ser realizada *in vivo* ou *ex vivo*. Na abordagem *in vivo*, ocorre a injeção direta do vetor no paciente para atingir as células endógenas, enquanto na abordagem *ex vivo*, ocorre modificação das células *in vitro*, que são então reintroduzidas no paciente (Santos et al.2021b).

A busca por novas ferramentas para a terapia gênica é um desafio constante. Diversos sistemas de entrega de ácidos nucleicos foram criados, visando melhorar a resposta terapêutica por meio de taxas de entrega mais altas, para também melhorar os resultados clínicos. A veiculação dos ácidos nucleicos pode ser realizada mediante a associação a vetores virais e não-virais (Schuh et al. 2016).

A entrega de genes às células pode ser feita por vetores virais como retrovírus, adenovírus e vírus adenoassociados, para citar alguns. Os vetores virais são partículas recombinantes que não possuem as sequências para replicação viral dentro do hospedeiro, mas carregam o transgene de interesse em seu genoma. Desta forma, eles podem efetivamente transduzir células, sem provocar sua morte. Embora muito eficientes para a entrega de genes, existem alguns problemas relacionados à segurança no uso desses vetores, como a resposta imune gerada (Santos et al2021b).

A entrega de genes às células também pode ser feita por vetores não-virais como micelas, nanopartículas e lipossomas. Os lipossomas são umas das classes mais bem sucedidas de vetores não virais. Eles são pequenas estruturas lipídicas que, ao se complexar com o DNA, formam estruturas semelhantes a vesículas, sendo possível citar como exemplo os lipossomas catiônicos, que contém um lipídio de carga positiva que formam um complexo com o DNA que possuem carga negativa. Os lipossomas possuem algumas vantagens como risco reduzido de rejeição imunológica, biocompatibilidade, baixo custo de produção, permitem uma produção em larga escala e são mais seguros sendo, no entanto, menos eficientes que vetores virais (Schuh et al. 2016).

A terapia gênica pode ser administrada sistemicamente ou pode ser aplicada em áreas pequenas e compartimentadas, visando tratar células ou tecidos específicos. A administração em um local específico é chamada de terapia gênica *in situ*. Essa abordagem reduz os efeitos fora do alvo e a toxicidade da terapia, pois as administrações sistêmicas podem levar à hepatotoxicidade, dependendo dos vetores e das doses utilizadas. Evidentemente, a terapia gênica *in situ* não é aplicável para tratar distúrbios multissistêmicos como EIM, de uma só vez, embora possa ser usada como complemento em tecidos de difícil tratamento, onde a terapia sistêmica não pode atuar de forma eficiente (Santos et al.2021b) como articulações, ossos e cérebro.

1.5.1 **Edição gênica**

Atualmente, outra estratégia para terapia gênica tem sido amplamente testada: a edição gênica. A edição gênica pode ser realizada por um conjunto de tecnologias consideradas disruptivas, entre as quais, a mais utilizada é a ferramenta CRISPR (Clustering Regularly Interspaced Short Palindromic Repeat) -Cas. Essa ferramenta foi descrita inicialmente como um mecanismo de defesa de procariotos e rapidamente foi transformada em ferramenta de edição genômica. O sistema CRISPR-Cas consegue editar o genoma das células permanentemente, para produzir um fator terapêutico através da correção ou indução de mutações pontuais ou inserção/exclusão de sequências de interesse. Ele consiste em uma nuclease, geralmente a Cas9, e um RNA guia (gRNA). Além disso, nos casos em que se deseja uma correção de mutações ou inserção de sequências específicas no genoma, também é fornecido um DNA com a sequência desejada, chamado de sequência doadora. Assim, o RNA guia direciona a Cas9, que cliva o DNA em um ponto específico (próximo a uma sequência chamada PAM). Posteriormente, a célula repara essa clivagem usando como molde a sequência doadora e assim a mutação pode ser corrigida (Figura 6) (Santos et al. 2021a; Santos et al.2021b).

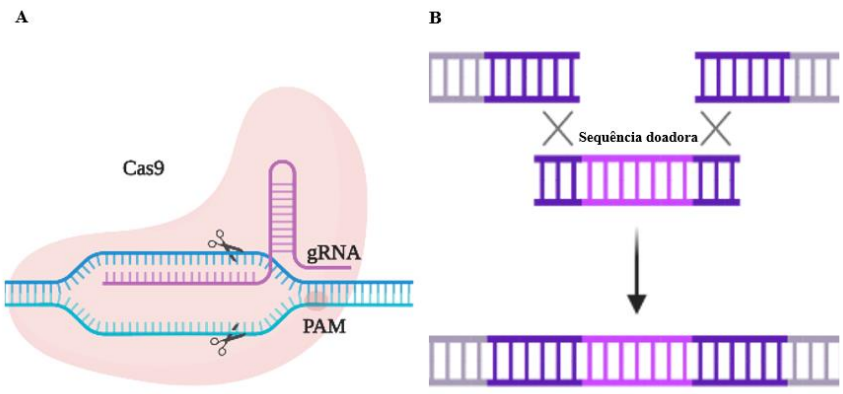

Figura 6: Ferramenta de edição gênica CRISPR (Clustering Regularly Interspaced Short Palindromic Repeat) - Cas9. **(A)** A nuclease Cas9 e o RNA guia (gRNA) reconhecem e clivam o ponto específico no DNA (Sequência PAM-Motivo adjacente protoespaçador). **(B)** Para correção de mutações ou inserção de sequências específicas no genoma, também é fornecido um DNA com a sequência desejada, chamado de sequência doadora.

Como na terapia gênica, uma das abordagens de entrega dos componentes necessários para a edição gênica também é através do uso de vetores (virais ou não virais). No entanto, nesta abordagem eles não carregam o transgene para o interior das células, mas sim, os componentes do sistema de edição gênica. Adicionalmente, esses componentes podem

ser entregues em diferentes constructos genéticos, como plasmídeo de DNA, mRNA ou complexo de ribonucleoproteína (RNP) (Sainz-Ramos et al. 2021).

Nas últimas décadas, os plasmídeos tornaram-se uma das ferramentas mais importantes em genética e biologia, e mais recentemente, na medicina com a terapia gênica e vacinas. Plasmídeos são pequenas moléculas de DNA circulares, de fita dupla, que contém informações genéticas extracromossômicas. Conduzem a expressão transitória de um transgene no núcleo das células alvo, esse transgene codifica uma proteína de interesse. São capazes de multiplicação autônoma, uma vez que estejam no núcleo da célula e a maquinaria celular esteja disponível (Schmeer et al. 2017).

Independentemente do vetor e do constructo genético utilizado, uma grande vantagem do uso do sistema CRISPR/CAS9 é a expressão controlada do transgene, uma vez que apenas um número limitado de cópias (frequentemente, apenas uma) pode ser integrado por alelo, pois a edição é direcionada para um locus específico da sequência no genoma (Sainz-Ramos et al. 2021; Santos et al. 2021a; Santos et al2021b.). Além disso, permitem a expressão continuada do transgene, após sua integração.

1.5.2 **Terapia gênica para MPS I**

A terapia gênica tornou-se uma alternativa em evidência para o tratamento de doenças monogênicas como a MPS I. Com o objetivo de alcançar a expressão gênica terapêutica estável e de longo prazo, tem sido aplicada com sucesso em uma variedade de modelos animais (camundongo, cachorro e gato), que permitiram que a terapia gênica para MPS I passasse das células dos pacientes para abordagens *in vivo* e *ex vivo*. A pesquisa para desenvolver terapias novas, seguras e eficazes para o tratamento da MPS I, bem como de outras doenças lisossômicas é rigorosa e incansável. A terapia gênica vem mostrando seu potencial para ser uma terapia adequada para pacientes com MPS I se iniciadas no estágio inicial da doença. Algumas estratégias chegaram aos ensaios clínicos, mas a tradução de resultados de laboratório para um cenário clínico ainda é um desafio (Vera and Baldo 2020).

Em trabalhos realizados pelo nosso grupo, foi testada a eficiência de lipossomas catiônicos como carregador não viral do sistema CRISPR/Cas9 associadas a um plasmídeo doador contendo cópia completa do gene *IDUA*, a ser inserido no lócus ROSA26, *in vitro* e *in vivo*. A incubação dos complexos com fibroblastos de pacientes

com MPS I aumentou a produção de enzima funcional e diminuiu a massa de lisossomos. A injeção hidrodinâmica contendo os complexos em camundongos com MPS I aumentou os níveis de enzima no soro por seis meses e causou diminuição do acúmulo de GAGs principalmente no fígado (Schuh et al. 2018b). No entanto, mais estudos são necessários para encontrar melhores estratégias de entrega para alcançar tecidos "difíceis de tratar" como ossos e articulações, pois esses tecidos geralmente não recebem níveis terapêuticos de enzima (Baldo et al. 2014; Schuh et al. 2020).

Outro trabalho realizado pelo nosso grupo de pesquisa avaliou a eficiência da terapia gênica intra-articular em camundongos com mucopolissacaridose tipo I (MPS I). Esse método demonstrou maior expressão gênica e atividade da IDUA nos joelhos e líquido sinovial, porém, essa atividade não foi duradoura, pois não houve edição do genoma, uma vez que o plasmídeo utilizado tinha apenas o gene de interesse (Bidone et al. 2018).

A edição gênica diretamente na articulação poderia aumentar a eficiência da terapia e corrigir os problemas articulares associados à doença. Além disso, a administração localizada seria uma abordagem segura, pois as articulações são cavidades fechadas e os órgãos não-alvo não seriam afetados (Santos et al. 2021b).

2. JUSTIFICATIVA

Considerando-se o exposto, pode-se notar que os estudos envolvendo terapia gênica e edição de genes para MPS ainda possuem limitações, sendo uma destas limitações a correção das alterações osteoarticulares. Para realizar esta correção são necessários que se conheça melhor as alterações osteoarticulares do modelo animal da doença (para poder utilizar os parâmetros alterados no estudo de novas terapias) bem como que se desenvolvam novas abordagens de entrega destas terapias, sendo uma destas potenciais formas de entrega, a injeção direta do sistema CRISPR/Cas9 no espaço articular.

3. OBJETIVOS

3.1 Objetivo Geral

O objetivo deste trabalho é elucidar as alterações presentes no tecido ósseo do modelo animal de MPS I e desenvolver um protocolo de edição gênica como terapia *in situ*, buscando corrigir as alterações articulares.

3.2 Objetivos específicos

- Revisar a literatura, buscando estudos sobre abordagens *in situ* de terapia gênica para distintas situações patológicas;
- Discutir os principais estudos envolvendo edição gênica para MPS;
- Estudar propriedades biomecânicas dos ossos de animais com MPS I e verificar o efeito de diferentes tratamentos (inibidor de catepsina B, inibidor da via do TGF-beta -losartana-, propranolol e edição de genes) sobre o sistema ósseo;
- Realizar um protocolo de edição de genes *in vitro* (em fibroblastos MPS I cultivados) e *in vivo* (em modelo animal), através da injeção *in situ* do sistema CRISPR/Cas9 na articulação do joelho;

4. RESULTADOS

Os resultados estão apresentados na forma de 4 trabalhos científicos, sendo 2 publicados e 2 em fase final de redação. A seguir, cada trabalho é apresentado de forma separada, em capítulos.

4.1 Artigo I: *In Situ* Gene Therapy

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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-

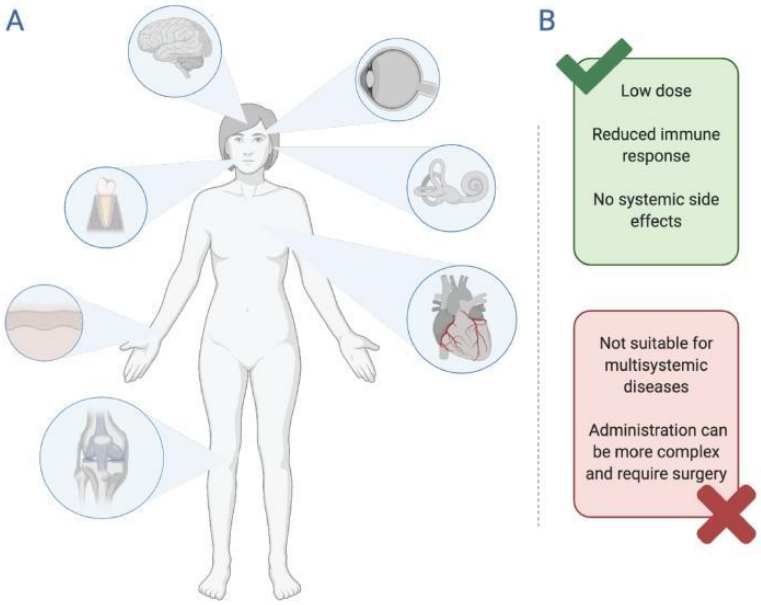

Fig. (1). Overview of *in situ* gene therapy. **A**) Local delivery of gene therapy products can be performed *via* different routes, including intracerebral, intraocular, in the inner ear, in the periodontal area, intracardiac, intradermal and intra-articular administrations. **B**) Advantages of *in 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-

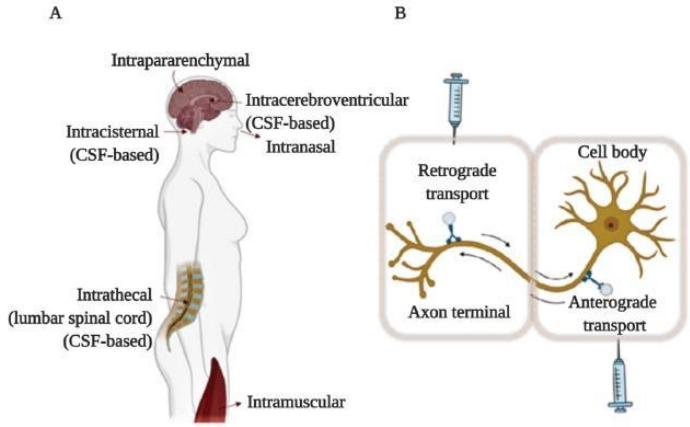

Fig. (2). Overview of intracerebral delivery routes and axonal transport. **A**) Vectors can be introduced into the central nervous system (CNS) directly into the brain parenchyma or *via* the cerebrospinal fluid (CSF). Intramuscular and intranasal injections can also lead to the transduction of CNS 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 virus; 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.

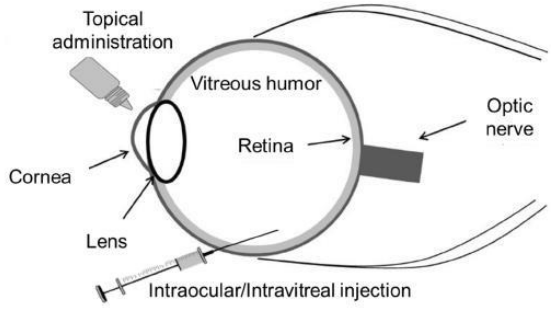

Fig. (3). Schematic representation of intraocular/ intravitreal injection and topical administration.

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	interferon 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].

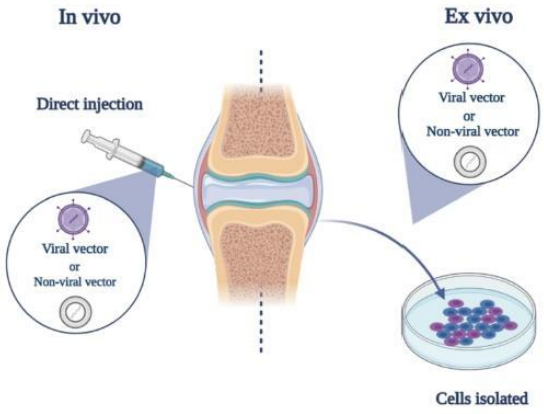

Fig. (4). Schematic representation of intra-articular gene therapy administration. The intra-articular gene therapy approach can be performed *in vivo* or *ex vivo* using viral and non-viral vectors. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

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-ɔ, 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 antitumor 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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CHAPTER TEN

Genome editing in mucopolysaccharidoses and mucolipidoses

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Abstract

Mucopolysaccharidoses (MPS) and mucolipidoses (ML) are disorders that alter lysosome function. While MPS are caused by mutation in enzymes that degrade glycosaminoglycans, the ML are disorders characterized by reduced function in the phosphotransferase enzyme. Multiple clinical features are associated with these diseases and the exact mechanisms that could explain such different clinical manifestations in patients are still unknown. Furthermore, there are no curative treatment for any of MPS and ML conditions so far. Gene editing holds promise as a tool for the creation of cell and animal models to help explain disease pathogenesis, as well as a platform for gene therapy. In this chapter, we discuss the main studies involving genome editing for MPS and the prospect applications for ML.

Abbreviations

AAVS	adeno-associated viruses
cDNA	complementary DNA
CNS	central nervous system
CRISPR	clustered regularly interspaced short palindromic repeat

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DSBs	double-strand breaks
GAGs	glycosaminoglycans
GBA	glucosylceramidase beta
GNPTAB	*N*-acetylglucosamine-1-phosphotransferase
gRNA	guide RNA
HDR	homology-directed repair
hIDS	human IDS
IDS	iduronate-2-sulfatase
IDUA	alpha-L-iduronidase
IPSCs	induced pluripotent stem cells
LSDs	lysosomal storage disorders
ML	mucolipidoses
MPS	mucopolysaccharidoses
NHEJ	error-prone non-homologous end joining
NSCs	neural stem cells
PAM	protospacer adjacent motif
pegRNA	prime editing guide RNA
RVDs	repeat-variable diresidues
TALENs	transcription activator-like effector nucleases
ZFNs	zinc finger nucleases

1. Clinical and molecular characteristics of mucopolysaccharidoses and mucolipidoses

1.1 Mucopolysaccharidoses

The mucopolysaccharidoses (MPS) are a group of 11 lysosomal storage disorders caused by a deficiency in one of the hydrolases that participate in the multistep degradation of glycosaminoglycans (GAGs).[1] They all have autosomal recessive inheritance, with exception of MPS II which is X linked. Table 1 summarizes the different types of MPS, their respective gene and enzyme deficiency and the partially metabolized GAG.[2]

GAGs are highly sulfated, complex, linear polysaccharides composed of repeated disaccharide units. They are constituents of the extracellular matrix where they play a key role in cell signaling and modulate several biochemical processes, including regulation of cell growth and proliferation, promotion of cell adhesion and wound repair.[3] Therefore, depending on which GAG degradation pathway is affected by each MPS type, different organs and tissues are more preeminently affected.[4]

MPS patients present a set of common features that include progressive multisystemic involvement, infiltrated faces, skeletal and joint problems.

Table 1 General features and key clinical features of mucopolysaccharidoses.

						Main affected organs		
MPS type	**OMIM**	**Eponym**	**Enzyme**	**Gene**	**Glycosaminoglycan**	**Brain**	**Bone/joint**	**Viscera (Liver and spleen)**
MPS I	#607014	Huler/ Scheie	alpha-L-iduronidase	*IDUA*	HS, DS	+++/++	++	+++
	#607016					+/–	++	++
MPS II	#309900	Hunter	Iduronate sulfatase	*IDS*	HS, DS	+++/–	++	++/+++
MPS III A	#252900	Sanfilippo A	Heparan N-sulfatase (sulfamidase)	*SGSH*	HS	+++	+	+
MPS III B	#252920	Sanfilippo B	alpha-*N*-acetyl-glucosaminidase	*NAGLU*	HS	+++	+	+
MPS III C	#252930	Sanfilippo C	Acetyl-CoA: alpha-glucosaminide acetyltransferase	*HGSNAT*	HS	+++	+	+
MPS III D	#252940	Sanfilippo D	*N*-acetylglucosamine 6-sulfatase	*GNS*	HS	+++	+	+
MPS IV A	#253000	Morquio A	Galactose 6-sulfatase	*GALNS*	KS, CS	–	+++	++
MPS IV B	#253010	Morquio B	beta-Galactosidase	*GLB1*	KC	–	+++	++
MPS VI	#253200	Maroteaux-Lamy	*N*-acetyl-galactosamine 4-sulfatase	*ARSB*	DS, CS	–	+++	+++
MPS VII	#253220	Sly	beta-glucuronidase	*GUSB*	HS, DS, CS	++	++	+++
MPS IX	#601492	Natowicz	Hyaluronidase	*HYAL1*	Hyaluronan	–	++	–

CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; KS, keratan sulfate; MPS, mucopolysaccharidosis.
-, no involvement; +, mild; ++, moderate; +++, severe.

They also may present heart and respiratory abnormalities, hepatosplenomegaly, and neurological impairment, although this last characteristic is highly variable between and within MPS types. Nevertheless, neurocognitive issues are restricted to those MPS in which heparan sulfate degradation is impaired, whereas skeletal and joint manifestations are present when dermatan or keratan sulfate degradation pathways are involved. It is important to notice, however, that the type of skeletal disease in MPS IV A/B is markedly different from that seen in other MPS that have impaired dermatan sulfate degradation. Table 1 presents a summary of key clinical findings in the different MPS.

Age at onset may be as early as prenatal (in MPS VII) to late childhood (in MPS IX) and varies greatly depending on where in the clinical spectrum the patient is situated. For historical reasons MPS I is clearly recognized as having severe and attenuated forms (Hurler and Scheie syndromes, respectively), but there is a continuum of clinical presentations within any given MPS that correlates with residual enzyme activity (Fig. 1). This variation can be mostly explained by the genotype of the patients. Usually, patients homozygous for nonsense mutations such as p.Trp402Ter and p.Gln70Ter in MPS I have the most severe form, due to the fact that no residual enzyme activity is produced.[5]

The incidence of MPS varies widely according to type and geographic region. Khan et al. (2017) based on epidemiological data, estimated frequencies between 7.85 (for MPS VI) to 0.01 (for MPS IV B) per 100,000 live births.[6] Borges et al. (2020), using the frequency of disease-causing variants in populational databases estimated incidences of 7.10 (for MPS I) to 0.05 (for MPS III D) per 100,000 live births.[7] Nevertheless, incidences can be

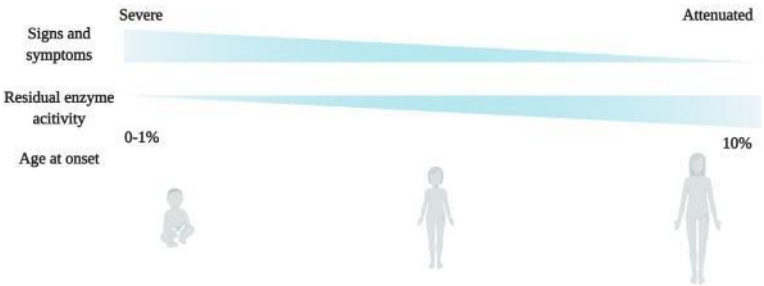

Fig. 1 Mucopolysaccharidoses symptoms and onset. Continuum of clinical presentation in the MPS correlates with residual enzyme activity and age at onset of symptoms.

higher in specific regions for particular MPS types, such as the cluster of MPS VI in Northeast of Brazil.[8] Allelic heterogeneity is common in all MPS, with a few predominant alleles, such as p.Trp402Ter in MPS I or p.Arg245His in MPS III A, that account for about 50% of the pathogenic alleles in different populations. However, these are rather the exception and many different pathogenic variants have been described for these diseases.

