UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Fatores que influenciam a edição gênica com CRISPR/Cas9: prevalência de anticorpos anti-SpCas9 e panorama de uso na América do Sul

Lariane Frâncio

Orientadora: Profa. Dra. Ursula da Silveira Matte

Porto Alegre, dezembro de 2023

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

Fatores que influenciam a edição gênica com CRISPR/Cas9: prevalência de anticorpos anti-SpCas9 e panorama de uso na América do Sul

Lariane Frâncio

Tese submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da UFRGS como requisito parcial para a obtenção do grau de ***Doutor em Genética e Biologia Molecular****.*

Orientadora: Profa. Dra. Ursula da Silveira Matte

Porto Alegre, dezembro de 2023

INSTITUIÇÃO E FONTE FINANCIADORA

Este trabalho foi desenvolvido nos laboratórios Células, Tecidos e Genes (CTG) e Unidade de Pesquisa Laboratorial (UPL) ambos no Centro de Pesquisa Experimental do Hospital de Clínicas de Porto Alegre, e vinculado ao Departamento de Genética do Instituto de Biociências da Universidade Federal do Rio Grande do Sul, Brasil.

O estudo foi financiado pelo Fundo de Incentivo à Pesquisa e Eventos do HCPA (FIPE) e pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (nº 149092/2019-9). Todos os experimentos apresentados nesta tese estão incluídos nos projetos de pesquisa aprovados pelo Comitê de Ética e Pesquisa do Grupo de Pesquisa e Pós-Graduação (GPPG) do Hospital de Clínicas de Porto Alegre, sob o registro nº 2020-0097.

AGRADECIMENTOS

Agradeço, em primeiro lugar, a Deus, por tudo que Ele colocou no meu caminho. Obrigado, Senhor, por todas as bênçãos!

Aos meus amados e queridos pais, Cláudio e Odete, pela vida, pelos meus primeiros passos, pela minha formação, pelo inabalável laço de amor que nos une, pela incansável luta e incentivo nos momentos difíceis e nas alegrias e principalmente, por acreditarem em meus ideais.

À minha querida irmã Larissa, que muito me incentivou e por todo o seu amor, em especial a minha sobrinha Anna Emanuelle por ser a alegria da nossa família.

Ao meu amado namorado Aléxis Kin, pelo constante apoio, compreensão, dedicação, companheirismo, carinho, amor e incentivo para lutar pelos meus sonhos. Obrigada por não soltar a minha mão e caminhar ao meu lado.

À UFRGS, CNPq e HCPA, pela excelente oportunidade. Em especial ao secretário do Programa de Genética e Biologia Molecular, Elmo por toda ajuda e paciência ao longo do meu doutorado.

À minha orientadora, Profa. Dra. Ursula Matte, pela oportunidade, confiança, apoio, orientação, disponibilidade e dedicação para realização deste trabalho. Agradeço também pelas horas dispendidas, pelas palavras encorajadoras e principalmente pela paciência, compreensão e críticas, certamente levarei para minha vida.

À Marina Siebert pelos ensinamentos e conhecimentos passados, amizade, risadas, positivismo, paciência, empenho e disposição constante para o êxito de minhas atividades.

Aos colegas e amigos do Laboratório CTG (Células, Tecidos e Genes) do Centro de Pesquisa Experimental do Hospital de Clínicas de Porto Alegre, pelas ajudas, mas principalmente pelas amizades, conselhos, risadas e reflexões positivas para a vida.

Aos demais familiares e amigos que estiveram ao meu lado durante esta jornada.

E a todos que de alguma maneira contribuíram para a realização deste trabalho.

Muito obrigada!

Deus abençoe todos!