1.2 Mucolipidoses

Lysosomal hydrolases require the addition of mannose-6-phosphate residues for a correct trafficking into the nascent lysosomes. This post-translational modification is performed by *N*-acetylglucosamine-1-phosphotransferase (GlcNAc-phosphotransferase; EC 2.7.8.17), an enzyme complex formed by six subunits: $a_2/b_2/g_2$. Both a and b subunits are encoded by the *GNPTAB* gene, located in chromosome 12q23.2, with 21 exons and spans over 85 kb. The non-catalytic G subunit is encoded by the *GNPTG* gene, located in chromosome 16p13.3 which has 11 exons and spans 11.44 kb. GlcNAc-phosphotransferase deficiency impairs trafficking and redirects lysosomal hydrolases from lysosomes to the extracellular milieu. This feature is used for diagnostic purposes, as patients will present reduced activity of several hydrolases in fibroblasts (but not in leukocytes) with increased enzyme activity in plasma.[9]

Mucolipidosis I is a distinct disorder caused by alterations in neuraminidase, being called Sialidosis, and therefore will not be discussed in this chapter. Mucolipidosis II (ML II disease, inclusion cell disease or I-cell disease) and III (ML III, pseudo-Hurler polydystrophy) are autosomal recessive disorders caused by defects in the GlcNAc-1-phosphotransferase complex. Although having a common biochemical basis, clinically there are three recognized types of ML. The severe phenotype is ML II (OMIM #252500), whereas milder forms are ML III α/β (OMIM #252600) and ML III gamma (OMIM #252605).

ML II is caused by mutations in *GNPTAB*, usually nonsense or frameshift, that cause complete loss of function of GlcNAc-1-phosphotransferase activity. The incidence is approximately 1:123,500 live births in Portugal[10] and an unusually high prevalence is found in the province of Quebec, Canada with 1:6184 live births and an estimated carrier rate of 1:39, probably due to a founder effect.[11] Symptoms are present at birth or even before, and death occurs in the first decade of life. Clinical features include

Table 2 General features of mucolipidoses (ML).

ML type	OMIM	Protein	Gene	Phenotype
ML II	#252500	GlcNAc-1-phosphotransferase (α/β subunits)	*GNPTAB*	Severe
ML III α/β	#252600	GlcNAc-1-phosphotransferase (α/β subunits)	*GNPTAB*	Intermediate
ML III δ	#252605	GlcNAc-1-phosphotransferase (δ subunit)	*GNPTG*	Attenuated

ML type I is Sialidosis, with different characteristics.

psychomotor retardation, coarse dysmorphic facial features, growth retardation, and restricted joint movement. Severe skeletal abnormalities, cardiac and pulmonary complications are also present and usually are the cause of death.[12]

ML III α/β is also due to mutations in *GNPTAB* but in this case it is usually associated to missense or splice site variants that retain some residual enzyme activity. Incidence is estimated to be similar to that of ML II and disease progression is slower, with clinical onset at approximately age 3 years and death in early-to-middle adulthood. Clinical features include joint stiffness, left and/or right ventricular hypertrophy in older individuals and death in early adulthood is often from cardiopulmonary causes.[13] Neuromotor development and intellect are the most variable features in ML IIIα/β.

ML III gamma is caused by mutations in *GNPTG*. No precise disease incidence is available, and it is considered an ultra-rare disease. Clinical onset is in early childhood and disease progress is slow (Table 2). It affects mainly the skeletal, joint, and connective tissues. Mild cardiac involvement is present.[14]

2. Genome editing: General concepts

Genome editing is a broadly used DNA engineering approach for biological research taking advantage of a new set of revolutionary technologies. The strategy involves making precise changes, such as insertion, deletion, or substitutions at a specific DNA locus. This technology can be used to edit diverse cell types and organisms, and it has been widely applied in pathophysiology studies or in the development of new gene-based therapies. Genome editing-based therapies are particularly promising for patients affected with genetic diseases,[15] especially monogenic diseases, like MPS and ML.

Currently, there is a wide variety of genome editing platforms. Among them, the most pertinent to treat diseases are the transcription activator-like effector nucleases (TALENs), zinc finger nucleases (ZFNs), clustered regularly interspaced short palindromic repeat (CRISPR)–associated (Cas) systems and the more recently described CRISPR-Cas9-based editors and prime editing.[15] To date, genome editing was performed in MPS using ZFN and CRISPR–Cas9. Although there are no reports of the application of genome editing tools in ML, the potential of the technique in this group of diseases is undeniable and will be discussed in this chapter as well.

Genome editing technologies are based on the use of engineered nucleases to create double-strand breaks (DSBs) at specific DNA sequences or genomic locations, to stimulate endogenous cellular DNA repair mechanisms. The error-prone non-homologous end joining (NHEJ) process incorporates random non-complementary nucleotides or delete nucleotides (indels). Depending on the type and position, it can change the open reading frame and generate non-functional proteins and, thus, gene knockouts. The homology-directed repair (HDR) needs a DNA donor template sequence to correct or insert the transgene. This template must have nucleotide sequences flanking the gene to be inserted that are homologous to those upstream and downstream of the break site[16] (Fig. 2).

The first programmable genome editing tools were ZFNs and TALENs (Fig. 3), which were designed by the fusion of a restriction enzyme and an unrelated DNA recognition domain. ZFNs consist of a nuclease domain of 196 amino acids, derived from the restriction enzyme FokI, and a DNA-binding domain made by consecutive Cys2His2 zinc finger units, each composed by ~30 amino acids. In the ZFN each zinc finger protein recognizes 3 base pairs, and the total recognized DNA sequence is about 9–18 nucleotides in length. TALENs are engineered by fusing an array of DNA-binding domain derived from transcription activator-like effectors (TALEs) with the FokI nuclease domain. The DNA recognition of TALENs is conferred by the repeat-variable diresidues (RVDs), which include multiple 33–35 amino acids repeat domains, each recognizes a single base pair for a total target site length between 15 and 30 nucleotides. For both systems, the recognition of two proximal DNA sequences is required for Fok1 dimerization and subsequent cleavage of the targeted region. Because DNA-binding domains derived from zinc fingers and TALE proteins can be customized they can recognize virtually any genomic DNA sequence.[17]

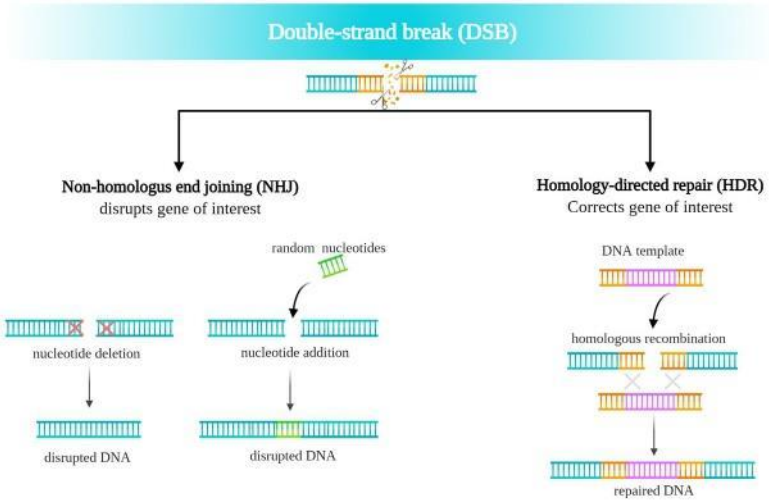

Fig. 2 Endogenous cellular DNA repair mechanisms exploited for gene editing. Following a DNA double-strand break (DSB), non-homologous end joining (NHEJ) joins two broken ends together, without using a homologous template for repair and leaving a disrupted DNA sequence with small insertion or deletions. This DNA repair pathway has been usually exploited to create gene knockouts. Homology-directed repair (HDR) is a template-dependent pathway for DNA DSB repair, which can be exploited for precise gene modification.

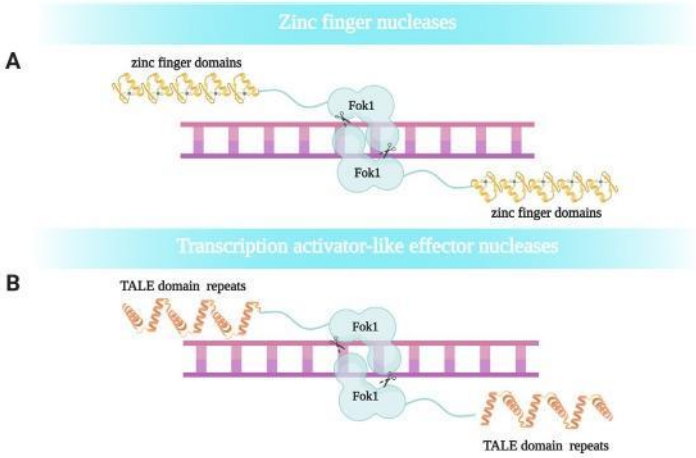

Fig. 3 Protein-based genome editing platforms. (A) Zinc finger nucleases (ZFNs) are formed by the DNA cleavage domain derived from the restriction enzyme *FokI* and a DNA-binding domain zinc fingers. (B) Transcription activator-like effector nucleases (TALENs) are formed by the same FokI nuclease domain present in ZFNs while their DNA-binding domain is derived from transcription activator-like effectors (TALEs) proteins.

The most recent genome editing tools are based on the CRISPR-associated (Cas) system, which consists of an endonuclease (usually Cas9) and a guide-RNA. The Cas9 nuclease cleaves DNA guided by the guide-RNA (gRNA). This complex recognizes the target sequence (protospacer) through RNA and DNA base pairing, in the presence of short (3–7 bp) protospacer-adjacent motif (PAM) sequence bound directly by the Cas9 protein.[18] Targeting different sequences can be easily programmed by altering its gRNA, due to RNA-based recognition. New genome editing tools based on CRISPR have recently appeared. Base editing and Prime editors are CRISPR-mediated platforms that do not rely on DSBs. Base editors utilize a modified Cas9 such as inactive Cas9 or Cas9 Nickase, complexed with base-modifying enzymes (cytosine deaminase or adenosine deaminase) and a gRNA for converting one target base or base pair to another (for example, A-T to G-C or C-G to T-A). The prime editing utilizes a reverse transcriptase fused to a Cas9 Nickase and a prime editing guide RNA (pegRNA). The pegRNA serves as gRNA and as a template for reverse transcription[15,18] (Fig. 4).

Genome editing requires delivery of the components to target cells, and there are two approaches to this: in vivo or ex vivo. In vivo genome editing involves direct delivery in the body, either by intravenous delivery or in situ injection. On the other hand, ex vivo genome editing involves removal of the target cell population from the body, modifying it in vitro, and then transplanting it back into the original host.[19] These cells can be from the patient, from a suitable donor, or from a cell bank[15] (Fig. 5).

Regardless of the chosen approach (in vivo or ex vivo), it is necessary to have a vector that can access the nucleus of the cell, so that the editing occurs. Genome editing components cannot efficiently pass-through cell membranes, due to their molecular features. Therefore, the viral or non-viral delivery vectors are used to encapsulate and transport them. In some instances, a non-viral physical method is used. For ex vivo studies, the most common approaches use mechanical deformation or electroporation as a strategy to create transient holes in cell membranes, allowing nucleic acids and proteins to enter the cell. For in vivo administration of the gene editing components in animals, some common approaches include the application of high-volume hydrodynamic injection of nucleic acid into the tail vein or by direct injection into the embryo or zygote to create animal models (Fig. 6).

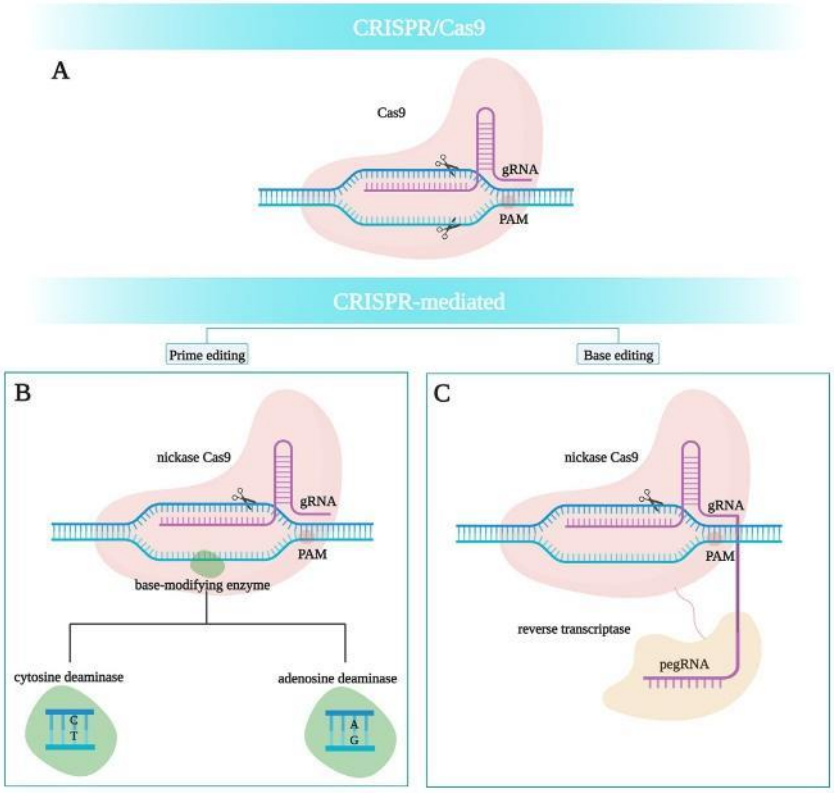

Fig. 4 CRISPR-based Genome Editing Platforms. (A) Clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system: guide RNA (gRNA), Cas9 and protospacer adjacent motif (PAM). (B) Base editors: Cas9 Nickase complexed with base-modifying enzymes and a gRNA. (C) Prime editors: Cas9 Nickase attached to reverse transcriptase and complexed with prime editing guide RNA (pegRNA).

The main viral vectors used for genome editing are retroviruses, adenoviruses, and adeno-associated viruses (AAVs). Currently, AAVs have received special attention in research and clinical studies. Viral vectors are very efficient for gene delivery, but important immune responses have been reported in the history of viral gene therapy. On the one hand, non-viral vectors involve cationic materials to form nanoparticles that can be electrostatically complexed with DNA and other nucleic acids. The most successful classes of cationic materials used are naturally occurring and synthetic polymers and lipids. The vectors based on non-viral materials are biocompatible, less toxic, and immunogenic and are well-tolerated, but have reduced delivery efficiency.

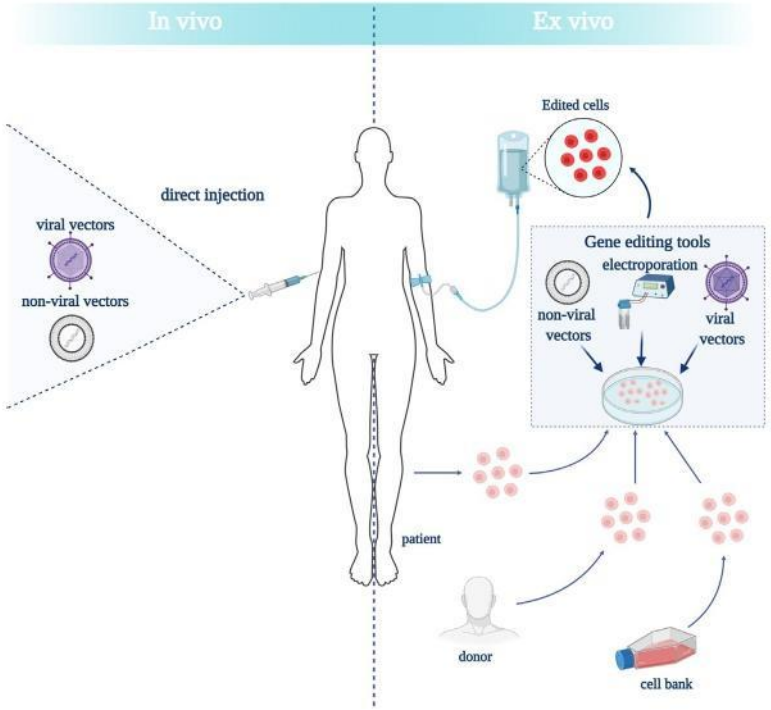

Fig. 5 Genome editing approaches: In vivo gene editing: direct injection of genome editing tools with viral or non-viral vectors. Ex vivo: removal of the target cell population from the body (patient, donor, or cell bank), in vitro modification (using non-viral, viral, or physical methods), and transplantation, usually back to the original host.

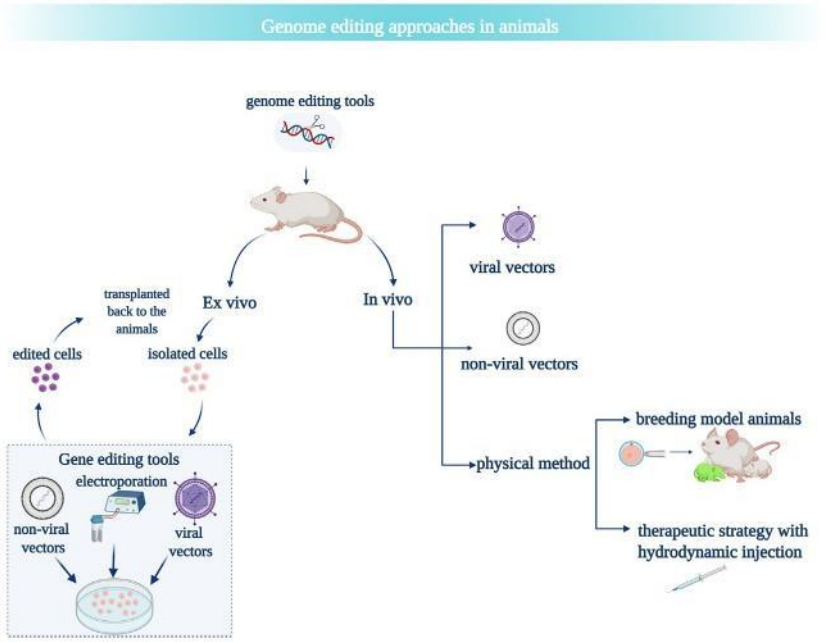

Fig. 6 Genome editing approaches in animal models. In vivo: direct injection of genome editing components with viral or non-viral vectors. Ex vivo: removal of the target cell population from the body, in vitro modification using non-viral, viral, or physical methods (hydrodynamic injection and direct injection) and injection of edited cells.

3. Genome editing for MPS and ML: In vitro studies

Preliminary studies involving pathophysiology or therapy development are traditionally done in patient-derived fibroblasts, as these cells present some disease features and are relatively easy to culture. When exposed to specific factors in the culture media, fibroblasts can even be differentiated into chondrocytes or osteoblast-like cells, becoming simple in vitro models of bone disease, for example. Stromal mesenchymal cells or hematopoietic stem cells also have the ability to differentiate into other cell types, as adipocytes or blood cells[20] (Fig. 7A). Lately, however, alternatives have been used to expand the possibilities of in vitro studies, mainly by means of using induced pluripotent stem cells (iPSCs), which can virtually be differentiated into many different cell types, as neural cells, cardiomyocytes or hematopoietic cells[21] (Fig. 7B).

In the last few years, several iPSCs models were created for Lysosomal storage disorders (LSDs) and MPS, including MPS I,[22] MPS II[23,24] (with a model from a heterozygote female patient with skewed X chromosome inactivation),[25] MPS IIIB,[26,27] MPS IVA[28]and MPS VII,[29] though none for ML. In 2011 the first iPSC MPS model reported was derived from keratinocytes and from mesenchymal stromal cells collected from two MPS I patients.[30] Once generated, alpha-L-iduronidase (IDUA)-deficient iPSCs were able to differentiate into hematopoietic and non-hematopoietic cells and the retroviral-mediated delivery of *IDUA* to correct the enzyme deficiency did not affect the differentiation pathway.

As the basis of MPS pathogenesis are still not completely understood in the central nervous system (CNS), patient-derived iPSCs are frequently differentiated into neural stem cells (NSCs). Indeed, despite MPS iPSCs maintain neural differentiation ability, MPS patient derived NSCs have a reduced self-renew capacity.[31] In MPS I NSCs, analysis of transcriptome revealed major alterations comparing to control cells, such as higher expression of autophagy genes and GAG biogenesis; in this study, cells derived from patients with the severe phenotypes present more pronounced alterations than cells from attenuated patients.[32] In the MPS II and MPS IIIB NSCs models, there are structural alterations in the Golgi complex and impaired autophagy.[32] When cultured in 3D organoids, MPS VII neural cells showed reduced neuronal activity and altered network connectivity.[29] Another interesting observation was done using MPS II cells, where recombinant enzyme administration did not normalize nor prevented GAG storage in

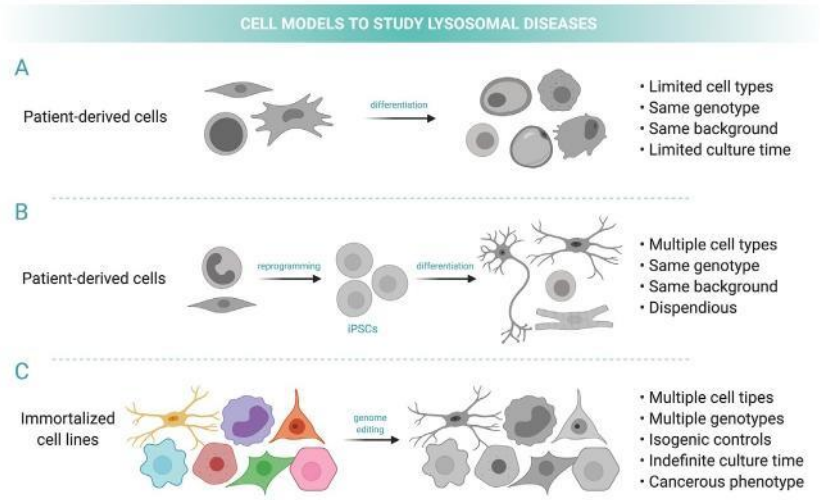

Fig. 7 Cell models to study lysosomal storage disorders (LSDs). (A) The obtention of patient-derived cells can be extremely invasive, thus not all cell types are available. Cells normally collected are fibroblasts, hematopoietic cells, and mesenchymal stromal cells. These cells can be differentiated into a small subset of cells, like osteoblasts, chondrocytes, adipocytes, and blood cells. (B) Alternatively, cells collected from patients can be reprogrammed into iPSCs cells, and afterwards be differentiated into virtually any cell type, like neurons or cardiomyocytes. In both patient-derived cells cases, all cells will harbor the patient genetic background and same disease-causing mutation and isogenic controls are only possible with genome editing. (C) Immortalized cell lines are derived from multiple individuals thus having different genetic marks. Multiple cell types can be chosen and edited to mimic the disease phenotype, with multiple genotypes. Gray cells: LSDs cells. Colored cells: wild-type cell lines.

neurons, astrocytes or oligodendrocytes, even when administered before GAG started to build up intracellularly[33]; these information from patient derived cells can give invaluable insights about therapeutic targets in the CNS.