SUMÁRIO

LISTA DE ABREVIATURAS

AAV: vetores adeno-associados

Cas: do inglês, *CRISPR associated*

Cas9: do inglês, *CRISPR Associated protein 9*

CRISPR: do inglês, *Clustered Regularly Interspaced Palindromic Repeats* / CRISPR-*associated protein 9*

crRNA: do inglês, *CRISPR-derived RNA*

DNA: Ácido Desoxirribonucleico

DSB: do inglês, *Double-Strand Break*

ELISA: do inglês, *Enzyme-Linked Immunosorbent Assay*

EMA: do inglês, *European Medicines Agency*

FDA: do inglês, *Food and Drug Administration*

GAGs: Glicosaminoglicanos

gRNA: RNA guia

HDR: do inglês, *Homology-Directed Repair*

MPS: Mucopolissacaridose

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

MPS III D: Mucopolissacaridose tipo III D

MPS IV A: Mucopolissacaridose tipo IV A

MPS IV B: Mucopolissacaridose tipo IV B

MPS IX: Mucopolissacaridose tipo IX

MPS VI: Mucopolissacaridose tipo V I

MPS VII: Mucopolissacaridose tipo VII

NGG: qualquer nucleotídeo seguido por duas guaninas

NHEJ: do inglês, *Non-Homologous End Joining*

PAM: do inglês, *Protospacer Adjacent Motifs*

RNA: Ácido ribonucleico

RNP: Ribonucleoproteína / Complexo Ribonucleoproteico

Strep A: Streptococcus do grupo A

***S. aureus*:** *Staphylococcus aureus*

***S. pyogenes*:** *Streptococcus pyogenes*

TALEN: do inglês, *Transcription Activator-like Effector Nucleases*

TCTH: Transplante de Células Tronco Hematopoiéticas

tracrRNA: do inglês, *Trans-activating CRISPR-derived RNA*

TRE: Terapia de Reposição Enzimática

ZFN: do inglês, *Zinc Finger Nucleases*

RESUMO

A edição gênica pelo sistema CRISPR/Cas9 é a ferramenta mais promissora para correção de doenças genéticas devido à sua simplicidade e precisão, principalmente quando comparada a outros métodos. Muitos recursos permitiram a rápida introdução desse sistema em laboratórios de pesquisa. Porém, como novas tecnologias são introduzidas em ritmos e propósitos diferentes em diversos países. No primeiro trabalho, realizamos uma revisão da literatura sobre o progresso da pesquisa usando o sistema CRISPR/Cas na América do Sul, com foco em aplicações relacionadas à saúde. O banco de dados PubMed foi utilizado para identificar artigos relevantes sobre edição de genes com CRISPR/Cas, enquanto as patentes foram pesquisadas no banco de dados Patentscope. Além disso, ClinicalTrials.gov foi usado para encontrar informações sobre ensaios clínicos ativos e de recrutamento. Foram encontrados 668 artigos não duplicados (extraídos do PubMed) e 225 patentes (nem todas relacionadas à saúde). Cento e noventa e dois artigos sobre aplicações do CRISPR/Cas na área da saúde foram analisados detalhadamente. Em 95 destes, mais de 50% dos autores eram filiados a instituições sul-americanas. Os estudos experimentais CRISPR/Cas visam diferentes doenças, especialmente câncer, distúrbios neurológicos e endócrinos. A maioria das patentes refere-se a pedidos genéricos, mas aquelas com indicações claras de doenças são para erros inatos do metabolismo, distúrbios oftalmológicos, hematológicos e imunológicos. Não foram encontrados ensaios clínicos envolvendo países sul-americanos. Embora as pesquisas sobre edição de genes na América do Sul estejam avançando, nossos dados mostram o baixo número de inovações nacionais protegidas por propriedade intelectual nesse campo. No entanto, como em todas tecnologias, existem desafios a serem superados. A Cas9 mais utilizada é derivada de *Staphylococcus aureus* e *Streptococcus pyogenes*, duas espécies bacterianas que infectam a população humana, podendo ocorrer com maior frequência em pacientes propensos a doenças respiratórias, como os portadores de Mucopolissacaridoses (MPS). Essa imunidade existente pode diminuir a eficiência do tratamento ou levar a abordagens *ex-vivo*. O segundo trabalho busca entender a prevalência de anticorpos anti-SpCas9 em pacientes brasileiros com MPS tipo I e tipo II, para os quais estratégias de edição de genes estão sendo testadas em modelos animais. Foram coletadas amostras de plasma de doadores saudáveis (n = 90), pacientes com diagnóstico bioquímico de MPS I (n = 27) e MPS II (n = 37). Uma amostra de um paciente com infecção confirmada por *S. pyogenes* (n = 1) foi usada como controle positivo. O título de anticorpos foi determinado por ELISA e as amostras foram consideradas positivas se acima de três vezes o ponto de corte (valor obtido para a amostra positiva). A idade mediana dos doadores saudáveis foi de 38 anos (interquartil 27,5–46,25). Para MPS I foi de 3 anos (intervalo interquartil 1–6) e para MPS II 4 anos (intervalo interquartil 2–9,5). A prevalência de amostras positivas foi de 3,33% no grupo controle, 7,4% (MPS I) e 10,8% (MPS II) ($p<0,05$ entre grupo controle e MPS I e II). Nossos resultados mostraram uma prevalência significativamente maior de anticorpos contra SpCas9 em pacientes com MPS I e II em comparação com o grupo controle, sugerindo implicações importantes para futuros ensaios clínicos.