An important aspect of using patient-derived iPSCs and healthy donor cells as controls is that these are not equivalent. One should be careful before drawing conclusions about what differences are due to the disease or simply due to distinct genetic background, epigenetic status or unmatched gender, age, and ethnicity. All these confounding factors can only be turned around by editing the cells—either by inducing pathogenic mutations in healthy cells or by correcting the disease-causing genotype in patient cells. In either way, both populations will harbor the same genome and epigenetic profile, except for the pathogenic variant in the specific gene of interest. Such approach was done using cells derived from MPS I mice, where isogenic

iPSCs were generated from murine fibroblasts by CRISPR-mediated deletion of the exogenous sequence on exon 6, originally introduced to create the MPS I mouse model. Edited cells had a restored wild-type sequence and were efficiently differentiated into IDUA-secreting fibroblast-like cells.[34]

Despite the clear importance of creating iPSCs from LSDs patients, the process can be challenging and requires special strategies. In LSDs cells, the reprogramming process may be impaired by the very nature of the metabolic dysfunction. For example, the first model for MPS IIIB required co-culture of iPSCs with a feeder-layer of alpha-*N*-acetyl-glucosaminidase (NAGLU)-expressing cells during reprogramming as the lack of enzyme and/or accumulation of undegraded metabolites were impeding the procedure.[35] An additional barrier for iPSCs development of LSDs models is the cost: besides reagents, the reprogramming process is labor-intensive and requires experienced personnel.[21]

One way to overcome some limitations to iPSCs use is to generate cell models from immortalized cell lines (Fig. 7C). Although they might normally have cancerous features that can interfere in some studies, these are accessible cells, easy to culture and frequently easy to engineer.[36]There are currently multiple cell types available, from epithelial to neural cells—like the neuroblastoma line SH-SY5Y (ATCC CRL-2266). This cell line can be differentiated into neuron-like cells and be used as a model for neurodegenerative diseases. Such model was developed for MPS II, where SH-SY5Y cells were knocked-out in the iduronate-2-sulfatase (IDS) gene using specific gRNA targeting exon 3 and Cas9, transfected to the cells as a single plasmid vector.[37] The MPS II SH-SY5Y cells presented undetectable IDS activity and accumulation of GAGs, thus were used to validate observations made in the mouse model, which had overexpression of the protease cathepsin B in the brain. Increased protease expression, including cathepsin B, has been previously demonstrated in MPS I mouse models[38,39] and is thought to be part of the pathogenesis of lysosomal diseases. To confirm the data observed in the MPS II mice, cathepsin B was measured in knockout SH-SY5Y cells. The immunofluorescence data shows increased signal for cathepsin B and leakage to the cytoplasm, as this protease was not co-localized with lysosomal markers, suggesting alteration in the lysosomal membrane possibly caused by GAG storage or other unknown mechanism.[37]

There are no reports of genome editing or generation of cell models for ML, and for MPS, the field is still in its beginning. First reports of genome editing for MPS were in vitro studies using patient fibroblasts. In these proof-of-concept studies[40,41] cells were collected from a patient harboring

the p.Trp402* mutation, the most common IDUA pathogenic variant.[5] Cationic nanoemulsions and liposomes were used to co-deliver a CRISPR-Cas9 and gRNA expression plasmid and a single-stranded oligonucleotide donor template containing a single nucleotide change (from tAg to tGg) to correct the p.Trp402Ter mutation. After 7 days of transfection, IDUA enzyme activity reached 5% of normal levels in the bulk population of edited cells; though seeming low, the achieved increased IDUA activity was enough to significantly reduce lysosome size in the whole cultured population, including non-edited cells, encouraging in vivo studies.[42,43]

Even though there are few studies using either iPSCs or genome editing for MPS, the applications of these two methodologies combined are huge. For example, it has been proposed the perspective of autologous neural stem cell transplantation, where iPSCs are edited, reprogrammed into NSCs and then transplanted back to the patient, in an attempt to serve as a permanent enzyme source in the brain.[44,45] While iPSCs can be efficiently edited,[46] human NSCs from biobanks can also be engineered to secrete lysosomal enzymes—as shown for Krabbe disease—and cross-correct enzyme-deficient cells.[47] A caveat of this approach, however, is the still remaining uncertainty regarding the safety of these cells once in vivo.

4. In vivo genome editing

Genome editing technologies have progressed to correct mutations that cause diseases through the use of nucleases such as ZFNs, TALENs, and more recently, CRISPR-Cas nucleases.[15] In vivo genome editing requires to tackle several critical aspects to achieve successful treatments. Indeed, the choice of gene delivery strategies[2,48] and biopharmaceutical aspects, such as route of administration, dose, therapeutic regimen, considering as well animal models or patient variables like age and other characteristics, lead to a very challenging path.[49–51]

Site-specific in vivo genome editing provides a promising approach for achieving long-term, stable therapeutic gene expression and has been successfully applied in a variety of preclinical models.[15] The first in vivo genome editing studies for MPS used ZFNs.[52] This first report describes a strategy for liver-directed protein replacement therapies using ZFN-mediated site-specific integration of either *IDUA* or *IDS* transgenes, delivered by an AAV vector, into the albumin locus. The study achieved

long-term expression of lysosomal enzymes at therapeutic levels for both MPS I and MPS II.[52]

A similar in vivo genome editing approach was described for the murine model of MPS I syndrome, delivering ZFNs along with a corrective copy of the *IDUA* gene, which was inserted at the albumin locus in hepatocytes. In these studies they co-delivered mouse albumin specific ZFNs with a donor construct encoding a partial complementary DNA (cDNA) for either human *IDS* (mutated in MPS II), *IDUA* (MPS I), or *GBA* (glucosylceramidase beta, Gaucher disease) using AAV in wild type mice.[53] They demonstrated stable integration of the transgenes at the albumin locus, which resulted in liver-specific expression and secretion of the enzymes to plasma. For IDS and IDUA, this correlated well with increased enzymatic activity and protein expression in the liver, also detected in secondary tissues. *IDS, IDUA* or *GBA* expression remained stable in mice for 1–2 months, suggesting that this procedure is well-tolerated. Another approach from the same group led to sustained enzyme expression, secretion from the liver to circulation, and subsequent systemic uptake at levels sufficient for correction of metabolic disease and prevention of neurobehavioral deficits in MPS I mice.[54]

Laoharawee et al.[55] used a ZFN-targeting system to mediate insertion of the human *IDS* (hIDS) coding sequence into a "safe harbor" site, the intron 1 of the albumin locus in hepatocytes of an MPS II mouse model, the same locus used in the MPS I model. Three dose levels of recombinant AAV2/8 vectors encoding a pair of ZFNs and a *hIDS* cDNA donor were administered systemically. Supraphysiological vector dose-dependent levels of IDS enzyme were observed in the circulation and peripheral organs of treated mice, as well as reduction in GAG content. Surprisingly, they also demonstrated that ZFN-mediated genome editing prevented the development of neurocognitive deficit in young MPS II mice (6–9 weeks old) treated with a high vector dose. This ZFN-based platform for expression of therapeutic proteins from the albumin locus is a promising approach for treatment of MPS II and other lysosomal diseases.

The promising results observed in these first studies in animals[53–55] suggested that this approach could be effective in humans,[56] and the strategy is being tested in two clinical trials for MPS I (ClinicalTrials.gov, NCT02702115[57]) and MPS II (NCT03041324[58]). However, the initial results suggest that a very discreet increase in serum enzyme activity, possibly due to the low transgene expression level or an immune response to the vector. So, the researchers designed a proprietary system (PS) gene

editing approach with CRISPR to insert a promoterless *IDUA* cDNA sequence in the albumin locus of hepatocytes. In this study, adeno-associated virus 8 (AAV8) vectors delivering the PS gene editing system were administered to newborn and adult MPS I mice.[59] As result, IDUA enzyme activity in the brain was significantly higher than control group, and neurobehavioral tests showed that treated mice had better memory and learning ability when compared to MPS I untreated mice. In addition, histological analysis showed efficacy and no vector-associated toxicity or increased tumorigenesis risk were observed. In summary, these results demonstrated the safety and efficacy of the PS in treating MPS I and paved the way for clinical studies. Additionally, as a therapeutic platform, the PS has the potential to treat other lysosomal diseases and new applications may rise from these studies.

Wang et al.[60] reported a genome-editing strategy to correct compound heterozygous mutations. The adeno-associated viral vector delivery of Cas9 gene and a guide RNA induced allelic exchange and rescued the disease phenotype in a mouse model of MPS I. This approach uses recombination of non-mutated genetic information present in compound heterozygous alleles into one functional allele without using donor DNA templates.[60] Neonatal MPS I mice were injected with a mixture of rAAV-SpCas9 and scAAV-sgRNA at 1:1 ratio in the facial vein. Six-week-old mice were injected with the same mixture of rAAV vectors in the tail vein. In the compound heterozygous MPS I mice, rAAV treatment at a young adult age restored IDUA activity to about 0.5% of the wild-type level, and substantially reduced GAG accumulation in the heart. Thus, Cas9-induced allelic exchange has the potential for gene correction in post-mitotic tissues. Several advantages distinguish this Cas9/sgRNA-mediated allelic exchange from other therapeutic in vivo genome-editing approaches such as Homologous Directed Repair, as no exogenous DNA repair template is needed. However, there are also important limitations to be considered, such as the low frequency of recombination events (lower than 1% of alleles) and the lack of information regarding safety of this approach, as no long-term study was published, and more than a single site in the genome needs to be cut, increasing the chance of off-target effects.

Another potential treatment approach is to engineer the patient's own hematopoietic system to express high levels of the deficient enzyme, thereby correcting the biochemical defect and halting disease progression. In this sense, Gomez-Ospina et al.[61] presented an efficient ex vivo genome editing approach using CRISPR/Cas9 that targeted alpha-L-iduronidase enzyme to the *CCR5* safe harbor locus in human CD34+ hematopoietic

stem and progenitor cells. The modified cells secreted supraphysiological enzyme levels, maintained long-term repopulation and differentiation potential, and improved biochemical and phenotypic abnormalities in an immunocompromised mouse model of MPS I. These studies provide support for the development of genome edited CD34+ hematopoietic stem cells and may be applicable to other lysosomal storage disorders as well.

Recently, our group also tested gene editing using the CRISPR/Cas9 system in a MPS I mouse model.[42] Cationic liposomes were used to carry a CRISPR/Cas9 plasmid and a donor vector carrying the entire *IDUA* cDNA, aiming at the *ROSA26* locus. We compared our treated mice with animals treated with naked plasmids, and with untreated controls. A single hydrodynamic injection of the liposomal complex in newborn MPS I mice led to a significant increase in serum IDUA levels which were maintained for up to 6 months. The biodistribution of liposomal complexes after hydrodynamic injection was markedly detected in lungs and heart, corroborating the results of increased IDUA activity and decreased GAG storage especially in these tissues, while the group that received the naked plasmids presented increased IDUA activity especially in the liver. Furthermore, animals treated with the liposomal formulation presented improvement in cardiovascular parameters, one of the main causes of death observed in MPS I patients.[42] In another study, we also reported the effects of gene editing in cardiovascular, respiratory, bone, and neurologic functions in MPS I mice. Bone morphology showed partial improvement, while 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.[43]

Genome editing is a potentially curative therapy for many genetic diseases, especially for MPS and ML. Ex vivo and in vivo genome editing platforms have been tested primarily on MPS, some approaches reaching clinical testing. The development of new genome editing platforms and the expansion to other diseases, may provide potential applications for both groups of disorders, which exceed the potential of current approaches.[15]

5. Clinical trials

Regarding MPS, the promising results in animal models suggested that gene editing could be effective in humans.[15] However, there are only two clinical trials involving gene editing for MPS to date, because clinical

research in rare diseases has many challenges, such as carrying out trials in small populations, difficult identification of affected patients and the almost inevitable international scale of rare disease studies involving different regulatory agencies.[62] Furthermore, since gene editing is a relative new technique, several preclinical studies are still ongoing to gather enough data to translate to the clinic.

Despite these limitations, as briefly mentioned before, to date there are two clinical studies for MPS (clinicaltrials.gov). One for MPS I (NCT02702115) (Table 3)[57] and the other for MPS II (NCT03041324).[58] Both studies are mediated by ZFNs and delivered by AAV2/6 vectors. Moreover, it is important to highlight that enzymes produced by the liver are not expected to cross the blood-brain barrier. In view of, only patients with mild forms of the diseases that have little or no involvement of the CNS were recruited or studies.[15] Below we describe the main features and findings of the two clinical trials for MPS:

The study called "CHAMPIONS" is a clinical trial for MPS II (NCT03041324), the first trial to attempt to edit the genome in vivo in humans. A Phase 1/2 clinical trial evaluating the safety, tolerability, and effect on IDS enzyme activity of SB-913, designed to insert an IDS transgene into the hepatocyte albumin locus and, this way, to provide the long-term expression of IDS. Nine participants were distributed in four different cohorts with rising therapeutic doses of SB-913. The first results showed generally well-tolerated and no drug-related adverse events were reported at any dose with up to 10 months of exposure. Plasma IDS values no were detected and 1 subject in cohort 2 showed an increase.[15,58,63] Additional analysis of the trial data is ongoing.

The second trial is called "EMPOWERS": The clinical trial directed to individuals with MPS I (NCT02702115). A Phase 1/2 clinical trial evaluating the safety and tolerability of SB-318, designed to insert IDUA transgene into the hepatocyte albumin locus in doing so, provide the long-term expression of IDUA. Three participants were distributed in three different cohorts with increasing therapeutic doses of SB-318. The primary outcome presented was generally well-tolerated, even the highest dose of the vector.[57,59] No serious adverse events related to the study drug were reported with up to 3 months of exposure. However, the plasma IDUA values unchanged from pre-treatment levels. Two patients showed a decrease in the excretion of GAGs, while one patient remained with GAGS values above the normal range[64]. Additional analysis is ongoing.

There are no clinical trials based on genome editing for ML to date. The development of future therapies for ML, such as those based on genomic

Table 3 Clinical trials involving genome editing for Mucopolysaccharidoses.

NCT number	Title	Status	Conditions	Interventions	Phase	Sponsor/ collaborators
NCT02702115	Ascending Dose Study of Genome Editing by the Zinc Finger Nuclease (ZFN)Therapeutic SB-318 in Subjects with MPS I	Active, not recruiting	MPS I	Biological: SB-318	I/II	Sangamo therapeutics
NCT03041324	Ascending Dose Study of Genome Editing by the Zinc Finger Nuclease (ZFN) Therapeutic SB-913 in Subjects with MPS II	Active, not recruiting	MPS II	Biological: SB-913	I/II	Sangamo therapeutics

MPS, mucopolysaccharidosis.
Adapted from clinicaltrials.gov.

editing, still needs to advance in experimental research and accumulate clinical experiences to learn about the natural history and pathophysiology of ML.

6. Conclusions

The mucopolysaccharidoses were one of the first group of diseases in which genome editing was employed. This is mainly due to the facts that (1) the mechanisms of disease are not completely understood and (2) patients with these disorders benefit from small increments in enzyme activity, making gene editing an alternative treatment approach. The escalation from animal models to clinical trials occurred fast in MPS disorders, but the initial results are still inconclusive regarding effectiveness of therapy, and better gene editing platforms are being tested. On the other hand, studies involving gene editing for mucolipidoses are still inexistent, also possibly due to the limitations of gene therapy for a disease which is not caused by a secreted hydrolase. However, the creation of cell lines for mucolipidoses can help elucidating mechanisms of disease and also allow drug screening and should be tested soon, especially considering the unmet medical needs for these diseases.

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4.3 Artigo III: Biomechanical properties and the effect of different treatments in MPS I bone disease. Artigo em preparação.

4.4 Artigo IV: Intra-articular nonviral gene editing in MPS I mice.

Artigo em preparação.

5. DISCUSSÃO

A MPS I é causada pela deficiência da enzima IDUA. A falha dessa enzima leva a um acúmulo progressivo de GAGs nos lisossomos. O acúmulo dessas moléculas afeta importantes processos celulares. Em consequência, desencadeia uma cascata de respostas metabólicas interrelacionadas. O resultado desses eventos é uma série de sintomas multissistêmicos. Existe um amplo espectro fenotípico, porém, um conjunto de características são comuns, como os problemas ósseos e articulares, como membros curtos e grossos e articulações inchadas que provocam dor e causam comprometimento do movimento, afetando a qualidade de vida dos pacientes (Santos et al. 2021b).

Apesar da MPS I ter sido descrita em 1919 (Kubaski et al. 2020)e desde então, ter sido bem estudada, a fisiopatologia osteoarticular ainda não é totalmente compreendida (Opoka-Winiarska et al. 2013; Borgo et al. 2018). Além disso, os tratamentos atualmente disponíveis para MPS I, a terapia de reposição enzimática (TRE) e transplante de células-tronco hematopoiéticas (TCTH), não melhoram as condições osteoarticulares da doença (Bidone et al. 2018). Isso incentiva a busca do melhor entendimento das alterações osteoarticulares da MPS I e novas abordagens terapêuticas que sejam efetivas para melhorá-las.

Nosso grupo de pesquisa tem contribuído com a descrição de alterações presentes na MPS I, a partir da utilização do modelo murino (Baldo et al. 2012; de Oliveira et al. 2013). Temos uma colônia de camundongos C57BL/6 geneticamente modificados que utilizamos para nossos estudos, sendo animais MPS I (*Idua* -/-) e wild type (normais) (*Idua*+/+ ou *Idua* +/-) os grupos estudados, em diferentes idades. Os camundongos MPS I mimetizam vários sintomas progressivos que também são encontrados em pacientes(Schuh et al. 2020). Um desses trabalhos prévios caracterizou a doença articular em camundongos MPS I, demonstrando que, a partir de 6 meses de idade, alterações como acúmulo de GAGs e proliferação fibrocartilaginosa são detectadas e são progressivas(de Oliveira et al. 2013) .No entanto, ainda não havíamos descrito as alterações ósseas deste modelo, e os relatos na literatura também são escassos. Conhecer a história natural da MPS I do modelo de camundongo MPS I oferece uma oportunidade para estudar a fisiopatologia deste distúrbio e determinar a eficácia de novas terapias (Baldo et al. 2012).

Adicionalmente, nosso grupo de pesquisa também vem explorando novas terapias, com foco na correção do defeito genético por meio de abordagens de terapias gênicas e, mais recentemente, de edição gênica com o sistema CRISPR/Cas9, ambas baseadas em vetores não-virais. Além disso, também focamos em tratamentos complementares baseados em alvos secundários que possam participar de eventos patológicos na MPS I.

Com base nestas considerações, a proposta da presente tese, parte de trabalhos prévios desenvolvidos pelo nosso grupo de pesquisa e teve o objetivo de elucidar as alterações presentes no sistema ósseo do modelo murino da MPS I e desenvolver um protocolo de edição gênica como terapia *in situ*, buscando corrigir as alterações encontradas na articulação.

No capítulo 1 realizamos uma revisão de literatura buscando protocolos que utilizaram a terapia gênica *in situ*, como abordagem para distintas situações patológicas. No capítulo 2 realizamos uma revisão de literatura para discutirmos os principais estudos envolvendo edição de genes para MPS e as aplicações prospectivas para outro grupo de doenças lisossômicas, as Mucolipidoses (ML). A revisão destes dados permitiu que o estudo de terapia gênica proposto fosse desenhado de forma otimizada, com abordagem *in situ* sendo testada.

No capítulo 3, caracterizamos as diferenças morfológicas e biomecânicas entre tecidos ósseos wild type (normais) e MPS I. Além disso, avaliamos os efeitos dos tratamentos previamente realizados no nosso grupo, como a edição de genes com o sistema CRISPR/Cas9 e tratamento farmacológico com Ca074Me (um inibidor da catepsina B), losartana (inibidor da via do TGF-beta) ou propranolol (bloqueador β-adrenérgico não seletivo da noradrenalina) nas características morfológicas e biomecânicas do tecido ósseo. Todas as análises foram realizadas com animais de 6 meses, que é quando as demais alterações da doença já estão estabelecidas.

Com relação à caracterização das diferenças morfológicas, existem dados na literatura que mostram que pacientes com MPS I comumente apresentam defeitos na formação e crescimento de ossos longos e ossos e cartilagens faciais achatadas. Essa constelação de anormalidades radiográficas, é denominada disostose múltipla e é uma característica prevalente nos grupos de MPS (Borgo et al. 2018). Assim como os pacientes, o modelo animal murino também apresenta essas características (Clarke et al. 1997; Schuh et al. 2020).

De acordo com a literatura, observamos alterações radiográficas nos tecidos ósseos dos camundongos MPS I, principalmente no fêmur e nos ossos zigomáticos. O osso zigomático dos camundongos MPS I é mais largo que os ossos zigomáticos normais assim como demonstrado por (Clarke et al. 1997; Schuh et al. 2020). Além disso, os fêmures dos camundongos MPS I eram mais largos quando comparados aos camundongos normais (Clarke et al. 1997).

Com relação à caracterização das diferenças biomecânicas, estudando a literatura, nós encontramos que o teste de flexão de três pontos é um ensaio bastante utilizado para caracterizar as propriedades biomecânicas dos ossos longos do esqueleto apendicular(Leppänen et al. 2006). No entanto, estudos sobre as propriedades biomecânicas dos ossos na MPS I utilizando o teste de flexão de três pontos são escassos. Esse estudo foi realizado apenas por um grupo, previamente ao presente trabalho (Ferreira et al. 2021), sendo uma importante fonte de informação para a realização do nosso estudo. Nós nos baseamos em alguns parâmetros descritos nesse trabalho para interpretar nossos dados. Segundo o trabalho de Ferreira et al. 2021 as propriedades biomecânicas são obtidas a partir da combinação das seguintes variáveis: máxima tensão, máxima força, elasticidade, tenacidade e resiliência. Juntas, essas variáveis fornecem informações sobre rigidez, força e a qualidade da matriz óssea. Individualmente, referem-se a aspectos específicos do tecido ósseo. Máxima tensão refere-se a máxima tensão suportada antes que ocorra fratura. Força máxima refere-se a carga necessária para causar fratura. A elasticidade é a capacidade do material de resistir à deformação e retornar a sua forma original quando a força exercida é interrompida. A tenacidade é o maior estresse suportado sem que haja fratura. A resiliência é a maior quantidade de energia absorvida até o limite elástico sem que haja deformação permanente.

Utilizando o mesmo modelo murino utilizado por nosso grupo, foi previamente demonstrado que há alterações em animais com 3 meses de idade, que tornam os ossos desses animais menos flexíveis e, portanto, mais suscetíveis a fraturas. Já aos 6 meses de idade, demonstraram alterações que concederam aos ossos desses animais maior resistência a forças exercidas, indicando que eles se tornam mais resistentes com o aumento da idade (Ferreira et al. 2021). Nós buscamos estudar os animais com 6 meses de idade, pois é nessa idade que a doença está estabelecida e quando os tratamentos são avaliados, no entanto,

reconhecemos que é importante para nosso grupo de pesquisa realizar estudo de história natural e avaliação dos tratamentos futuramente.

Nossa análise biomecânica demonstrou que os fêmures dos camundongos MPS I apresentaram maior carga até a falha, comparado aos fêmures dos camundongos normais, conforme relatado anteriormente por Ferreira et al. 2021. Esse parâmetro refere-se à carga necessária para causar uma fratura. Nossa análise biomecânica também demonstrou que os fêmures dos camundongos normais apresentaram maior resistência à flexão do que os fêmures dos camundongos MPS I. Diferente do que foi demonstrado anteriormente por Ferreira et al. 2021. Neste trabalho prévio, o número de amostras era menor que o do nosso estudo, as amostras de fêmures mostraram uma grande variação nesse parâmetro e nenhuma diferença estatística foi observada. A resistência à flexão refere-se à tensão máxima suportada pela amostra, antes das fraturas ósseas. Esses resultados sugerem que a infiltração de GAGs neste tecido, além de outras possíveis alterações, como diferenças na composição óssea, alterações em componentes da matriz extracelular, incluindo colágeno, elastina e cálcio, podem estar alteradas na MPS I e ser responsáveis pelas alterações nas propriedades mecânicas. O estudo da composição óssea na MPS I era parte dos objetivos iniciais desta tese, mas, devido à pandemia, as análises ainda não puderam ser realizadas, devendo ser investigadas em breve.

Com relação à avaliação dos efeitos dos tratamentos previamente realizados no nosso grupo, como a edição de genes com o sistema CRISPR/Cas9 e tratamento farmacológico com Ca074Me (um inibidor da catepsina B), losartana (inibidor da via do TGF-beta) ou propranolol (bloqueador β-adrenérgico não seletivo da noradrenalina) nas características morfológicas e biomecânicas do tecido ósseo, é importante ressaltar que o princípio dos tratamentos testados varia bastante: o sistema CRISPR/Cas9 cliva o DNA da célula em um ponto específico e quando uma sequência homóloga é fornecida junto com o sistema (denominada “sequência doadora”), poderá ocorrer a recombinação homóloga e a sequência doadora é inserida no genoma celular. Neste caso, num trabalho prévio inserimos uma cópia do gene *IDUA* por administração intravenosa de plasmídeos, utilizando lipossomos como vetor. Este tratamento elevou os níveis de IDUA sérica a valores cerca de 5-6% dos valores encontrados em animal *wild type*, sendo o suficiente para que fossem obtidas melhoras em múltiplos aspectos da doença, como a doença cardíaca e pulmonar (Schuh et al. 2018b). No

presente trabalho, os ossos deste estudo que haviam sido coletados foram analisados, sendo que a análise de raios-X mostrou que os ossos zigomáticos tinham larguras reduzidas em comparação com camundongos MPS I não tratados, embora não estatisticamente significativos. A largura do osso longo foi estatisticamente menor, em comparação com os camundongos MPS I não tratados, mas não foi normalizada. Embora os resultados de melhora sejam apenas discretos, é importante ressaltar que os tratamentos atuais, como a TRE, não melhoram os aspectos ósseos da doença(Bidone et al. 2018; Schuh et al. 2020) de forma que uma melhora, mesmo que discreta, é algo já considerado promissor, aos nossos olhos.

Já a catepsina B é uma protease lisossomal que participa dos processos de remodelação controlada dos compostos da matriz extracelular (MEC), e está aumentada em múltiplos tecidos na MPS I(Gonzalez et al. 2018). Nossa análise de raios-X nesse grupo, mostrou uma pequena redução estatisticamente significativa na largura zigomática em comparação com camundongos MPS I não tratados, o que pode sugerir que o bloqueio de certas enzimas capazes de degradar substâncias como o colágeno podem também evitar ou ao menos retardar o aparecimento de certas manifestações ósseas na MPS. O mecanismo pelo qual a catepsina B poderia estar agindo ainda é incerto, mas estudos demonstram que esta protease é essencial para ativação do inflamassomo em macrófagos (Chevriaux et al. 2020), que está relacionado a diversas doenças osteoarticulares com achados semelhantes à MPS, como a artrite reumatoide (Tong et al. 2014). Uma vez que recentemente nosso grupo demonstrou ativação da mesma via no cérebro de animais com MPS II (Azambuja et al. 2020) é possível que a inibição da catepsina B possa inibir o inflamassomo e gerar benefício terapêutico. No entanto, este não deve ser o único mecanismo, pois nenhuma outra diferença morfológica foi encontrada em camundongos tratados com inibidor de catepsina B.

A losartana é um fármaco bloqueador do receptor de angiotensina (BRA) e do TGF-beta. O sistema renina angiotensina (RAS) regula pressão sistêmica e equilíbrio de fluidos do corpo e o uso deste composto em estudo prévio mostrou melhoras em aspectos cardiovasculares da doença, bem como em alterações craniofaciais (Osborn et al. 2017). Uma vez que melhoras parciais foram observadas após o tratamento com losartana, nossos resultados estão de acordo com o trabalho acima citado e sugerem que a via do TGF-beta também possa ser um alvo terapêutico na MPS. No entanto, uma vez mais cabe ressaltar que

nem todas as alterações foram prevenidas, o que dá indícios que múltiplas vias estão alteradas e contribuem de forma somatória para a progressão da doença óssea.

Já o propranolol é um agente bloqueador β-adrenérgico não seletivo da noradrenalina (Al-Majed et al. 2017; Gonzalez et al. 2017). Ao se estudar os benefícios da losartana na dilatação da aorta, também se utilizou um segundo anti-hipertensivo como controle, que não agisse na mesma via. Desta forma, por ter o material dos animais disponíveis, resolvemos verificar se existia algum benefício do uso deste fármaco na doença óssea. Além disso, vários estudos com animais mostram que o propranolol modula negativamente a reabsorção óssea ao inibir a inflamação e a diferenciação de osteoclastos, aumenta a massa óssea e previne a osteoporose (Sato et al. 2010; Rodrigues et al. 2012; Zhang et al. 2013). Nossa análise de raios-X nesse grupo, mostrou que os ossos zigomáticos e osso longo femoral tinham larguras um pouco reduzidas em comparação com camundongos MPS I não tratados, embora não estatisticamente significativos, de modo que este tratamento parece ser ineficaz neste aspecto.

Infelizmente, nossa análise morfológica e biomecânica demonstrou que nenhum dos tratamentos testados foi capaz de normalizar completamente as alterações ósseas. Em conjunto, esses resultados evidenciam a dificuldade em corrigir completamente tal aspecto da mucopolissacaridose. No entanto, cabe ressaltar que, com exceção da edição de genes, os demais tratamentos foram realizados em animais jovens adultos, quando alguns aspectos da doença óssea já estão estabelecidos. Possivelmente, a modulação de vias em um período anterior (desde o nascimento) poderia prevenir o aparecimento de sintomas ósseos ou atenuá-los e deverá ser testado no futuro próximo.

No capítulo 4 realizamos um protocolo de edição de genes *in vitro* (em sinoviócitos semelhantes a fibroblastos-FLS MPS I cultivados) e *in vivo* (no modelo animal), através da injeção *in situ* do sistema CRISPR/Cas9 na articulação do joelho.

Na busca por novas abordagens terapêuticas que melhorem os problemas osteoarticulares, a terapia gênica *in situ* aparece como uma metodologia potencialmente mais eficaz e segura. Recentemente, outra estratégia para a terapia gênica tem sido amplamente testada, a edição de genoma, principalmente pelo sistema CRISPR-Cas9. Essa ferramenta também pode ser aplicada em áreas pequenas e compartimentadas, visando tratar células ou tecidos específicos como a articulação(Santos et al. 2021a).

Com a utilização do sistema CRISPR/Cas9, o genoma das células é editado, em uma região específica, para produzir permanentemente um fator terapêutico através da correção ou indução de mutações pontuais ou inserção/exclusão de sequências de interesse (Santos et al. 2021a). A entrega eficiente do sistema nas células é um processo crítico. Superar barreiras extracelulares e intracelulares para alcançar o local de ação ainda é um grande desafio. Diferentes estratégias de entrega a partir de vetores virais e não virais têm sido aplicadas para entrega desse sistema. Os vetores virais, embora muito eficientes, podem induzir um importante resposta imune, portanto, devem ser usados com cautela. Uma alternativa promissora aos equivalentes de entrega de genes baseados em vírus são os vetores não virais. O aumento do conhecimento adquirido nos últimos anos, nas áreas de biologia molecular e nanotecnologia, contribuiu para o desenvolvimento de diferentes tipos de sistemas de vetores não virais (Sainz-Ramos et al. 2021).

O desenvolvimento de vetores não virais ganhou atenção da comunidade científica e, atualmente, a entrega de genes mediada por esses sistemas é considerada um dos alicerces da terapia gênica moderna devido a vantagens relevantes como baixa toxicidade, baixa imunogenicidade e alta capacidade de empacotamento(Sainz-Ramos et al. 2021). Como mencionado anteriormente, nosso grupo de pesquisa tem focado no desenvolvimento terapias gênicas alternativas baseadas em vetores não-virais para o tratamento da MPS I.

O trabalho realizado por Bidone et al. 2018 visando à redução ou prevenção das complicações ortopédicas características da MPS I, utilizou terapia gênica intra-articular em camundongos MPS I. Esse método demonstrou maior expressão gênica e atividade da IDUA na articulação e líquido sinovial, porém, essa atividade não foi duradoura, pois o plasmídeo utilizado continha apenas o gene terapêutico, sem ser capaz de editar o genoma. Mais recentemente, nosso grupo tem utilizado o sistema CRISPR-Cas9 como uma nova estratégia para o tratamento de doenças lisossômicas (de Carvalho et al. 2018; Schuh et al. 2018a; Schuh et al. 2018b; Schuh et al. 2020; Vera et al. 2022). O trabalho desenvolvido por Schuh et al. 2018b realizou uma injeção hidrodinâmica, contendo lipossomas catiônicos carregando o plasmídeo CRISPR/Cas9 e um vetor doador para edição do gene MPS I, em camundongos neonatos, levando a um aumento nos níveis séricos de IDUA por até seis meses.

Esses trabalhos anteriores foram muito importantes como aporte de informações para darmos início ao protocolo testado aqui. Iniciamos isolando e cultivando os sinoviócitos

semelhantes a fibroblastos (FLS) das articulações de camundongos MPS I e normais. Os FLS são importantes na manutenção da homeostase articular, pois representam a principal fonte de ácido hialurônico e outras glicoproteínas, principais componentes do líquido sinovial(Stebulis et al. 2005; Bartok and Firestein 2010). Essas células desempenham um papel crucial na patogênese da MPS I e juntamente com os condrócitos da articulação do joelho, são as células alvo para edição gênica *in situ* intra-articular.

Após o estabelecimento da cultura nós iniciamos os ensaios *in vitro*. Fizemos a avaliação da citotoxicidade dos complexos e assim como demostrado anteriormente por Bidone et al. 2018 e Schuh et al. 2018b obtivemos baixa citotoxicidade, sendo esse resultado altamente desejável para estudos de transferência gênica.

Investigamos a eficiência da edição gênica com lipossomas catiônicos que transportam plasmídeos que codificam o sistema CRISPR\Cas9 mais um vetor doador para inserir o gene *IDUA* no lócus *ROSA26* dos FLS MPS I (Figura 6). Com o objetivo de apenas realizar a prova de conceito de que os complexos conseguiriam realizar a correção gênica e que a produção da enzima seria duradoura, nós medimos a atividade enzimática de IDUA 3, 7, 15 e 30 dias de cultivo após a transfecção. Para comparação, utilizamos a Lipofectamina 3000^{TM}, que é um reagente comercial utilizado amplamente para transfecção. Esse reagente contém subunidades de lipídeos, que podem formar lipossomas em ambientes aquosos, semelhante ao vetor produzido pelo nosso grupo (THERMO FISHER SCIENTIFIC, 2022).

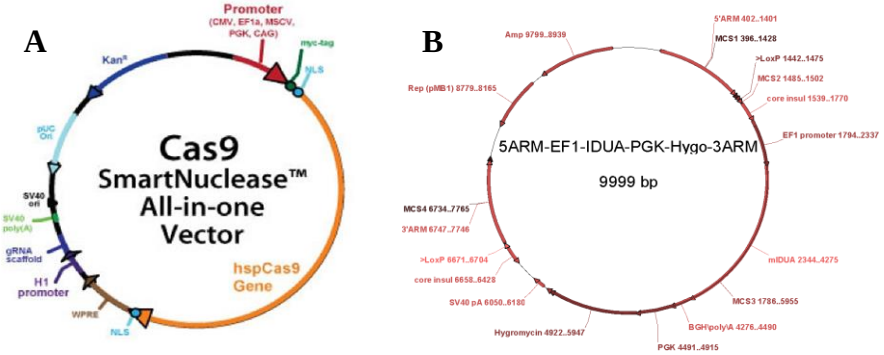

Figura 7: Plasmídeos utilizados para edição gênica. **(A)**Vetor CRISPR-Cas9. **(B)**Vetor doador da IDUA. O vetor A produz a Cas9, que cliva o DNA no locus ROSA26, guiado pelo gRNA. O Vetor B promove reombinação homóloga nesta região, inserindo uma cópia do gene IDUA neste locus.

A transfecção dos FLS MPS I foi realizada com: lipossoma + CRISPR/Cas 9 + doador IDUA (LP+C+D) ou lipossoma + doador IDUA (LP+D) ou lipofectamina+ CRISPR/Cas9 +doador IDUA (LF+D +C) ou lipofectamina+doador IDUA (LF+D). O tratamento com a LF+C+D ou com LP+C+D levou a um aumento na atividade da IDUA em todos os tempos avaliados, sendo que os tratamentos não foram significativamente diferentes entre si. Como esperando, os tratamentos apenas com os reagentes de transfecção e o plasmídeo doador (sem o vetor com o gene Cas9 e o gRNA) mostrou atividade de IDUA aumentada apenas em 3 dias. A atividade enzimática IDUA aumentada nesse tempo, é provavelmente resultante da transcrição do plasmídeo doador, sem, no entanto, se manter estável, pois não há sua inserção no genoma (Sharma et al. 2015; Schuh et al. 2018a; Ou et al. 2019).

A premissa da edição gênica para MPS I é permitir que as células doentes produzam a enzima IDUA, que podem ser exportadas para o fluido extracelular e captadas pelas células vizinhas através de receptores de manose-6-fosfato (M6P) (um mecanismo chamado correção cruzada (Giugliani 2012; Vera and Baldo 2020). Devido a essas modificações pós-traducionais, a edição gênica diretamente a um pequeno número de células poderia fornecer enzima suficiente para melhorar as condições pretendidas da MPS I.

A edição gênica plasmidial só ocorre quando os plasmídeos atravessam as membranas celular e nuclear, entram no núcleo onde as sequências de DNA da Cas9 e o RNA guia são transcritas e finalmente o complexo gerado pela enzima Cas9 e o RNA guia induz a quebra da fita dupla de DNA no local alvo. Por esse motivo, esse tipo de sistema tende a ser menos eficiente quando comparado com outros formatos utilizados do sistema CRISPR/Cas9 como o mRNA ou RNP, pois nele a eficiência da edição é menor e a eficácia terapêutica é mais lenta (Sainz-Ramos et al. 2021).

Tendo em vista essas informações, podemos completar que na edição de genes *in vitro*, nem todas as células presentes no poço são editadas, além disso, as que são editadas podem morrer por terem recebido maior quantidade de lipossoma, resultando em uma baixa eficiência de edição. Nesse cenário, as células editadas conseguem produzir a enzima e por correção cruzada fornecê-las para as células não editadas. Isso explica o aumento da atividade enzimática que foi observado nos nossos experimentos bioquímicos, mas, provavelmente, com o passar do tempo o número de células não editadas supera o número

de células editadas e a atividade enzimática vai diminuindo. Isso poderia explicar a aparente atividade enzimática mais baixa com 30 dias.

Adicionalmente, o plasmídeo e o lipossoma utilizados no estudo não possuía fluorescência, então não fizemos a seleção das células editadas. Com a seleção das células editadas por citometria de fluxo poderíamos avaliar melhor o efeito do aumento da atividade enzimática de IDUA na morfologia dos lisossomos. Nós consideramos fazer a seleção das células editadas por antibiótico, pois nosso plasmídeo possui um gene de resistência à canamicina para que as células possam ser selecionadas, porém não tínhamos previsto esses experimentos inicialmente e não tivemos tempo de repeti-los.

Após observamos que a atividade enzimática de IDUA foi aumentada e mantida em todos os tempos avaliados nós começamos a realizar os experimentos *in vivo*. Nós realizamos um estudo piloto, onde foi administrada uma injeção intra-articular do complexo lipossoma + CRISPR/Cas 9 + doador IDUA (LP+C+D) ou solução salina tamponada com fosfato (PBS) nos joelhos de camundongos com 2 meses de idade. Após 7 dias, nós realizamos a eutanásia para realização das análises. Nessa etapa nós queríamos avaliar a eficiência da edição gênica com lipossomas catiônicos que transportam plasmídeos que codificam o sistema CRISPR\Cas9 mais um vetor doador para corrigir o gene *IDUA* dos camundongos MPS I. Como objetivo de realizar a prova de conceito de que os complexos conseguiriam realizar a correção gênica *in vivo*, nós medimos a atividade enzimática de IDUA nas articulações e no líquido sinovial dos camundongos tratados. Além disso, também fizemos essas medições no soro, fígado e rim como prova de conceito que a correção gênica ocorreu apenas *in situ*. Nós também fizemos a avaliação dos níveis de GAGs nas articulações, porém os animais deste estudo eram jovens e os níveis de GAG ainda não se mostraram aumentados neste momento.

Nosso estudo piloto revelou que a injeção intra-articular de LP+C+D em animais MPS I aumentou a atividade de IDUA na articulação e no líquido sinovial sem aumento no soro, fígado e rim, conforme observado anteriormente por Bidone et al. 2018. Esses resultados sugerem que a administração do sistema CRISPR/Cas9 nas articulações promove a edição gênica apenas *in situ*, sem a liberação do vetor para soro ou outros órgãos em níveis significativos. Isso é particularmente importante por razões de segurança e abre a possibilidade de usar o mesmo vetor para tratar também outras condições articulares.

Após resultados extremamente satisfatórios, iniciamos um novo tratamento para avaliar os efeitos do tratamento a longo prazo. Nessa etapa os camundongos receberam 3 administrações do produto de edição gênica contendo o complexo LP+C+D ou complexo lipossoma + doador *IDUA* LP+D e comparamos com camundongos não tratados. As injeções foram realizadas uma vez por mês, de 2 a 4 meses de vida, e os animais foram eutanasiados aos 8 meses para realização das análises. Vale ressaltar que a possibilidade de múltiplas administrações é possível apenas utilizando vetores não virais, uma vez que vetores virais geram uma resposta imune pelo organismo receptor (Vera and Baldo 2020). Nós então medimos a atividade enzimática, avaliamos os níveis de GAGs nas articulações, líquido sinovial, soro, fígado e rins. Além disso, medimos a eficiência de edição de genes na articulação.

Nosso estudo a longo prazo revelou que os animais tratados com LP+C+D apresentaram atividade de IDUA aumentada na articulação, enquanto os animais tratados com LP+D e os animais não tratados apresentaram níveis igualmente baixos de atividade de IDUA, como demonstrado anteriormente por Sharma et al. 2015. Assim como no estudo *in vitro*, esse resultado era esperado, pois os animais tratados com LP+D, não receberam o plasmídeo com a Cas9 e o gRNA. A Cas9 é o componente do sistema responsável pelo corte na fita dupla de DNA e o gRNA é o componente responsável por guiar a Cas9 para o sítio alvo para inserção da sequência doadora *IDUA* no genoma. Isso demonstra, mais uma vez, a importância de todo os componentes do sistema para o tratamento funcionar(Schuh et al. 2018a).

Dependendo de qual via de degradação de GAGs é afetada por cada tipo de MPS, diferentes órgãos e tecidos são mais afetados. Como a IDUA é responsável pela hidrólise dos resíduos dos GAGs dermatan sulfato (DS) e heparan sulfato (HS) na MPS I, esses são os GAGs acumulados (Santos et al. 2021a). Na MPS I, as manifestações clínicas osteoarticulares estão associadas principalmente ao acúmulo do DS enquanto a patologia do sistema nervoso central está associada ao acúmulo de HS (Opoka-Winiarska et al. 2013). Avaliamos se a atividade da IDUA foi suficiente para diminuir os níveis específicos de GAGs na articulação, líquido sinovial, soro, fígado e rins. Nossos dados mostraram que as injeções reduziram os níveis de GAGs apenas nas articulações e líquido sinovial, mas não foram suficientes para normalizá-los completamente. Estudos anteriores mostraram que TRE e terapia gênica às articulações de animais com MPS, visando o tratamento de

problemas articulares, resulta em atividade de IDUA sinovial e articular rápida e redução dos níveis de GAGs sem efeitos em órgãos não-alvo. No entanto, a repetida administração da TRE na articulação acaba sendo incômoda e pouco aplicável aos pacientes, algo que pode ser evitado pela terapia gênica(Auclair et al. 2006; Wang et al. 2014; Raymond Y. et al 2019).

A biodistribuição de agentes terapêuticos no espaço intra-articular é uma questão relevante para a tradução clínica. Embora as articulações sejam espaços isolados, existe a preocupação com vazamento desses agentes terapêuticos e efeitos adversos em órgãos não-alvo. Uma literatura substancial tem demonstrado um perfil de segurança favorável, indicando que a transfecção ou transdução de vetores ocorre majoritariamente *in situ*, sem efeito sistêmico(Santos et al. 2021a). Aqui nós mostramos importantes informações sobre a segurança e viabilidade de tal tratamento para pacientes com MPS, que pode ser extrapolado para outras doenças articulares.