ABSTRACT

Gene editing by CRISPR/Cas9 system is the most promising tool for correcting genetic diseases because of its simplicity and precision, especially when compared to other methods. Many resources allowed the rapid introduction of this system into research laboratories. However, as new technologies are introduced at different paces and purposes in distinct countries. In our first study, we carried out a literature review on research progress using the CRISPR/Cas system in South America, focusing on health-related applications. The PubMed database was used to identify relevant articles about gene editing with CRISPR/Cas, whereas patents were searched in the Patentscope database. In addition, ClinicalTrials.gov was used to find information on active and recruiting clinical trials. A total of 668 non-duplicated articles (extracted from PubMed) and 225 patents (not all health-related) were found. One hundred ninety-two articles on health-related applications of CRISPR/Cas were analyzed in detail. In 95 out of these, more than 50% of the authors were affiliated with South American institutions. Experimental CRISPR/Cas studies target different diseases, particularly cancer, neurological, and endocrine disorders. Most patents refer to generic applications, but those with clear disease indications are for inborn errors of metabolism, ophthalmological, hematological, and immunological disorders. No clinical trials were found involving South American countries. Although research on gene editing in South America is advancing, our data show the low number of national innovations protected by intellectual property in this field. However, there are challenges to be overcome. The most used Cas9 is derived from *Staphylococcus aureus* and *Streptococcus pyogenes*, both bacterial species that infect the human population at high frequencies. This number could be higher in patients prone to respiratory diseases, such as those with Mucopolysaccharidoses (MPS). This existing immunity could decrease treatment efficiency or lead to *ex-vivo* approaches. In the second study, we aimed to understand the prevalence of anti-SpCas9 antibodies in Brazilian patients with MPS type I and type II. Plasma samples from healthy donors (n = 90), patients with biochemical diagnosis of MPS I (n = 27) and MPS II (n = 37) were collected. A sample from a patient with confirmed *S. pyogenes* (n = 1) infection was used as positive control. Antibody titer was determined by ELISA and samples were considered positive if above three times the cutoff point (value obtained for the positive sample). The median age of healthy donors was 38 years (interquartile 27.5–46.25). For MPS I it was 3 years (interquartile range 1–6), and for MPS II 4 years (interquartile range 2–9.5). The prevalence of positive samples was 3.33% in the control group, 7.4% (MPS I) and 10.8% (MPS II) ($p<0.05$ between control and MPS type I and type II). Our results showed a significantly higher prevalence of antibodies against SpCas9 in patients with MPS I and II compared to the control group, suggesting important implications for future clinical trials.

1. INTRODUÇÃO

1.1. O Sistema CRISPR/Cas9

As Repetições Palindrômicas Curtas Regularmente Espaçadas, conhecidas como CRISPR (do inglês, *Clustered Regularly Interspaced Short Palindromic Repeats*) foram inicialmente identificadas em *Escherichia coli* por Ishino e colaboradores em 1987. Em 2000, o grupo de Mojica identificou essas sequências em espécies microbianas (Mojica et al., 2000). Inicialmente chamadas de Repetições Curtas Regularmente Espaçadas (SRSRs, do inglês *Short Regularly Spaced Repeats*), essas sequências foram posteriormente denominadas CRISPR em 2002 (Jansen et al., 2002). Elas são compostas por 24 a 40 pares de bases (pb) e estão presentes em diversos grupos de procariontes, incluindo *archaea* e diferentes linhagens de bactérias gram-negativas e gram-positivas (Jansen et al., 2002; Mojica et al., 2000).