A atividade de IDUA no líquido sinovial foi supreendentemente alta. É sabido que o acúmulo de GAGs nas células sinoviais causa aumento dos níveis de citocinas pró-inflamatórias, levando as células sinoviais MPS a se proliferarem a uma taxa mais rápida do que as células sinoviais normais (Hiperplasia) (Opoka-Winiarska et al. 2013). Nós observamos, em nossos experimentos *in vitro*, na curva de crescimento, a hiperproliferação dos FLS MPS I em comparação com células normais. Adicionalmente, o sistema está desenhado para gerar um reparo por homologia direta. Sendo assim, para que a edição gênica ocorra, é preciso induzir a quebra da fita dupla no local alvo, ou seja, é necessário que a célula esteja em divisão. Nesse cenário, pode ter ocorrido uma elevada taxa de edição no líquido sinovial dos animais tratados. Essa surpreendente atividade enzimática levantou algumas dúvidas com relação à edição gênica e sobre a inflamação sinovial que ocorre na MPS I. Porém, a quantidade de líquido sinovial que conseguimos coletar de um camundongo é muito pequena e não foi suficiente para fazer análises adicionais, sendo necessário novos animais para esse fim.

Com relação à eficiência da edição de genes na articulação do joelho, os dados de qPCR mostraram cópias de higromicina nas articulações dos camundongos tratados, esses resultados indicam que a edição realmente aconteceu (Joshi et al. 2008). A partir desses dados, pudemos concluir que houve baixa porcentagem de correção. Infelizmente, os

condrócitos têm um potencial limitado de replicação (Tanikella et al. 2020), um fator limitante para a edição gênica. Adicionalmente, foi reportado previamente que a administração intra-articular de TRE nas articulações do modelo felino da MPS VI resultou na redução no material de GAGs nos tecidos articulares superficiais, pois a enzima não foi capaz de penetrar na cartilagem até o nível mais profundo adjacente ao osso subcondral, o que pode ter acontecido no presente trabalho, limitando o número de células atingidas pelo tratamento (Auclair et al. 2006).

Considerando nossos resultados, juntamente com essas informações, acreditamos que a edição tenha acontecido apenas nos condrócitos localizados mais próximos da superfície articular e que as ferramentas de edição gênica, ou até mesmo as enzimas, produzidas pelas células editadas, não conseguiram penetrar na cartilagem até o nível mais profundo adjacente ao osso subcondral. Serão necessários estudos adicionais, como análises histológicas, estudos sobre difusão molecular através da matriz extracelular da cartilagem para que possamos desenhar uma nova estratégia para aumentar a eficiência da edição nas células mais profundas do tecido cartilaginoso. Adicionalmente, esse resultado pode ter acontecido devido ao sistema plasmidial que foi utilizado. Como mencionado anteriormente, esse sistema possui eficiência de edição menor e eficácia terapêutica mais lenta devido a necessidade de transcrição e tradução das sequências de DNA da Cas9 e do RNA guia e todas as dificuldades inerentes a esse processo. Sendo assim, talvez seja interessante a utilização de outros formatos do sistema CRISPR/Cas9 como o mRNA ou RNP (Liang et al. 2015; Gibson and Yang 2017). Ainda, estudos de clivagens em sítios off-target estão em andamento.

Com base nesses resultados, temos em vista a realização de novos experimentos. Pois temos algumas dúvidas a sanar. Nós gostaríamos de avaliar a edição gênica a partir de um experimento onde a terapia é feita *in vivo*, seguida da coleta de fibroblastos dos animais tratados e seu cultivo *in vitro*, para verificar se os FLS coletados dos animais teriam grande proporção de células editadas, pois são células altamente proliferativas.

Ainda são poucos os estudos no campo da edição gênica com uso da ferramenta CRISPR/Cas9 intra-articular como abordagem de tratamento para os problemas articulares da MPS. Evidentemente, a edição gênica *in situ* não é aplicável para tratar todos os sintomas de distúrbios multissistêmicos como a MPS, sozinha, embora tenha mérito para ser usada

como complemento em tecidos de difícil tratamento, onde a edição não pode atuar de forma eficiente, como articulações, ossos e cérebro. Além do mais, pode ser combinada com outras abordagens, como a terapia ou edição gênica sistêmica, TRE intravenosa ou TCTH. Como a cartilagem é muito difícil de reparar uma vez danificada, a edição gênica intra-articular precisa ser idealmente iniciada bem antes da instalação das condições articulares da MPS. Nós apresentamos uma abordagem terapêutica complementar direcionada aos tecidos articulares, que poderá ser utilizada para tratar os problemas articulares da MPS, bem como, outras doenças articulares.

Ao longo da realização dos trabalhos presentes nessa tese, nós enfrentamos algumas dificuldades. O trabalho referente ao capítulo 3 é realizado em parceria com o professor Vicente Leitune, da Faculdade de Odontologia da UFRGS, onde algumas análises são realizadas. Em razão da pandemia, o acesso à faculdade foi limitado, atrasando na execução do planejamento inicial do trabalho. Por esse motivo, esse trabalho ainda não foi finalizado como planejado. Para finalizá-lo, precisamos realizar medida das características morfológicas e biomecânicas do grupo Ca074Me e finalizar análises de alguns parâmetros das propriedades biomecânicas dos ossos. Para finalizarmos as análises das propriedades biomecânicas dos ossos, ainda precisamos das análises de elasticidade, tenacidade e resiliência, que serão realizadas tão logo, ocorra a liberação de acesso.

Quanto ao trabalho referente ao capítulo 4, durante a realização dos estudos *in vivo*, nós enfrentamos algumas dificuldades. Inicialmente, nenhum membro da equipe possuía experiência na coleta de líquido sinovial, bem como na administração das injeções intra-articulares. Adicionalmente, a colônia de camundongos enfrentou problemas com a genotipagem dos animais. Por esse motivo, por um bom tempo não tivemos animais MPS I. Além disso, em razão da pandemia, o acesso à Unidade de Experimentação Animal e ao Centro de Pesquisa Experimental do HCPA foi limitado, atrasando a execução do planejamento inicial da pesquisa, só tendo sido normalizado em fevereiro de 2021. Apesar dessas dificuldades, conseguimos finalizar o tratamento longo e obtivemos resultados promissores. Nós também gostaríamos de realizar a avaliação dos níveis de GAGs após a edição gênica *in vitro*, para saber se a atividade enzimática IDUA foi suficiente para reduzir ou normalizar os níveis de GAGs. Porém, no final de 2021, foi iniciada uma reforma em nosso laboratório, esperada por nós desde o início de 2020. Isso impossibilitou que essas análises fossem realizadas a tempo de termos os resultados para apresentar na presente tese.

Ainda assim, foi possível realizar o projeto e atingir conclusões importantes acerca do tema da tese.

Essa tese apresentou informações importantes sobre algumas alterações presentes no sistema osteoarticular do modelo animal de MPS I e desenvolveu um protocolo de edição gênica *in situ* (intra-articular) e servirá como base para o melhoramento e desenvolvimento de novos experimentos necessários para que essa terapia alcance a tradução clínica, uma contribuição importante para nosso grupo pesquisa.

6. CONCLUSÕES

Neste trabalho, pudemos constatar que existem parâmetros que podem ser mensurados para avaliar alterações ósseas na MPS I e que podem ser usados para testar futuras abordagens terapêuticas. Além disso, a edição gênica intra-articular, utilizando vetores não virais para a entrega do sistema CRISPR-Cas9, pode ser utilizada como alternativa para tratar os problemas articulares da MPS I.

Portanto, concluímos que:

- As características morfológicas e biomecânicas ósseas são diferentes entre os camundongos selvagens (normal) e MPS I, aos 6 meses de idade.
- A edição gênica com o sistema CRISPR/Cas9 intravenoso e os tratamentos farmacológicos com inibidor de catepsina B, losartana ou propranolol não foram capazes de normalizar completamente as alterações ósseas presentes no modelo animal de MPS I.
- Os lipossomas catiônicos carregando plasmídeos com o sistema CRISPR-Cas9 aplicado por via intra-articular levaram a um aumento localizado na atividade da IDUA e redução no acúmulo de GAGs neste tecido.

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8. ANEXOS

Produção cientifica relacionada: Neste item constam 2 artigos publicados durante o período do doutorado na área de estudo**.**

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ORIGINAL RESEARCH
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Progression of Cardiovascular Manifestations in Adults and Children With Mucopolysaccharidoses With and Without Enzyme Replacement Therapy

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Background: Cardiovascular involvement is among the main features of MPS disorders and it is also a significant cause of morbidity and mortality. The range of manifestations includes cardiac valve disease, conduction abnormalities, left ventricular hypertrophy, and coronary artery disease. Here, we assessed the cardiovascular manifestations in a cohort of children and adults with MPS I, II, IV, and VI, as well as the impact of enzyme replacement therapy (ERT) on those manifestations.

Methods: We performed a chart review of 53 children and 23 adults with different types of MPS that had performed echocardiograms from January 2000 until October 2018. Standardized Z scores were obtained for heart chamber sizes according to the body surface area. When available, echocardiographic measurements that were performed before ERT and at least 18 months after that date were used for the assessment of pre- and post-treatment parameters.

Results: Left side valvular disease was a frequent finding, with mitral and aortic thickening being reported in most patients in all four MPS types. Left atrium dilatation was present in 26% of the patients; 25% had increased relative wall thickness; 28% had pulmonary hypertension. The cardiovascular involvement was, in general, more prevalent and more severe in adults than in children, including conduction disorders (40 vs. 16%), mitral stenosis (26 vs. 6%), aortic stenosis (13 vs. 4%), and systolic dysfunction (observed in only one adult patient). ERT promoted a significant reduction of the left ventricular hypertrophy parameters, but failed to improve valve abnormalities, pulmonary hypertension, and left atrial dilatation.

Conclusions: Adult patients with MPS may develop severe cardiovascular involvement, not commonly observed in children, and clinicians should be aware of the need for careful monitoring and timely management of those potentially life-threatening complications. Our results also confirm the impact of long-term ERT on left ventricular hypertrophy and its limitations in reversing other prevalent cardiovascular manifestations.

Keywords: mucopolysaccharidoses, enzyme replacement therapy, pulmonary hypertension, left ventricular hypertrophy, left atrium, heart valve disease

INTRODUCTION

The mucopolysaccharidoses (MPS) are a group of eleven disorders characterized by impaired catabolism of glycosaminoglycans (GAGs) as a consequence of a deficiency of lysosomal enzymes directly involved in their degradation, resulting in the accumulation of one or more of five different types of GAGs: heparan sulfate, dermatan sulfate, keratan sulfate, chondroitin sulfate, and hyaluronan (1). According to the accumulated substrate and clinical features, the MPS disorders are classified into seven main types (I, II, III, IV, VI, VII, and IX), with MPS III and MPS IV being further subclassified in four (IIIA, IIIB, IIIC, and IIID) and two (IVA and IVB) subtypes, respectively, according to the enzymatic defect (2).

Cardiovascular involvement is among the main features of MPS disorders and it is also a significant cause of morbidity and mortality. As GAGs are a significant normal component of cardiac structures, enzymatic deficiencies related to GAG degradation result in prominent storage of undegraded GAGs in heart structures, which may cause tissue damage through the activation of cell proteases (3, 4). This results in many different manifestations, including cardiac valve disease, conduction abnormalities, hypertrophy of the left ventricle, and coronary artery disease (3).

Replacing the deficient enzyme, either through Enzyme Replacement Therapy (ERT) or Hematopoietic Stem Cell Transplantation (HSCT), is the current paradigm of targeting the primary defect in MPS. HSCT is widely used in the severe form—Hurler phenotype—of MPS I and ERT is available for the treatment of MPS types I, II, IVA, VI, and VII (5). Nevertheless, real-world experience with those disease-modifying therapies has unveiled several limitations, especially regarding their ability to reverse or even stop the progression of some cardiovascular manifestations in MPS patients (5, 6). In recent years, as the management of patients of MPS is resulting in an increased life expectancy, those progressing complications are a growing concern for healthcare providers (7).

In this study, we aimed to assess the cardiovascular manifestations in children and adults with different MPS types, as well as the impact of enzyme replacement therapy on those manifestations.

METHODS

Data Collection

Following institutional ethical approval (17-0013; Grupo de Pesquisa e Pós Graduação, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil), we performed a chart review of patients

TABLE 1 | Characteristics of the subjects according to MPS type.

	Overall ($n = 76$)	MPS I ($n = 27$)	MPS II ($n = 22$)	MPS IVA ($n = 19$)	MPS VI ($n = 8$)	*p*-value
Sex (*n*)	M: 46 F: 30	M: 12 F: 15	M: 22 F: 0	M: 8 F: 11	M: 4 F: 4	<0.001[†]
Age (years)	11.3 (12.7)	10.2 (20.4)	11.8 (15.5)	10.1 (8.5)	16.2 (8.3)	0.491
Number of adults (*n*)	23/76 (30%)	8/27 (29%)	5/22 (23%)	7/19 (37%)	3/8 (38%)	0.757
Weight (kg)	25.6 (21.0)	23.5 (23.4)	28.0 (36.5)	21.0 (16.6)	22.3 (14.9)	0.103
Height (cm)	111.0 (35.0)	108.0 (8.4)	129.0 (36.0)	99.5 (19.7)	106.5 (18.4)	0.003[‡]
BSA (m^2)	0.9 (0.5)	0.9 (0.6)	1.0 (0.7)	0.8 (0.3)	0.8 (0.4)	0.057
Treated with ERT (*n*)	47/76	16/27*	15/22	8/19	8/8	0.029[§]
Age at the start of ERT (years)	8.3 (10.1)	4.0 (15.4)	6.6 (8.9)	8.3 (8.1)	9.7 (2.3)	0.538
Time on ERT (months)	41.0 (77.0)	52.0 (79.5)	64.0 (76.0)	22.5 (22.3)	73.5 (79.0)	0.317

Continuous variables are reported as median and interquartile range.

**Four patients who received ERT as a concomitant therapy for HSCT are not included in the ERT-treated group. Statistical analysis with chi-squared and partitioning for categorical variables and Kruskal-Wallis test with Dunn's post-hoc test for continuous variables.*

[†] *MPS II is different from other MPS types.*

[‡] *MPS IVA is different from MPS II.*

[§] *MPS IVA is different from MPS VI.*

with MPS that had performed echocardiograms from January 2000 until October 2018. When available, electrocardiographic data were also recorded. MPS III patients were excluded from the study because there is an insufficient number of individuals in our center with available echocardiograms or electrocardiograms results.

Body surface area was calculated using the geometric method of Haycock (8). Left ventricular mass (LVM) was obtained using

TABLE 2 | Characteristics of the subjects according to the age group.

	Overall ($n = 76$)	Children ($n = 53$)	Adults ($n = 23$)	*p*-value
Sex (*n*)	M: 46 F: 30	M: 34 F: 19	M: 12 F: 11	0.444
Age (years)	11.3 (12.7)	9.2 (7.3)	26.7 (9.8)	<0.001†
Weight (kg)	25.6 (21.0)	21.5 (10.0)	40.0 (32.0)	<0.001†
Height (cm)	111.0 (35.0)	104.0 (22.0)	128.5 (42.0)	<0.001†
BSA (m^2)	0.9 (0.5)	0.80 (0.29)	1.21 (0.62)	<0.001†
Treated with ERT (*n*)	47/76*	32/53*	15/23	0.799
Age at the start of ERT (years)	8.3 (10.1)	6.3 (6)	17.1 (6.25)	<0.001†
Time on ERT (months)	41.0 (77.0)	33.5 (58)	107.06 (65)	<0.001†

Continuous variables are reported as median and interquartile range.
**Four children who received ERT as a concomitant therapy for HSCT are not included in the ERT-treated group. Statistical analysis with chi-squared for categorical variables and Mann-Whitney U test for continuous variables.*
†$p < 0.05$.

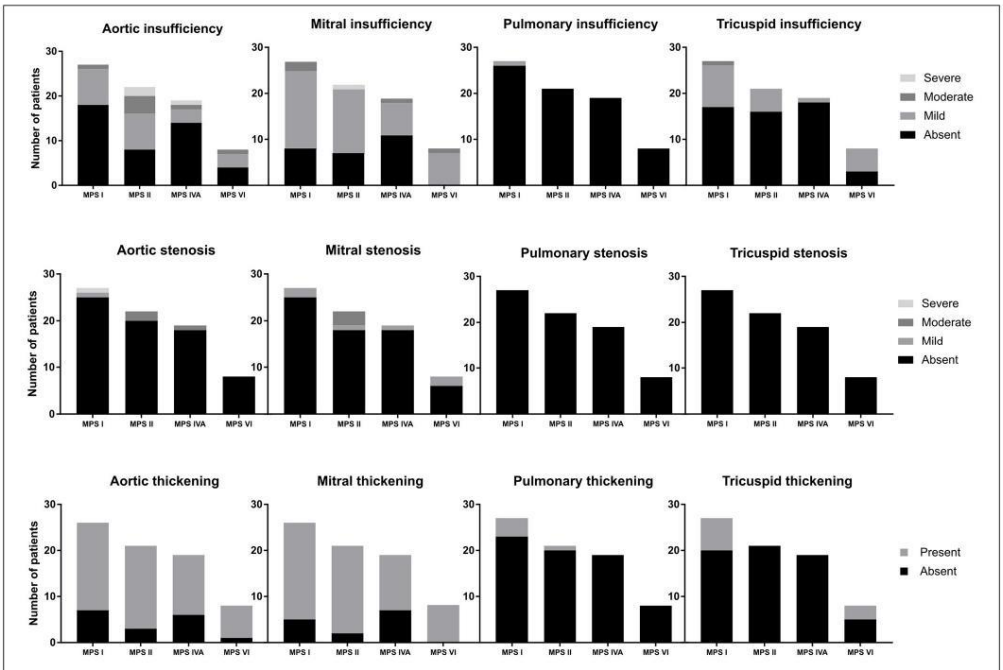

FIGURE 1 | Prevalence of heart valve abnormalities in MPS types I, II, IVA, and VI, including both treated and untreated subjects.

the formula of Devereaux (9). Standardized Z scores for left ventricle posterior wall thickness (LVPWT) and interventricular septum thickness (IVST) were calculated using the methods described by Lopez et al. (10), while, for the left ventricular mass (LVM) and left atrium parasternal long axis anteroposterior dimension (LAD) an online resource from Boston Children's Hospital Heart Center was used (11, 12). Relative wall thickness was obtained using the formula (2 × LVPWT)/(LV internal diameter at end-diastole) and a cut-off value of 0.41 was used for classification of the left ventricle geometry, as previously described (13). Left ventricle ejection fraction (LVEF) was obtained through the method of Teichholz (14) and systolic pulmonary artery pressure was obtained with doppler flow studies. For the analysis of the prevalence of the cardiac manifestations, the last available echocardiogram was used. When available, echocardiographic measurements performed before ERT (up to 18 months before ERT was started) and at least 18 months after that date (up to 166 months after ERT was started) were used for the assessment of pre- and post-treatment measurements. Those patients who received ERT in the peri-transplant period or as a concomitant therapy after transplantation were not included in that assessment. Changes in echocardiographic measurements were also analyzed for treatment naïve patients that had at least two echocardiograms performed with a minimum interval of 18 months.

For those who also had an available electrocardiogram, the presence or absence of left atrial enlargement, repolarization disorder, and left ventricular hypertrophy according to the clinical report were recorded. Interval measurements and Z scores for heart rate vs. age were also obtained (11, 12). Corrected QT (QTc) interval was calculated using the Bazett formula (15).

Statistical Analysis

All data were entered into PASW Statistics 18.0 for Windows (SPSS Inc., Chicago, IL, USA) and submitted to specific statistical analysis. Graphics and part of the statistic tests were generated using the GraphPad Prism version 7.0. The normality of the samples was assessed with Shapiro-Wilk and D'Agostino and Pearson tests. Non-parametric Kruskal-Wallis and Dunn's *post-hoc* tests were used for the comparison of quantitative measurements among different MPS types. Mann-Whitney U test was used for comparisons of quantitative measurements

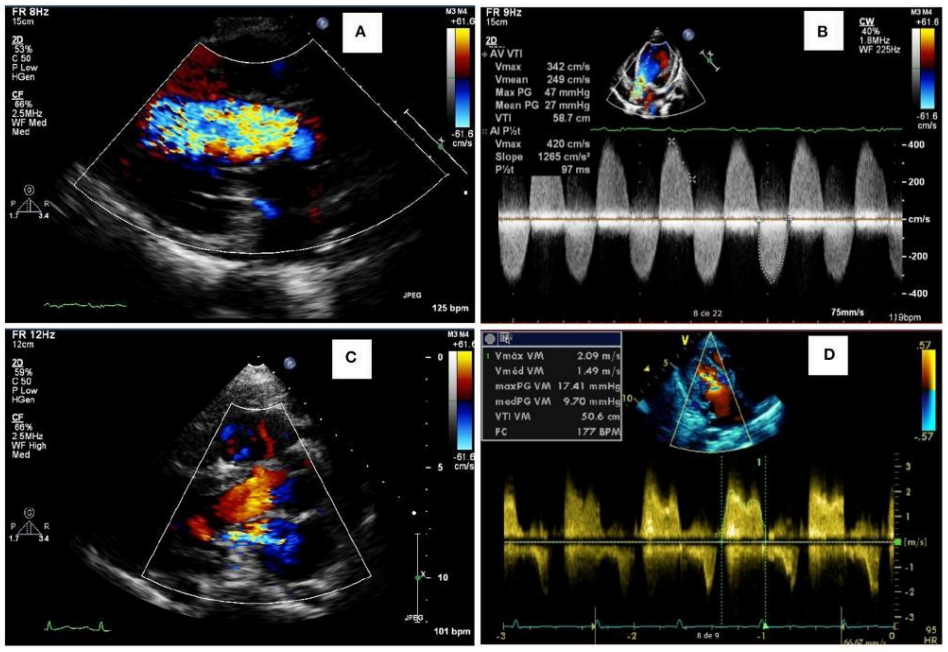

FIGURE 2 | Representative abnormalities identified in the valves of the patients of this study. Aortic insufficiency **(A)** and aortic stenosis **(B)** in a female in the age range of 26–30 years with MPS IVA, who also had a reduced ejection fraction (35%). Mitral insufficiency **(C)** in a male in the age range of 21–25 years with MPS VI. Mitral stenosis **(D)** in an adolescent male with MPS II.

between adults and children. A chi-squared test was used for comparison of the frequencies of abnormalities among MPS types and between children and adults. For the comparison between baseline and follow-up parameters after ERT, the parametric paired *t*-test was used to assess Z-scores of cardiac structures and the Wilcoxon matched-pairs signed-rank test was used to assess cardiac valves. A *p*-value of <0.05 was considered significant.

RESULTS

A total of 76 patients (27 MPS I, 22 MPS II, 19 MPS IVA, and 8 MPS VI), 46 males and 30 females, with a mean age of 14.2 years, being 53 children and 23 adults, were included in this study (**Tables 1**, **2**). Most of those patients were analyzed in a previous publication, which focused on the aortic root dimension (16). All MPS II patients were males. MPS IVA patients had a lower median height, when compared to MPS II patients and were less frequently treated with ERT when compared to MPS VI patients.

Valvular Disease

Among the 76 patients included in this study, left side valvular disease was a frequent finding, with mitral and aortic thickening being reported in most patients in all four MPS types (**Figure 1**). Furthermore, mitral and aortic insufficiency, mostly mild, were frequently found in patients with MPS I, II, and VI, but were also observed in a significant proportion of patients with MPS IVA. To a lesser extent, tricuspid valve thickening and insufficiency were also present in patients with MPS types I, II, and VI. Heart valve involvement was both more prevalent and more severe in adult patients (**Figure 2**; **Table 3**).

Other Echocardiographic Parameters

Left ventricular hypertrophy parameters, including LVPWT, IVST Z scores, and RWT, were above average in a significant proportion of patients in all subgroups (**Figure 3**). Nevertheless, LVM Z scores were normal in the last available echocardiogram of most of the patients (**Table 2**). Signs of left ventricular hypertrophy were more commonly observed in children than in adults (**Table 3**).

When assessed the last available echocardiogram, the median left atrium diameter (LAD) and the estimated systolic pulmonary artery pressure (SPAP) were increased in patients with all types of MPS (**Figures 3D,E**). A total of 26 and 28% of the patients had LAD or SPAP above normal reference limits, respectively (**Table 4**). The median value of SPAP was significantly lower in MPS IVA than in MPS I (**Figure 3D**). Left ventricular ejection fraction was preserved in most patients, except for one adult patient with MPS IVA, who also had eccentric hypertrophy, severe aortic insufficiency, and moderate aortic stenosis (**Figures 2A,B**, **3F**).

Electrocardiogram

Electrocardiograms were performed in 65 patients (45 children and 20 adults) at some point during follow-up. All patients had a sinus rhythm, although transient junctional rhythm was observed for one female with MPS IVA in the middle childhood. The most frequently observed finding was the presence of repolarization anomalies (29%) (**Table 5**; **Figure 4A**). Left ventricular hypertrophy (LVH) and left atrium enlargement were present in 5 and 8% of the patients, respectively. Atrioventricular block and long QT intervals were also occasionally observed (**Figure 4C**), as well as intraventricular blocks. As compared to children, adults had a higher prevalence

TABLE 3 | Prevalence of echocardiographic abnormalities in children and adults with MPS.

	Overall ($n = 76$)	Children ($n = 53$)	Adults ($n = 23$)	*p*-value
Aortic insufficiency	32/76 (42%)	17/53 (32%)	15/23 (65%)	0.011*
Aortic stenosis	5/76 (7%)	2/53 (4%)	3/23 (13%)	0.159
Pulmonary insufficiency	0/76 (0%)	0/53 (0%)	1/23 (4%)	0.303
Pulmonary stenosis	0/76 (0%)	0/53 (0%)	0/23 (0%)	n/a
Mitral insufficiency	50/76 (66%)	31/53 (59%)	19/23 (83%)	0.064
Mitral stenosis	9/76 (12%)	3/53 (6%)	6/23 (26%)	0.019*
Tricuspid insufficiency	21/76 (28%)	16/53 (30%)	5/23 (22%)	0.580
Tricuspid stenosis	0/76 (0%)	0/53 (0%)	0/23 (0%)	n/a
LVM Z score > 2	6/76 (8%)	5/53 (9%)	1/23 (4%)	0.661
RWT > 0.41	19/76 (25%)	15/53 (28%)	4/23 (17%)	0.395
Concentric remodeling	16/76 (21%)	12/53 (16%)	4/23 (17%)	0.622
Concentric hypertrophy	3/76 (4%)	3/53 (6%)	0/23 (0%)	
Eccentric hypertrophy	3/76 (4%)	2/53 (4%)	1/23 (5%)	
LAD Z score > 2	20/76 (26%)	11/53 (21%)	9/23 (39%)	0.155
SPAP > 35 mmHg*	11/40 (28%)	8/28 (29%)	3/12 (25%)	1.000
LVEF < 55%	1/76 (1%)	0/53 (0%)	1/23 (5%)	0.303

LAD, Left atrial diameter; LVEF, Left ventricle ejection fraction; LVM, left ventricle mass; RWT, relative wall thickness; SPAP, systolic pulmonary artery pressure. Statistical analysis with Fisher's exact test.
*$p < 0.05$.

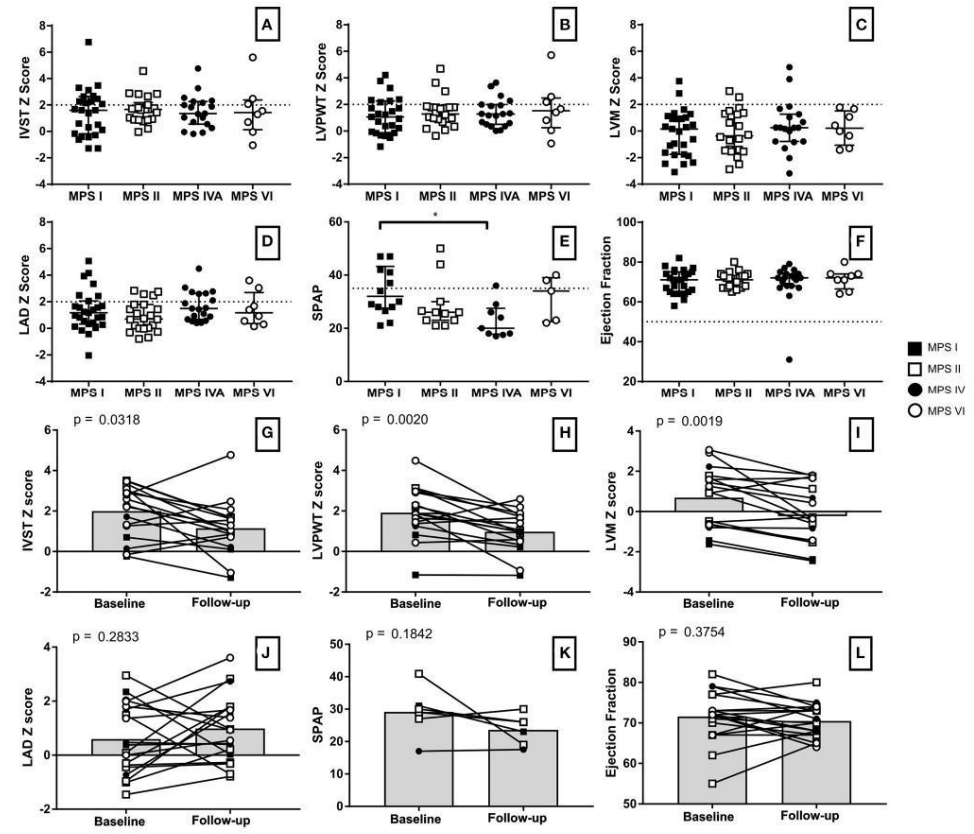

FIGURE 3 | Measurements of echocardiographic parameters among MPS types **(A–F)** as well as before and after ERT for single individuals **(G–L)**, including the Z scores of interventricular septum thickness **(A,G)**, left ventricle posterior wall thickness **(B,H)**, left ventricle mass **(C,I)**, left atrium diameter **(D,J)**, systolic pulmonary artery pressure **(E,K)** and ejection fraction **(F,L)**. A school-aged girl with severe MPS I fell outside the axis of both IVST **(A)** and LVPWT, with Z scores values of 9.0 and 9.7, respectively. Another MPS I patient (a female in the age range of 26–30 years with Hurler-Scheie phenotype) fell outside the axis range of SPAP **(E)**, because she had an SPAP of 110 mmHg. Statistical analysis with Kruskal-Wallis and Dunn's *post-hoc* test for comparisons among MPS types **(A–F)** and with paired *t*-test for before and after analyses **(G–L)**. $^{*}p < 0.05$. MPS IVA is different from MPS I.

of most of the ECG abnormalities (**Table 6**), particularly ventricular repolarization abnormalities (**Figure 4A**) and left atrial enlargement (**Figure 4B**).

Effects of the Enzyme Replacement Therapy

For those 19 patients whose echocardiographic measurements were available before and after ERT start (11 children and 8 adults), a significant reduction of LVH parameters (including IVST, LVPWT, and LVM) was observed after ERT was started (**Figures 3G–I**), a finding that was not identified in 18 patients that remained untreated with ERT (**Supplementary Figure 1**). However, no statistically significant changes in SPAP, LAD, or LVEF were observed after ERT (**Figures 3J–L**). Moreover, ERT did not lead to significant improvements in valvular disease (**Figure 5**).

As only 4 patients had available electrocardiograms in an adequate time frame before and after the start of ERT, we could not explore the impact of ERT in conduction anomalies.

TABLE 4 | Prevalence of echocardiographic abnormalities at the last available echocardiogram, including both ERT treated and untreated subjects.

	Overall ($n = 76$)	MPS I ($n = 27$)	MPS II ($n = 22$)	MPS IV ($n = 19$)	MPS VI ($n = 8$)	*p*-value
LVM Z score > 2	6/76 (8%)	2/27 (7%)	2/22(9%)	2/19 (11%)	0/8 (0%)	0.821
RWT > 0.41	19/76 (25%)	7/27 (26%)	7/22 (32%)	3/19 (16%)	2/8 (25%)	0.701
Concentric remodeling	16/76 (21%)	5/27 (19%)	6/22 (27%)	3/19 (16%)	2/8 (25%)	0.697
Concentric hypertrophy	3/76 (4%)	0/27 (0%)	1/22 (5%)	2/19 (11%)	0/8 (0%)	
Eccentric hypertrophy	3/76 (4%)	2/27 (7%)	1/22 (5%)	0/19 (0%)	0/8 (0%)	
LAD Z score > 2	20/76 (26%)	7/27 (26%)	4/22 (18%)	7/19 (37%)	2/8 (25%)	0.605
SPAP > 35 mmHg*	11/40 (28%)	6/14 (43%)	2/12 (17%)	1/9 (11%)	2/5 (40%)	0.265
LVEF < 55%	1/76 (1%)	0/27 (0%)	0/22 (0%)	1/19 (5%)	0/8 (0%)	0.385

LAD, Left atrial diameter; LVEF, Left ventricle ejection fraction; LVM, left ventricle mass; RWT, relative wall thickness; SPAP, systolic pulmonary artery pressure.
**SPAP was measured in 40 of the total 76 available last echocardiograms of the participants. Statistical analysis with chi-squared.*

TABLE 5 | Prevalence of electrocardiographic abnormalities at the last available electrocardiogram, including both ERT treated and untreated subjects.

	Overall ($n = 65$)	MPS I ($n = 20$)	MPS II ($n = 19$)	MPS IV ($n = 19$)	MPS VI ($n = 7$)	*p*-value
Ventricular repolarization abnormalities	19/65 (29%)	4/20 (20%)	6/18 (33%)	8/19 (42%)	1/8 (13%)	0.311
Left atrial enlargement	5/65 (8%)	2/20 (10%)	2/18 (11%)	0/19 (0%)	1/8 (13%)	0.514
Right axis deviation	5/65 (8%)	2/20 (10%)	0/18 (0%)	1/19 (5%)	2/8 (25%)	0.159
Left ventricular overload	3/65 (5%)	1/20 (5%)	1/18 (5%)	1/19 (5%)	0/8 (0%)	0.930
Right ventricular overload	3/65 (5%)	0/20 (0%)	2/18 (11%)	1/19 (5%)	0/8 (0%)	0.377
Intraventricular block	3/65 (5%)	1/20 (5%)	0/18 (0%)	0/19 (0%)	2/8 (25%)	0.025*
Atrioventricular block	3/65 (5%)	2/20 (10%)	1/18 (5%)	0/19 (0%)	0/8 (0%)	0.447
Right atrial enlargement	2/65 (3%)	2/20 (10%)	0/18 (0%)	0/19 (0%)	0/8 (0%)	0.200
Prolonged QRS	1/65 (2%)	0/20 (0%)	1/18 (5%)	0/19 (0%)	0/8 (0%)	0.448

Statistical analysis with chi-squared.
**$p < 0.05$.*

DISCUSSION

In this study, we assessed the prevalence of cardiac manifestations in a cohort of patients with MPS I, II, IVA, and VI; which included mostly patients treated with ERT. In agreement with previous reports (3, 17–20), valve involvement comprised mainly left-sided valves and affected a high proportion of patients. Mitral valve involvement was more common than the aortic valve in the four MPS types. Valve involvement, especially aortic insufficiency and mitral stenosis, was more commonly observed in older patients. We could not identify any significant worsening or improvement of valve pathology after ERT. It is widely accepted that ERT has limited impact on valve abnormalities of patients with MPS (13, 21–25), probably due to poor tissue penetration and irreversibility of the valvar damage. Nevertheless, it may have a role in preventing or delaying its appearance when treatment is started very early, as suggested by studies with sibling pairs and animal models (26, 27).

The finding of normal Z scores for LVM in a significant proportion of the patients in our cohort may reflect the effects of long-term therapy in this parameter. Accordingly, significant reductions of IVST, LVPWT, and LVM were observed in the follow-up measurements, which is in agreement to prior studies and generally acknowledged as a well-established effect of ERT (3, 5, 13, 22–24).

Hypertrophic cardiomyopathy (HCM) has been described in different inherited metabolic disorders and it is relatively milder in MPS as compared to other conditions such as infantile Pompe Disease or Danon disease (13, 28, 29). In patients with MPS, an increase in echocardiographic LVH parameters may reflect storage of undegraded substrate, without actual increase in muscle mass (pseudohypertrophy) and/or the activation of complex pathways triggered by the GAG deposition, causing HCM (28–30). Moreover, LVH in MPS may also be secondary to an increased workload associated with valve disease or arterial hypertension (3, 31).

In both humans and murine models of MPS, it was observed that GAG deposits occur mainly in histiocytes, while there is scant evidence of deposit in cardiomyocytes (32, 33). Furthermore, in MPS mice treated with bone marrow transplantation, no abnormal storage is detected in the heart tissue (32). Effects of ERT in left ventricular (pseudo-) hypertrophy are also likely to be secondary to its ability to clear GAG storage in the heart wall, as there is no evidence of improvement of valve disease or hypertension with the treatment.

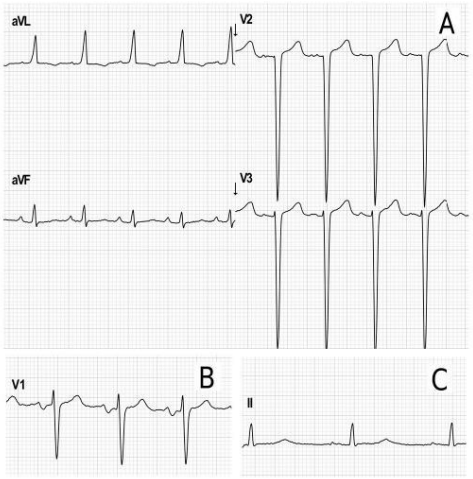

FIGURE 4 | Representative electrocardiographic abnormalities identified in the adult patients of this study. **(A)** Electrocardiographic criteria for left ventricular hypertrophy (Cornell index: 40 mm) and repolarization abnormalities in a female in the age range of 26–30 years with MPS IVA. **(B)** Left atrium enlargement in a male in the age range of 21–25 years with MPS II (terminal negative deflection within the P wave in lead V1). **(C)** Increased PR interval (203 ms) and transient prolonged QTc (464 ms) in female in the age range of 31–35 years with MPS I during postoperative care for aortic valve and mitral valve replacement.

TABLE 6 | Prevalence of abnormalities in the resting electrocardiogram in children and adults with MPS.

	Overall ($n = 65$)	Children ($n = 45$)	Adults ($n = 20$)	p-value
Ventricular repolarization abnormalities	19/65 (29%)	7/45 (16%)	12/20 (60%)	0.001*
Left atrial enlargement	5/65 (8%)	0/45 (0%)	5/20 (25%)	0.002*
Right axis deviation	5/65 (8%)	2/45 (4%)	3/20 (15%)	0.165
Left ventricular overload	3/65 (5%)	2/45 (4%)	1/20 (5%)	1.000
Right ventricular overload	3/65 (5%)	3/45 (7%)	0/20 (0%)	0.547
Intraventricular block	3/65 (5%)	1/45 (2%)	2/20 (10%)	0.222
Atrioventricular block	3/65 (5%)	2/45 (4%)	1/20 (5%)	1.000
Right atrial enlargement	2/65 (3%)	2/45 (4%)	0/20 (0%)	1.000
Prolonged QRS	1/65 (2%)	1/45 (2%)	0/20 (0%)	1.000

Statistical analysis with Fisher's exact test.
*$p < 0.05$.

We found a high proportion of patients with left atrial diameter Z scores above normal limits (26%), but a lower prevalence of left atrial enlargement criteria on electrocardiogram (8%). This difference may be attributed to a lower sensitivity of the latter method as an indicator of left atrial enlargement (34). Previous studies have reported the presence of left atrial dilatation in 6.3% of patients with MPS IVA (35) and 2.3% of patients with MPS VI (13). Another study reported 10.7% of MPS patients with biatrial enlargement criteria on electrocardiogram (25). As there is a higher proportion of aortic root dilatation in patients with MPS (6, 36), the use of left atrium to aortic root ratio may underestimate the true prevalence of atrial enlargement (37). Left atrial enlargement was more frequently identified in adults, and the impact of ERT in the parameter has not been established.

Systolic pulmonary artery pressure (SPAP) was increased in 28% of the patients. Other authors have reported a prevalence of 36% of pulmonary hypertension (PH) in a sample of 28 pediatric patients and emphasized that it was the main cause of death in their cohort (17). Chronic hypoxemia secondary to obstructive sleep apnea is a known cause of pulmonary hypertension among MPS patients, but it cannot explain all cases, and a role for the left heart dysfunction in its pathogenesis is also likely (17, 38, 39).

When analyzing the whole sample, we could not demonstrate a statistically significant improvement or worsening of the left ventricle ejection fraction. Nevertheless, ejection fraction was already preserved in most patients. Also, the adult MPS IVA patient reported with low LVEF in **Figure 3F** has never received ERT. In the literature, the impact of ERT in ejection fraction is less clear, with some studies pointing to stabilization or reduction of the mean value over time (13, 40) and others showing improvements in those patients who already had reduced LVEF on baseline (41). Although those inconsistencies need further clarification, it is possible that ERT may improve LVEF in a subset of patients with prior systolic dysfunction, while not being able to prevent a slow long-term deterioration.

In our study, all subjects had a sinus rhythm and none was on pacemakers. However, our results support the conclusions of previous authors that noticed that cardiac conduction abnormalities may increase strikingly in older adult MPS patients (42). It is also important to notice that, while in this study no MPS III patient was included, a high prevalence of first-degree AV block was described in that MPS type (43). Although most MPS patients will not have a clinically significant conduction abnormality, resting electrocardiogram and 24 h Holter monitoring, when indicated, should be an integral part of the care of MPS patients, considering that the early recognition may have a high impact on morbidity and mortality (42).

This work has some limitations. The measurements were performed by more than one echocardiographer and the time frame of the study involved a period before the establishment of new guidelines for echocardiographic measurements in our center when newer quantification methods for LA size, ejection fraction, and LVM were included in the routine echocardiogram protocol. Nevertheless, by including data from a wide period of time, we were able to estimate the prevalence of cardiovascular abnormalities in a large sample of MPS patients as well as the long-term effectiveness of ERT.

In summary, we identified a high proportion of MPS patients with cardiac abnormalities, which ranged from isolated mitral or aortic thickening to a more severe clinical picture including moderate to severe valvular insufficiency or stenosis, left ventricular hypertrophy, pulmonary hypertension, and, rarely, systolic dysfunction. We also confirmed the impact

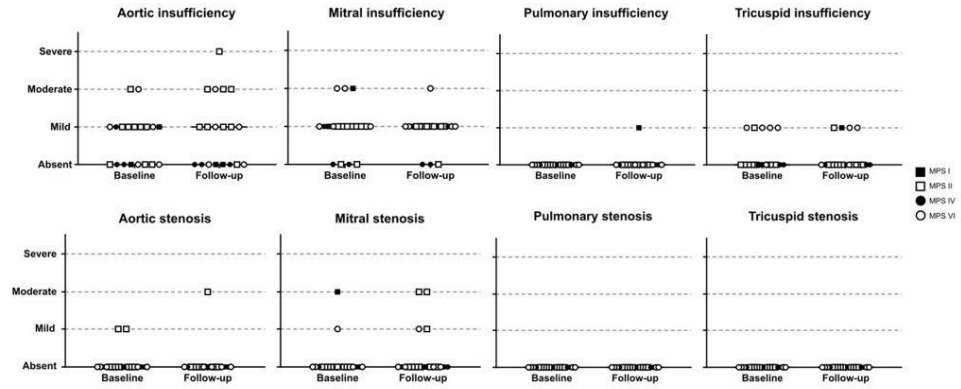

FIGURE 5 | Comparison of valve abnormalities before and after ERT. In the statistical analysis, with Wilcoxon matched-pairs signed rank test, no significant difference was identified.

of long-term ERT on left ventricular hypertrophy in the mucopolysaccharidoses and its limitations in reversing other cardiovascular manifestations, such as valvular involvement.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Grupo de Pesquisa e Pós-Graduação, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

FP, RG, and GB designed the study. HS and FP obtained the clinical data from chart review. AS and SB reviewed the echocardiographic and electrocardiographic data and analysis. CS reviewed the clinical data. FP drafted the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.801147/full#supplementary-material

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RESEARCH ARTICLE

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Brain and visceral gene editing of mucopolysaccharidosis I mice by nasal delivery of the CRISPR/Cas9 system

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Abstract

Background: Mucopolysaccharidosis type I (MPS I) is an inherited disease caused by deficiency of the enzyme alpha-L-iduronidase (IDUA). MPS I affects several tissues, including the brain, leading to cognitive impairment in the severe form of the disease. Currently available treatments do not reach the brain. Therefore, in this study, we performed nasal administration (NA) of liposomal complexes carrying two plasmids encoding for the CRISPR/Cas9 system and for the *IDUA* gene targeting the *ROSA26* locus, aiming at brain delivery in MPS I mice.

Methods: Liposomes were prepared by microfluidization, and the plasmids were complexed to the formulations by adsorption. Physicochemical characterization of the formulations and complexes, in vitro permeation, and mucoadhesion in porcine nasal mucosa (PNM) were assessed. We performed NA repeatedly for 30 days in young MPS I mice, which were euthanized at 6 months of age after performing behavioral tasks, and biochemical and molecular aspects were evaluated.

Results: Monodisperse mucoadhesive complexes around 110 nm, which are able to efficiently permeate the PNM. In animals, the treatment led to a modest increase in IDUA activity in the lung, heart, and brain areas, with reduction of glycosaminoglycan (GAG) levels in serum, urine, tissues, and brain cortex. Furthermore, treated mice showed improvement in behavioral tests, suggesting prevention of the cognitive damage.