Em 2005, Mojica e colaboradores estabeleceram a conexão entre as sequências CRISPR e o sistema de defesa bacteriano contra elementos genéticos estrangeiros, como os vírus. Descobriu-se que essas sequências se incorporam ao genoma bacteriano como uma forma de memória imunológica. Quando a bactéria é reinfectada pelo vírus, por exemplo, as sequências CRISPR guiam as proteínas Cas (proteínas associadas ao CRISPR) para desativar o DNA viral, proporcionando imunidade (Mojica et al., 2005). Essa descoberta foi fundamental para a compreensão do sistema CRISPR e sua subsequente aplicação na edição gênica.

O mecanismo de ação das sequências CRISPR envolve a incorporação dos fragmentos do DNA viral como espaçadores entre as sequências repetidas específicas. Em seguida, a matriz CRISPR é transcrita em um único RNA então é processado em sequências mais curtas, o crRNA (CRISPR RNA), contendo cerca de 18 a 20 bases. No entanto, apenas o reconhecimento pelo crRNA não protege a bactéria, é necessário que a sequência exógena seja inativada por meio da clivagem pela proteína Cas. Os crRNAs se ligam ao RNA CRISPR transativador (tracRNA) e são reconhecidos e ligados pela proteína Cas9, formando um complexo ribonucleoproteico (RNP). A Cas9 age como uma nuclease, criando uma quebra da fita dupla (DSB, do inglês *double strand break*) ao cortar o DNA, inibindo assim o ciclo de vida do invasor (Jinek et al., 2012).

O sistema CRISPR-Cas, apresenta uma alta variabilidade em relação às sequências da proteína Cas. Com base na estrutura e funções das proteínas Cas, pode ser dividido em duas categorias e vários subtipos: Classe I (tipo I, III e IV) e Classe II (tipo II, V e VI). Os sistemas de classe I consistem em complexos de proteínas Cas de múltiplas subunidades, enquanto a Classe II requer apenas uma única proteína Cas como, por exemplo, a Cas9 (Makarova et al., 2011; Nishimasu et al., 2014). Devido à sua estrutura relativamente simples, o sistema CRISPR/Cas9 tipo II foi extensivamente investigado e empregado na engenharia genética (Liu et al., 2020).

Para simplificar e facilitar o acesso à técnica de edição gênica utilizando o sistema CRISPR/Cas9, dois componentes importantes, crRNA e tracrRNA, foram combinados em uma única molécula chamada RNA guia (sgRNA). O sgRNA geralmente contém cerca de 20 pb, com a sequência complementar ao sítio alvo do DNA de forma específica (Jinek et al., 2012). Nesse sistema simplificado, a proteína Cas9 age como uma "tesoura molecular", criando uma DSB. O sgRNA desempenha um papel crucial, guiando a Cas9 até um local genômico específico, previamente escolhido. Em 2012, Charpentier e Doudna purificaram a Cas9 de bactérias como *Streptococcus thermophilus* e *Streptococcus pyogenes* (Jinek et al., 2012; Gasiunas et al., 2012). A Cas9 extraída de *S. pyogenes*, chamada SpCas9, é uma enzima complexa composta por 1.368 aminoácidos (Anders et al. 2014; Jinek et al. 2014; Nishimasu et al. 2014; Jiang et al. 2016). Ela possui várias partes importantes: as regiões de reconhecimento (REC1 e REC2) que se conectam ao RNA guia, a região de nuclease (NUC) com os domínios RuvC e HNH, que cortam as fitas simples do DNA, e o domínio *Protospace Adjacent Motif* (PAM), essencial para a atividade da enzima e reconhecimento do local de ligação ao DNA alvo (Nishimasu et al., 2014).

O mecanismo de edição dessa ferramenta pode ser dividido em três etapas: reconhecimento, clivagem e reparo. O RNA guia específico (sgRNA) direciona a proteína Cas9 para a sequência alvo, no gene de interesse, reconhecendo-a através do seu pareamento complementar com o componente 5' do crRNA. Quando o local com a sequência alvo e o padrão PAM adequado (5′-NGG-3′) é encontrado, a Cas9 cria DSBs três bases à montante da PAM. O domínio HNH corta a cadeia complementar, enquanto o domínio RuvC cliva a cadeia não complementar do DNA, produzindo DSBs predominantemente com extremidades cegas. Posteriormente, o DSB é reparado pela maquinaria celular hospedeira (Jiang and Doudna, 2017).