Conclusion: Nonviral gene editing performed through nasal route represents a potential therapeutic alternative for the somatic and neurologic symptoms of MPS I and possibly for other neurological disorders.

KEYWORDS
CRISPR/Cas, genome editing, hurler syndrome, liposome, lysosomal storage disease, Mucopolysaccharidosis type I, nonviral vector

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1 | INTRODUCTION

Mucopolysaccharidosis type I (MPS I; OMIM 252800), or Hurler syndrome, is an autosomal recessive disorder caused by mutations in the *IDUA* gene. Mutations cause deficiency of *alpha-L-iduronidase* (IDUA) (EC 3.2.1.76) function or a total absence of this enzyme. IDUA is one of the lysosomal enzymes responsible for breaking down the glycosaminoglycans (GAG) dermatan and heparan sulfate.[1] These molecules accumulate vastly in lysosomes of different tissues and organs, causing a progressive multisystemic dysfunction in patients.[2]

Patient phenotype can vary from attenuated to severe. Patients with the severe form present with neurological impairment, while other somatic manifestations are common in all phenotypes.[1,2] These abnormalities include bone deformities, corneal opacity, hepatosplenomegaly, and respiratory and cardiovascular manifestations. They can appear early or later in life, depending on the phenotype.[1]

Disease-modifying therapies are currently available but have limitations. Depending on the clinical presentation, both allogeneic hematopoietic stem cell transplantation (HSCT) or enzyme replacement therapy (ERT) can be performed.[3,4] For patients with severe MPS I diagnosed before 2–3 years of age, HSCT is preferred as it arrests the neurological impairment and improves some somatic complications and survival.[4,5] Limitations of this treatment include finding a suitable donor, the invasive and high-risk medical intervention, as well as limited efficacy in correcting some aspects of the disease, such as heart valve thickening and osteoarticular impairment.[3] ERT, on the other hand, is more likely to be an option when the patient has an attenuated form, and for older patients when HSCT is not indicated. As a gold standard of care in patients, this strategy is commonly used as a monotherapy, but sometimes has been used as a cotherapy in patients that undergo HSCT.[1,4] As monotherapy, ERT has shown normalization of urinary GAG and reduction of organomegaly. Despite this, there are limited or no improvements in the brain, bone, cardiac valves, and corneal clouding, for example.[1,3,6]

Gene editing has been studied as a strategy to overcome the limitations of the available treatments,[7,8] as it holds the promise of editing the genome in a specific location.[8] The CRISPR/Cas9 system is a gene editing platform that employs a nuclease (Cas9) guided by a short RNA (gRNA) to cleave a specific target DNA sequence.[9,10] Despite its advantages, a reliable and efficient delivery system is still one of the main challenges for gene editing. Most studies use viral vehicles to deliver the system,[11] while liposomes have been considered a suitable option as well.[12] The easy manufacturing, low cost, low immunogenicity, and the ability to carry and protect large nucleic acid constructs remains the main advantages of these nonviral vectors.[12]

Previously, our research group showed the potential of these liposomal carriers as nonviral vectors of the gene editing system to correct MPS I mice in vivo.[13,14] Through a single hydrodynamic injection of liposomes containing plasmids with the CRISPR-Cas9 components in newborn mice, we achieved an increase in IDUA activity and reduction in GAG storage in visceral tissues. Moreover, mice showed improvement in cardiovascular and respiratory manifestations. Despite these positive results, brain tissue did not show improvements after intravenous treatment.[13,14]

Among other routes that may favor delivery into the central nervous system (CNS), a noninvasive strategy is nasal administration (NA). This route has been demonstrated to bypass the blood–brain barrier in both preclinical and clinical tests delivering several molecules and DNA.[15,16] Thus, the aim of the present study was to assess the effectiveness of NA of a liposome carrying the CRISPR/Cas9 system and a plasmid encoding for the *IDUA* gene to correct both brain and peripheral tissues in the murine model of MPS I.

2 | MATERIALS AND METHODS

2.1 | Vectors

PrecisionX CRISPR/Cas9 SmartNuclease system (System Biosciences, USA) was used for in vivo gene editing experiments. The target sequence 5′ggattctcccaggcccaggg3′ at the *ROSA26* locus of the mouse genome was inserted into the vector for gRNA transcription.[13] For homologous recombination, a plasmid vector containing the *Idua* cDNA customized by System Biosciences (USA) was used. The construct contains the mouse *Idua* cDNA sequence regulated by an EF promoter and two homologous regions (approximately 1 Kb each) to the *ROSA26* locus of mice, in the region that Cas9 recognizes and cleaves.[13] It also contains a copy of the hygromycin resistance gene **(Supplementary Figure S1)**.

2.2 | Preparation of formulations

Liposomes and complexes were prepared according to Schuh et al.[13] Briefly, blank liposomes were composed of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE; Lipoid, Germany), 1,2-dioleoyl-sn-glycero-3-trimethylammonium propane (DOTAP; Lipoid, Germany), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [amino (polyethylene glycol)- 2000] (DSPE-PEG; Lipoid, Germany). Lipids were dissolved in chloroform and evaporated under reduced pressure (40°C). The thin lipid film was hydrated in an aqueous glycerol solution, vortexed for 1 min and left to stand overnight at 4°C. The following day, formulations were sonicated for 10 min at 38°C. Finally, formulations were microfluidized (Microfluidics, Canada). Complexes were prepared by adsorption of DNA on blank formulations through the addition of previously established amounts of DNA to blank liposomes (L) at a +4/−1 charge ratio.

2.2.1 | Fluorescent liposome

The NBD-PE (N-[7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl]-1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine, Triethylammonium Salt) fluorescent labeled phospholipid (Thermo Fisher Scientific, USA) was dissolved in chloroform with the other lipids in

the proportion of 1 mg/10 mL of formulation replacing the same amount of DOPE.[13] The preparation of the formulation continued as described to the blank liposome ("FL") and adsorption of "CRISPR +Donor" labeled plasmids producing "FLA" complexes.

2.2.2 | Droplet size and zeta potential

Mean droplet size, polydispersity index (PDI), and ζ-potential of the formulations and complexes were determined by photon correlation spectroscopy (PCS) at 90° and electrophoretic mobility measurements (3000HS Zetasizer, Malvern Instruments, UK). The samples were diluted in water or 1mM NaCl.[13]

2.2.3 | Mucoadhesive properties in porcine nasal mucosa

Mucoadhesion assessments were estimated using a texture analyzer TAXTplus (Stable Micro Systems, UK). Porcine nasal mucosa (PNM) was obtained from a local slaughterhouse. Heads of animals were divided by an incision along nasal septum and respiratory mucosa was carefully removed from nasal turbinates and stored at −20°C for a maximum period of 1 month. PNM was hydrated with artificial nasal mucus, as previously described,[17] and attached to a upper movable probe (10 mm diameter) of a mucoadhesion ring, and samples were kept in the lower platform of the instrument. The measurements were performed with a triggered force of 2 mN, and no force was applied for 60 s, then the probe was raised at a constant speed of 0.5 mm/s. The maximal force (mN) required for detachment of the sample from PNM was determined.

2.2.4 | Porcine nasal mucosa permeation/retention assays

Nasal mucosal permeation/retention of the fluorescent complexes with labeled plasmids was evaluated in a Franz-type diffusion cell through an excised PNM membrane. PNM (Harmonia, Brazil) was prepared and previously immersed in potassium phosphate solution with pH 5.8 for 15 min. Then, the nasal mucosa membrane was mounted between the donor and recipient compartments. The receptor compartment was filled with 12 mL of pH 5.8 potassium phosphate. The bath solution was stirred at 450 rpm and maintained at a controlled temperature (34 ± 1°C). In the donor compartment, 500 μL of formulation was placed, maintaining sink conditions. After 4 h, the mucosa was wiped with a cotton swab, removed from the cell, and cut into small pieces. This study was performed with formulations containing the fluorescent complexes (fluorescent liposomes containing the fluorescent phospholipid NBD-PE associated with the fluorescent plasmids, FLA). DNA plasmids were dyed with CAL Fluor Red 590 (Biosearch Technologies). Fresh PNM were observed under a fluorescence microscope (Olympus BX51TF, Japan) equipped with a digital camera using an objective (Olympus-DP 71, Japan) (200 × for overview images). Green channel was detected at Exc/Em 463 nm/536 nm and red channel at Exc/Em 560 nm/590 nm. PNM pieces were fixed in 10% buffered formalin, processed, and embedded in paraffin wax. Thin cross sections were submitted for routine histological processing, stained with hematoxylin–eosin (H&E) and analyzed for signs of tissue damage.

2.3 | In vivo assays

Thirty-day-old C57BL/6 MPS I mice (Idua-KO, donated by Dr. Elizabeth Neufeld, UCLA, USA) and normal C57BL/6 mice (wild type) were used for the experiments. MPS I mice were genotyped by PCR reaction as previously described,[18] and maintained under standard conditions according to the animals facility regulations. Briefly, animals were placed in plastic boxes with a maximum of five animals/box, in a controlled environment temperature (20–24°C, relative humidity 40–60%, and air exhaust systems) with cycles of 12 h light and 12 h dark, and standard commercial food for the species and water ad libitum. Treated mice received nasal administrations of the liposomal complex for 30 days (from 30–60 days old). Daily, animals were held in a reverse Trendelemburg position, and with a sterile 10 μL tip the treatment was instilled into each nostril every 15 min, six times, up to 120 μL per day (40 μg total DNA per day). The animals were anesthetized prior to each administration by inhaling isoflurane at 0.5 L/min (5% for induction and 3% for maintenance). After euthanasia, organs, blood, and urine were collected and stored at −80°C for further assays.

2.3.1 | Pilot (short-term) study

One-month-old MPS I animals ($n = 5$) were treated with the complex for 30 days and euthanized 48 h after the last administration. Main organs and fluids were collected to assess IDUA activity.

2.3.2 | Extended (long-term) study

One-month-old MPS I mice ($n = 7$) were treated with the complex for 30 days and euthanized at 6 months of age, soon after behavioral tests were performed. Main organs (Brain, heart, lung, liver, spleen, and kidney), urine, and serum were collected and IDUA activity and GAG levels were assessed. An additional group of MPS I mice received the liposomes with the donor vector only, without the Cas9 plasmid ($n = 5$). These mice were compared with wildtype (WT) mice and untreated MPS I mice (MPS I).

2.4 | IDUA activity

To assess IDUA serum levels, a sample of blood from treated and untreated MPS I mice was collected at day 15 and monthly after the

treatment started. Serum was collected after centrifugation and stored at −80°C. IDUA levels in organs were assessed by homogenizing 20 mg of frozen tissue in distilled water and collecting the supernatant after centrifugation (4°C at 5,000 rpm for 10 min), and frozen at −20°C. A fluorometric assay was performed to assess IDUA activity using 4-methylumbelliferyl α-L-iduronide (Glycosynth #44076).[19] Fluorescence was measured using a Spectramax M3 microplate reader (Molecular Devices) with excitation at 355 nm and emission at 460 nm. IDUA activity in serum was calculated as nmol/h/ml of serum. Results from IDUA activity in tissues were normalized by total protein content measured by the Lowry method[20] and calculated as nmol/h/mg protein. All enzyme activities are shown as the percentage of IDUA activity found in wild-type mice.

2.5 | GAG levels

Specific GAG levels were assessed by tandem mass spectrometry from serum, urine, and tissues (brain total cortex, heart, lungs, liver, spleen, and kidney). GAGs were extracted from tissues after acetone precipitation. Dermatan sulfate (DS), heparan sulfate with *O- or N*-sulfation (HS–*OS* and HS–*NS*) disaccharides were obtained through digestion of samples with chondroitinase B, heparitinase, and keratanase II, followed by quantification through liquid chromatography tandem mass spectrometry (LC/MS/MS) as previously described.[21] Briefly, 10 μL of extracted tissues, urine, or serum were mixed with 90 μL of 50mM Tris HCL (pH 7) and added to Omega 10 K filter plates (Pall Co., MI, USA) and centrifuged for 15 min. Samples were incubated in a shaker overnight at 37°C with 60 μL of 50mM Tris HCL, 10 μL of 5 μg/mL internal standard (chondrosine), 10 μL of 0.6 mU chondroitinase B (in bovine serum albumin [BSA] 1%), 10 μL of 1 mU heparitinase (in BSA 1%), and 10 μL of 1 mU keratanase II (in BSA 1%) (enzymes and internal standards (IS) were provided by Seikagaku Co., Tokyo, Japan).[21] For detection, samples were injected into Xevo TQ-S micro Triple Quadrupole Mass Spectrometry (Waters Tech) 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 (138 HS–*NS*; 378.3, 175.1 HS–OS, DS) The concentration of each disaccharide was calculated using QQQ Quantitative Analysis software.

2.6 | Cytokine levels

To assess long-term peripheral inflammation, interleukins 6 (IL-6) and 1-beta (IL-1b) levels were measured in 6-month mouse serum (1:2 dilution) from the extended study using commercial enzyme-linked immunosorbent assays (ELISAs) (Finetest, USA, #EM0121 and #EM0109) following the manufacturer's instructions, adapting to a 3 h sample incubation. Absorbance was analyzed using a Spectramax M3 microplate reader (Molecular Devices) at 650 nm.

2.7 | Efficiency of gene editing

To estimate the efficiency of gene editing, quantification of hygromycin gene present in the donor sequence was performed by real-time PCR. Genomic DNA was extracted from olfactory bulb, brain frontal cortex, brain total cortex, lung, and heart tissues with the Easy-DNA gDNA Purification Kit (Thermo Fisher Scientific, USA) according to manufacturer's instructions, and quantified with NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). The donor plasmid, which contains a single copy of hygromycin sequence, was used as a standard. The number of copies per nanogram of plasmid was calculated with the following formula (http://cels.uri.edu/gsc/cndna.html):

$$\text{number of copies} = (\text{amount in ng} \times 6.02 \times 10\text{e}23)/(\text{length in bp} \times 1 \times 10\text{e}9 \times 650).$$

Reactions were carried out in a StepOne Real-Time PCR System (Thermo Fisher Scientific, USA) with 5 μL of master mix SybrGreen, 0.1 μL of each primer (0.1 mM final concentration), 1.8 μL of nuclease free water, and 60 ng of DNA in a final volume of 10 μL. The following primers were used: forward 5′- CAA GAC CTG CCT GAA ACC GA-3′ and reverse 5′-ATT GAC CGA TTC CTT GCG GT-3′. All reactions were performed in standard conditions at an annealing temperature of 60°C. A standard curve of serial dilutions of the donor plasmid in triplicate was included in each run (1:4 serial dilutions from 36,000 to 140 copies of hygromicin gene). Samples were considered as containing the transgene if amplification occurred before the Ct30 (cycle to threshold). Copy number in tissues was calculated according to previously described.[22] The correction efficiency was expressed as the percentage of transgene hygromycin copy number found in each tissue analyzed.

2.8 | Behavioral tests

2.8.1 | Open field and repeated open field

Before euthanasia, exploratory behavior was assessed using a simple open field test. Mice were placed in one of the corners of the open field arena (Monitor de Atividade-IR EP 14. INSIGHT, Brazil) and (a) ambulation (crossings) and (b) exploratory behavior (rearings) were observed over 5 min. Nonaversive memory was assessed using the same open field test, but mice repeated the test three times, spaced by 30 min between each test. Memory was assessed by comparing the numbers of crossings and rearings from the third test with the results from the first test. All tests were held at the same time of day by the same observer.

2.9 | Ethics statement and statistics

This study was approved by the authors' institutional ethics review board (CEUA HCPA permit numbers #20150215 and #20180035), and all animal procedures were carried out following the

recommendations of the Guide for Care and Use of Laboratory Animals of the Brazilian National Council for Animal Experimentation Control (CONCEA), monitored by the veterinarians and designed to minimize animal suffering. Results were compared using ANOVA and Tukey or Student's *t*-test as indicated. *P* values lower than 0.05 were considered statistically significant. Pearson's test was used for correlation analysis. GraphPad Prism 5 (GraphPad, USA) software was used for graphic design and statistical analyses.

3 | RESULTS

3.1 | Physicochemical properties of the formulations

The physicochemical properties of the formulations are listed in Table 1. The average vesicle size of the liposomes before complexation (L) was close to 85 nm. However, the average diameter tended to increase after association with DNA (LA). The polydispersity index was less than 0.14 in all formulations. The formulations demonstrated a positive zeta potential, and the addition of DNA decreased the zeta potential of the liposomes.

TABLE 1 Physicochemical properties of formulations

Formulation	Mean diameter (nm)	PDI	ζ-Potential (mV)
L	85.2 ± 8.8	0.14 ± 0.06	+42.4 ± 8.1
LA	112.4 ± 11.7[a]	0.10 ± 0.08	+26.4 ± 4.7[b]

Results represent the mean ± standard deviation of three experiments; difference before and after complexation. Student's *t*-test, $p < 0.05$.
PDI, polydispersity index; L, blank liposome; LA, liposome associated with CRISPR/Cas9 plasmid and *Idua* donor plasmid for in vivo mouse correction.
[a]Mean diameter.
[b]ζ-Potential.

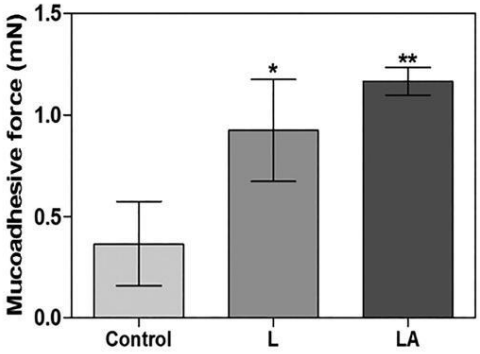

FIGURE 1 Mucoadhesive force of L and LA, compared with water (used as control). *Significant difference in comparison with water (*$p < 0.05$ and **$p < 0.0001$). L: Blank liposome; LA: Liposome associated with CRISPR/Cas9 plasmid and *Idua* donor plasmid for in vivo mouse correction

Mucoadhesion assessments (Figure 1) were performed using a detachment test. The mucoadhesive force of the formulations L and LA was significantly higher ($p < 0.0001$) when compared with the control, and this may be due to the presence of the cationic lipid DOTAP in the formulations, which interacts with the negatively charged mucin.

A fluorescent formulation was used to demonstrate permeation through porcine nasal mucosa (PNM), as well as H&E staining to observe any tissue damage. As can be seen in Figure 2 in the bottom pictures, both fluorescent liposome (FL) and liposome associated with plasmids (FLA) formulations were retained in the PNM, while the merged image shows the presence of the fluorescent liposomes and CRISPR/Cas9 plasmid at the same sites of PMN. In addition, there were no signs of evident tissue damage when FL and FLA were compared with the untreated PNM, as observed in the upper pictures of PMN stained with H&E (Figure 2).

3.2 | In vivo studies

3.2.1 | Pilot (short-term) study

Aimed at prospecting the treatment approach, a pilot assay with short-term outcomes was conducted. Five animals received six NA

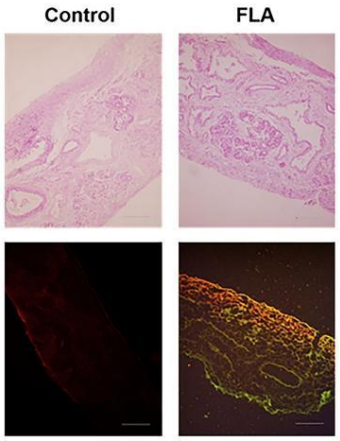

FIGURE 2 Permeation/retention of fluorescent formulations in porcine nasal mucosa (PNM, lower images) and H&E staining (upper pictures). Bottom images were acquired in fluorescence (green channel: Exc/Em 463 nm/536 nm, and red channel: Exc/Em 560 nm/590 nm) at 200× magnification, representative of three experiments. Control: Untreated PMN; FLA: Fluorescent liposome associated with CRISPR/Cas9 plasmid and *Idua* donor plasmid for in vivo mouse correction. Green channel: *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine phospholipid; red channel: CAL Fluor red 590 DNA dye. PNM: Porcine nasal mucosa

per day of the liposomal CRISPR/Cas9 complex for 30 days and were euthanized 48 h after the last administration. The overall results show that serum IDUA levels in treated MPS I mice increased significantly ($p < 0.005$) after 15 days of NA and doubled after 30 days (Figure 3A). It can be observed that IDUA activity was detected in all brain regions analyzed ($p < 0.05$), representing about 0.4–0.8% of normal mice IDUA activity (Figure 3). In addition, there was a small increase in enzyme activity in the lung, liver, kidney, and spleen (an average of about 1.5%, $p < 0.05$), and it significantly increased in the heart (by almost 3%, $p < 0.005$) of treated animals.

3.2.2 | Extended (long-term) study

Serum IDUA levels

Based on the results from the pilot study, we treated another group of animals in a similar way, but kept the mice alive up to 6 months of age. Serum IDUA levels after day 15 were already detectable in treated mice, with an average of 2.4% of wild-type mice enzyme activity. These levels remained constant over time ($p > 0.05$ compared with untreated mice), suggesting that gene editing occurred in visceral organs to a certain level. At 6 months, serum IDUA levels corresponded to an average of 3.6% of those found in wild-type mice (Figure 4A). Animals treated with only the donor plasmid showed no increase in IDUA activity, and therefore further analyses were not performed in this group (data not shown).

Urine and serum GAGs levels

Urinary GAGs were quantified using tandem mass spectrometry at 6 months. Heparan sulfate disaccharides (HS–OS and HS–*NS*) were significantly reduced in the treated group ($p < 0.05$ versus MPS I) but still not normalized ($p < 0.05$ versus wild type, (Figure 4B,C). Dermatan sulfate disaccharides (DS) were not statistically different among groups (Figure 4D). Serum GAGs levels were also assessed through tandem mass spectrometry. At 6 months, DS, HS–OS, and HS–*NS* were significantly reduced in the treated group when compared with untreated MPS I mice (Figure 4E–G).

Tissue IDUA activity and efficiency of gene editing

IDUA activity was assessed in tissues of 6-month-old mice. In visceral tissues, a three-fold increase in enzyme activity levels was found in the heart and the lungs, representing approximately 0.7% and 0.5% of

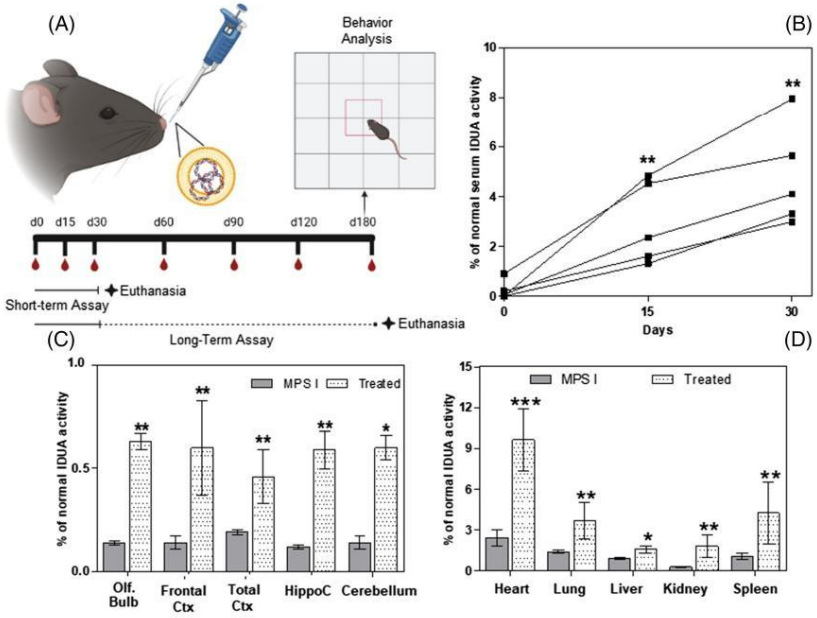

FIGURE 3 Experimental design and results of in vivo short-term assay. (A) Schematic timeline of short- and long-term studies; (B) serum enzyme activity after 15 and 30 administrations. $t(5) = 5.485$, $p < 0.005$. (B) IDUA activity in brain areas 48 h after the 30th administration; (C) IDUA activity in visceral tissues 48 h after the 30th administration. Untreated MPS I (MPS I, $n = 5$), and treated MPS I mice (treated, $n = 5$). Values were expressed as a percentage of normal (wild-type mice) IDUA activity. ANOVA and Tukey post hoc, where $^*p < 0.05$ and $^{**}p < 0.005$, versus untreated MPS I mice. Treatment: Liposome associated with CRISPR/Cas9 plasmid and *Idua* donor plasmid for in vivo mouse correction

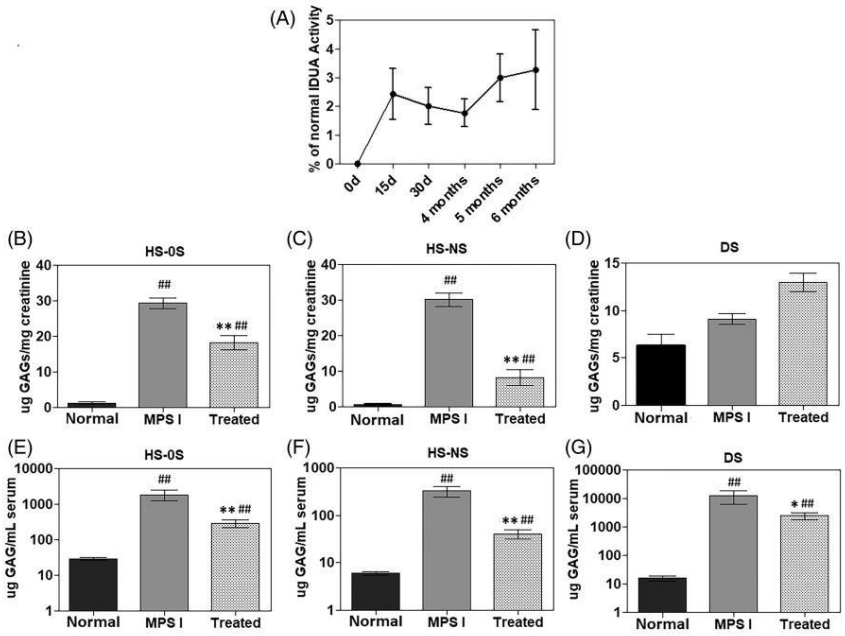

FIGURE 4 Urine and serum biochemical assessments: Results from long-term study. (A) Serum enzyme activity in treated mice after gene editing. IDUA activity in mouse serum was assessed at 15 days and monthly after treatment started. Values are shown as a percentage of normal (wild-type mice) activity. Untreated mice have undetectable IDUA levels. (B–D) urinary GAG levels expressed as μg GAG/mg creatinine. (E–G) serum GAG levels expressed as μg GAG/mL serum. (B–G) Dermatan sulfate (DS), heparan sulfate (HS–NS and HS–OS) disaccharides were determined in serum and urine using tandem mass spectrometry from normal (wild-type, $n = 3$), untreated (MPS I, $n = 5$), and treated MPS I mice ($n = 5$) at 6 months. ANOVA and Tukey post hoc, where $^{*}p < 0.05$ and $^{**}p < 0.005$, versus untreated and $\#p < 0.05$ and $\#\#p < 0.005$, versus wild type (normal). Averages for all animals in each group ± the standard error of the mean (SEM) are shown. The *Y*-axis in serum GAGs graphs is shown in log scale. Treatment: Liposome associated with CRISPR/Cas9 plasmid and *Idua* donor plasmid for in vivo mouse correction

wild-type mice levels, respectively (Figure 5A). Brain tissue was assessed separately by area, and activity was significantly increased in the olfactory bulb, frontal cortex, and total cortex compared with untreated mice ($p < 0.05$). In all cases, values corresponded to approximately 0.7% of wild-type mice (Figure 5B).

Percentage of gene editing was investigated in tissues that showed an increase in enzyme activity at DNA level. The olfactory bulb, heart, and lung tissues presented 0.04–0.06% of hygromycin copy number. Meanwhile, editing was not observed in the frontal and total cortex (Figure 5C), which suggests that the enzyme is produced in the olfactory bulb and is distributed to other brain areas. As expected, untreated mice showed undetectable gene editing levels (data not shown).

Tissue GAG levels

Specific GAGs (DS, HS–OS, and HS–NS) levels were determined in all visceral organs and total brain cortex from treated mice using tandem mass spectrometry. At 6 months of age, DS levels in the treated group were reduced by half in the heart, liver, kidney, and spleen when compared with untreated MPS I mice. Furthermore, brain cortex DS was 70% lower, compared with untreated MPS I mice ($p < 0.05$) (Figure 5D). Heparan sulfate disaccharides HS–NS and HS–OS also showed a significant reduction in the lung, liver, and spleen, but not in the heart or kidney ($p < 0.05$; Figure 5E,F). Heparan sulfate levels in the brain cortex were significantly reduced, being normalized in some animals ($p < 0.05$, Figure 5E,F). As observed in Figure 5D–F, GAG levels were reduced significantly in most tissues, although not completely normalized. A negative correlation between tissue IDUA levels and heparan sulfate levels in the brain cortex was observed. All correlations can be observed in supplementary Figures S2–S4.

Behavioral tests

Two behavioral tests were performed at 6 months. The open field test evaluates exploratory and vertical activities, while the repeated open field test evaluates nonaversive memory. As shown in Figure 6,

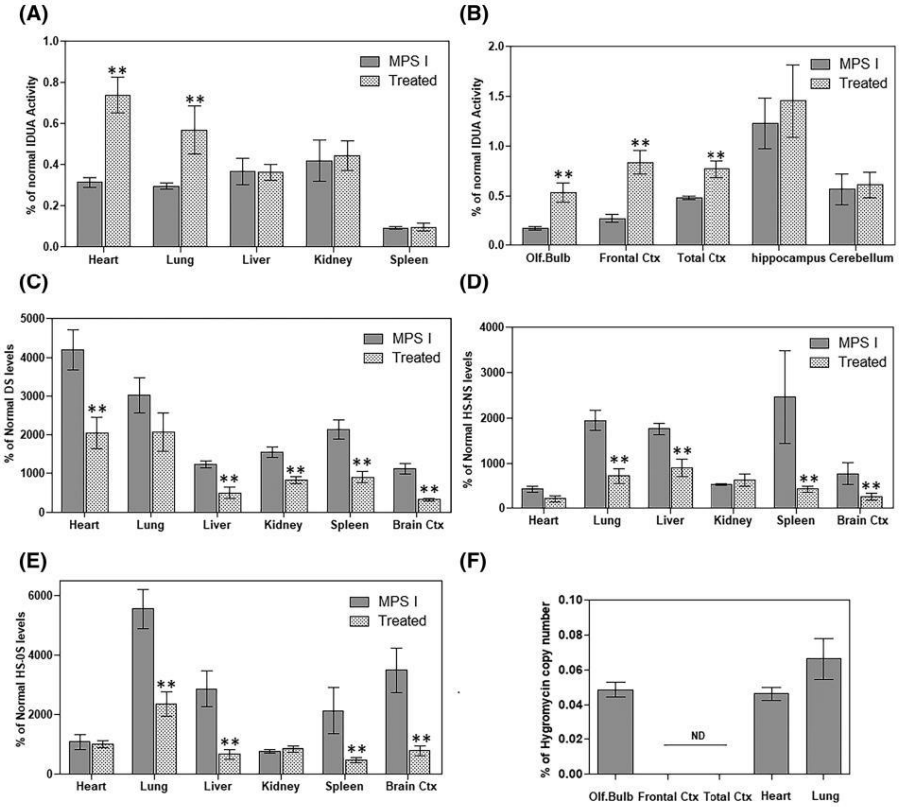

FIGURE 5 Biochemical and molecular results in tissues after long-term study. The efficacy of the treatment was demonstrated by IDUA activity and GAG levels. (A) IDUA activity in visceral organs and (B) brain tissue at 6 months. (C) Gene editing efficiency in treated mice expressed as a percentage of transgene (*hygromycin*) copy number found in each tissue analyzed from treated mice. (D) Dermatan sulfate (DS), (E) heparan sulfate (HS–*NS*), and (F) HS–*OS* disaccharide levels. Results from enzyme activity and GAGs levels are expressed as a percentage of normal (wild-type mice) activity. Untreated MPS I mice (gray bars), MPS I treated mice (dot bars). ND, not detected. ANOVA and Tukey post hoc, where $^{*}p < 0.05$ and $^{**}p < 0.005$, versus untreated MPS I mice. Averages for all animals in each group ± the standard error of the mean (SEM) are shown. Treated: Animals treated with liposome associated with CRISPR/Cas9 plasmid and *Idua* donor plasmid from 30–60 days sacrificed at 6 months

untreated MPS I mice had, on average, a 40% reduction in locomotor activity compared with normal mice ($p < 0.01$). Treated mice had an exploratory behavior similar to normal (Figure 6A). The number of rearings, which denotes the curiosity and response to stimuli, was also reduced by almost 60% in MPS I mice ($p < 0.01$). Treated mice presented an improvement in this parameter (75% of normal mice), but without statistical differences to either of the other groups (Figure 6B).

We also observed a slight improvement in the memory test. While untreated MPS I mice did not habituate to the environment, treated mice behaved similarly to wild-type mice in the number of crossings ($p < 0.05$, Figure 6C). On average, treated mice also performed similarly to wild-type mice when crossings were evaluated, however, due to higher variability, there were no significant differences of treated mice compared with both normal or untreated MPS I mice and also compared with the donor group (Figure 6D and **Supplementary Figure S5**).

Assessment of inflammation

To analyze whether the repeated administration of the liposomes can cause long lasting cytotoxicity or permanent injury after treatment, peripheral inflammatory cytokines were measured. Both IL-6 and IL-1b were increased in untreated MPS animals but treatment did not influence interleukin levels (Figure 7A,B).

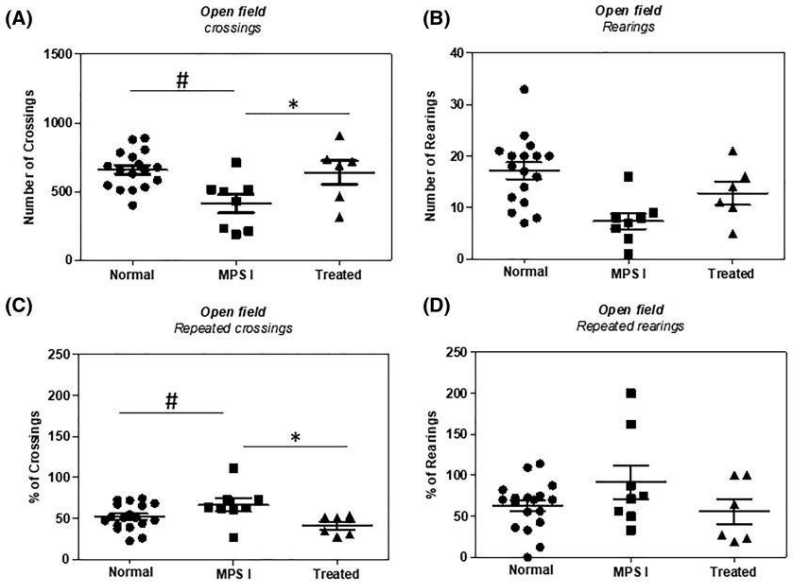

FIGURE 6 Behavior analysis: Open field and repeated open field test. Mice were analyzed at 6 months and exploratory behavior was compared among groups, considering (A) number of crossings and (B) number of rearings of normal (wild-type, circles, $n = 15$), untreated MPS I mice (squares, $n = 8$), and treated MPS I mice (triangles, $n = 6$). Memory was studied according to the reduction in the percentage of (C) crossings and (D) rearings in the third trial, compared with the first in the repeated open field. Dots represent individual mice and trace indicate average and standard deviation. ANOVA and Tukey post hoc, where $^{*}p < 0.05$ shows difference from untreated MPS I group and $\#p < 0.05$ difference from wild-type group. Treated: Animals treated with liposome associated with CRISPR/Cas9 plasmid and *Idua* donor plasmid from 30–60 days, sacrificed at 6 months

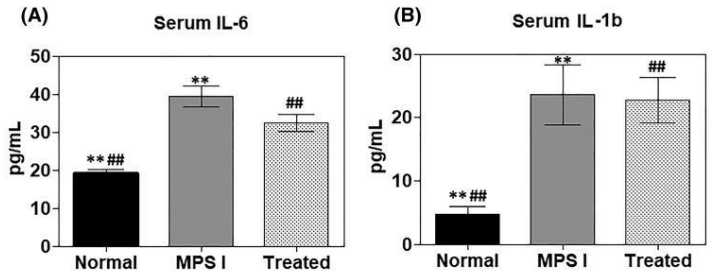

FIGURE 7 Serum interleukin levels. (A) Interleukin 6 (IL6) and (B) interleukin 1-beta (IL-1b) in mouse serum at the moment of sacrifice. $^{**}p < 0.05$ and $^{\#\#}p < 0.05$ compared with normal mice

4 | DISCUSSION

In this study, we evaluated the efficacy of NA of liposomes complexed with the CRISPR/Cas9 system and a *IDUA* donor plasmid as an approach for the treatment of neurological and somatic features in an MPS I murine model.

Physicochemical results showed that the obtained droplet size (80–120 nm) and PDI (≤0.15) were as expected and desirable for NA. In addition, all formulations exhibited a positive ζ-potential value due to the presence of the cationic lipid DOTAP. A positively charged surface is a crucial element for the interaction with the superficial nasal mucosa, as possible electrostatic interactions may occur with negatively charged sialic groups of mucin.[16,23] PNM was chosen to investigate nasal permeation/retention profiles of the complexes because of its morphological similarities to human mucosa.[24] The vector was retained in PNM because of the mucoadhesive characteristics and the presence of DOTAP.[25] These results demonstrated the high retention potential of the complexes and reinforced the presence of a

strong interaction of the liposome with nasal mucosa, which is highly desirable considering the mucus turnover.[26] In addition, in latest studies, we performed some experiments showing that these complexes are relatively stable in simulated biological milieu, and demonstrated the ability of these nanostructures to efficiently protect the nucleic acids from nuclease degradation.[13,16,27]

Recent publications from our group have shown that a single hydrodynamic intravenous injection of liposomal/CRISPR-Cas9 complex could lead to a correction that positively modifies biochemical and functional characteristics of the disease. This includes cardiovascular and skeletal impairments in MPS I animals when performed at an early stage of the development.[13,14] Nevertheless, hydrodynamic injections are hard to scale up if the treatment aims to reach clinical trials. Furthermore, intravenous injection of the liposome does not reach the brain, and therefore new delivery routes need to be tested.

Considering this limitation, we initially performed a pilot study to verify if the treatment could reach the brain and other organs when administered through the nasal route. As we have shown in the present study, the treatment increased IDUA activity[16] in several brain regions, as well as in some peripheral organs. This route has been extensively studied as an alternative to deliver different drugs to the central nervous system (CNS).[14,20] We have shown that the complexes were retained and permeated the PNM, which allowed the CNS to be reached, possibly via olfactory and trigeminal pathways.[28] The pilot study was highly positive, showing increased IDUA activity in the serum, the main organs, and especially in brain areas. As these results were favorable, we decided to perform an extended study to verify if these changes would be long lasting.

For this, a different subset of animals that received the treatment was euthanized 4 months after the last NA, at 6 months of age. To follow the enzyme production during and after treatment, blood samples were collected 15 and 30 days after administration, and then monthly until 6 months. Serum IDUA levels were stable over time. Since NA was performed daily during the first 30 days, we hypothesized that a cumulative increase in the activity could occur during that time, which was shown in our pilot study but was not reproduced long term. The fact that IDUA activity was detected in serum throughout the 6 months along with the gene editing efficiency results, suggests that gene editing indeed occurred in peripheral organs of treated mice.

Urinary and serum GAGs were also quantified. These molecules are the gold standard biomarkers to follow treatment in MPS. Their reduction also suggests the therapeutic efficacy of the treatment.[21] Despite the low enzymatic activity detected in serum, specific GAG levels of DS, HS–*OS*, and HS–*NS* were significantly reduced in serum samples at 6 months. In urine, this reduction was also observed for HS–*OS* and HS–*NS*.

IDUA and GAG levels were also assessed in tissues. An increase in enzyme activity was observed in different regions in the brain, mostly the olfactory bulb, but also in the frontal cortex and total cortex. Also, among the visceral tissues, the enzyme was detected in the heart and the lungs. These results confirm the findings from our previous studies, that this vector edits cells mainly in the heart and the lungs.[13,14] Administration through the nasal route also results in gene editing of cells from the olfactory bulb. IDUA activity was detected in other brain areas, but results from quantitative PCR suggest that the enzyme is transported from the olfactory bulb to other brain regions. It was previously shown that IDUA can be efficiently transported in an axonodendritic fashion,[29] and it provides a rationale for gene editing based on the release of therapeutic enzyme from the olfactory bulb.

In nose-to-brain delivery, the olfactory and trigeminal pathways are the only connection between the outside environment and brain tissue, as large molecules are prone to fail the entrance due to the low permeability of the blood–brain barrier.[15,28] Large molecules transported include big proteins or compounds, but also large plasmids such as the ones used in this study. Therefore, using this strategy, olfactory nerves and bulb are more likely to be edited. The enzyme produced by olfactory nerves and olfactory bulb may be rapidly diffused by olfactory and trigeminal pathways into the nearest regions in the brain, such as the frontal and total cortex.

Belur et al. (2017) observed a similar distribution of an Adeno-associated virus 9 (AAV9)-IDUA in brain tissue using the nasal approach to deliver the vector in MPS I mice. By the end of the treatment, high IDUA expression and activity were detected in the nasal epithelium and olfactory bulb. However, in the brain, only enzyme activity was detected. IDUA expression was also only detected in lungs and liver, even though AAV9 had a broad viral genome distribution.[30,31]

Gene editing of the heart and lungs can be facilitated, compared with other visceral tissues, as both are connected through the respiratory pathway. Moreover, it has been shown that the liposomal carrier has affinity for these organs when administered intravenously, which can contribute to the editing efficiency.[13] These factors can explain why enzyme activity in the heart and lungs was observed, which agrees with our correction efficiency data. Nevertheless, a significant reduction in GAGs in most of the tissues showed a cross-correction effect. Even though only few tissues produced enzyme—with IDUA levels corresponding to about 1% of a normal mouse—this may be enough to elicit a metabolic improvement in disease manifestations of MPS I mice. However, the significance of such improvement must be regarded with caution as no functional study in peripheral organs was performed.

The CRISPR/Cas9 system still has limitations. Not only the nature of Cas9 nuclease can activate the adaptive and innate immune response, but also hairpins present in the guide RNA have shown to activate Toll-like receptors.[11] However, the production of a new protein in the organism could also trigger an immune response. These hypotheses could help explaining the discrepancies observed in our short- and long-term studies, and these aspects will be addressed in future studies.

We also wanted to address if repeated administrations of the vector could cause permanent damage to the nasal tissue, since we have previously shown that a single administration causes a mild transient inflammation in the nasal tissue, detected by histology and increased serum IL-6.[16] Inflammation was assessed through IL-6 and IL-1b

levels. In this study, we found that both treated and untreated MPS I mice displayed similar levels of cytokines; therefore, we assume that the treatment itself causes no long-term damage to tissues. Nevertheless, the metabolic improvements found in animals were not sufficient to normalize cytokine levels, which were reported to be increased in MPS animal models as well as in Lysosomal storage diseases patients due to the natural course of the disease.[32–35]

Treatment of neurocognitive and behavioral manifestations in severe Hurler patients is still a challenge. The possibility to develop an efficient, noninvasive therapy that also enhances patient compliance needs to be further investigated. In this study, the prevention of neurocognitive impairment of treated mice was also evaluated. Previous work has shown that 6-month-old MPS I mice and wild-type mice already present significant differences in brain function.[36,37] This makes it possible to assess functional correction of neurological symptoms. Our results showed that, even though tissue enzyme activity was low, we still observe benefits in behavioral tests. However, the possibility of multiple administrations (unlike AAV vectors) allows a cumulative effect of the therapy. Furthermore, animals with higher brain IDUA activity and lower GAG levels tended to perform better in the tests **(as shown in Supplementary Figure S2).** Thus, to improve behavior and memory, the enzyme levels could be as low as 1% of a wild-type mouse, as suggested by our biochemical and behavioral tests.

5 | CONCLUSIONS

This study shows that NA of our liposomal complexes resulted in a significant increase in IDUA levels in some brain areas, as well as in peripheral organs. The enzyme produced was able to reduce GAG levels and improve behavioral parameters, suggesting a functional benefit. In this sense, we present a suitable strategy that could be used as a base for the development of a noninvasive therapy for MPS I patients with neurological impairment. Further studies need to be developed, aiming for a better biodistribution of the vector and higher editing efficiency, potentially leading to total normalization of GAGs and delaying or avoiding the progression of functional impairment.

DECLARATION OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

G.B. and H.F.T. were responsible for the study conceptualization and design. L.N.P.V. and R.S.S. were responsible for the experiments. L.N.P.V., R.S.S., E.P., E.P., and G.R. were responsible for nasal administrations. R.S.S. and F.N.S.F. were responsible for formulation preparation. L.N.P.V., E.C., B.B. and H.S. were responsible for DNA preparations. F.K. was responsible for tandem mass spectrometry analysis. L.N.P.V., R.S.S., and G.B. wrote the manuscript. G.B., R.G., U.M., and H.F.T. were responsible for funding acquisition. All authors approved the final version of the manuscript submitted for publication.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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