Existem duas vias principais de reparo utilizadas: via de reparo dirigido por homologia (HDR, do inglês *homology directed repair*) ou pela via de junção de extremidades não homólogas (NHEJ, do inglês *non-homologous end joining*). O NHEJ é um mecanismo eficiente, mas propenso a erros, unindo fragmentos de DNA na ausência de uma sequência homóloga. É ativo em todas as fases do ciclo celular e pode resultar em pequenas inserções ou deleções aleatórias (INDELs) no local de clivagem. Assim, essa técnica pode ser utilizada para nocaute gênico. Por outro lado, o HDR é altamente preciso e pode ser usado para deleção, inserção ou correção direcionada de genes. O HDR utiliza modelos de DNA doador como homologia de sequência no local da DSB previsto na realização da edição gênica (Figura 1) (Liu et al., 2019; Yang et al., 2020).

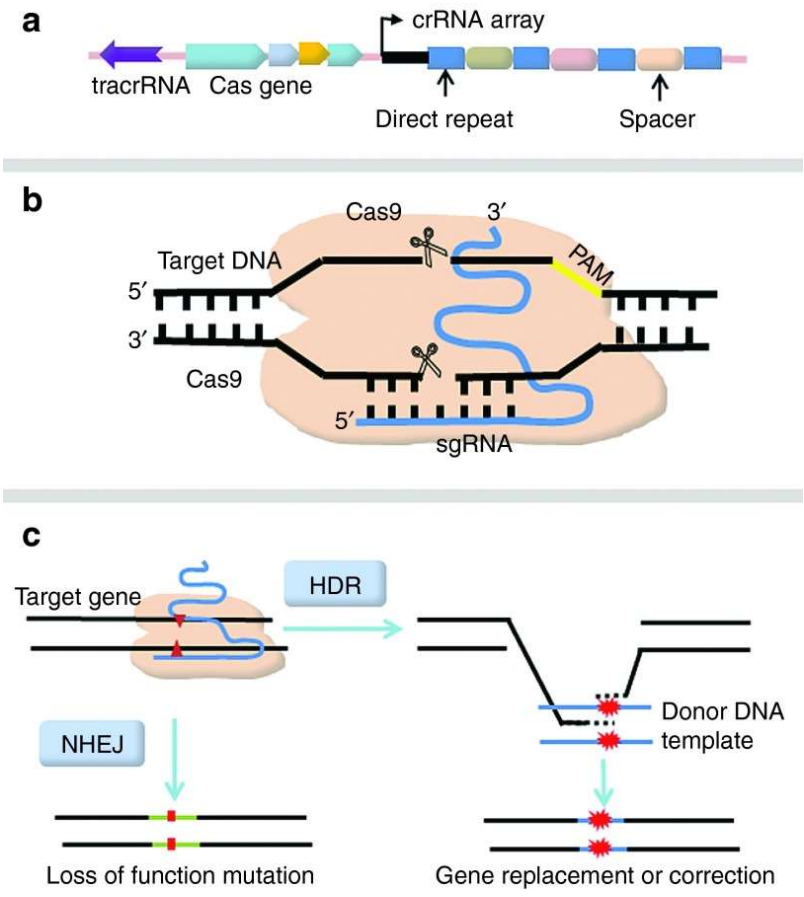

Figura 1: Representação esquemática da edição gênica pelo sistema CRISPR-Cas9. (a) Esquema do locus CRISPR (de *Streptococcus pyogenes*). (b) Clivagem específica do DNA pela Cas9, dirigida pelo RNA guia (sgRNA) e a sequência PAM. (c) Quebra da fita dupla (DSBs) subsequentemente reparada pela junção de extremidades não homólogas (NHEJ) ou pelo reparo dirigido por homologia (HDR) (Dai et al., 2016).

Apesar da existência de uma variedade de ortólogos da enzima Cas9 já caracterizados, as mais comumente utilizadas na edição gênica são derivadas das bactérias *S. pyogenes* (SpCas9) e *Staphylococcus aureus* (SaCas9) (Jinek et al., 2012; Ran et al., 2015). Ambas essas variantes, embora sejam patogênicas para os humanos, têm a notável capacidade de clivar o DNA em qualquer local específico do genoma desejado. A SpCas9 foi a primeira a ser adaptada para a edição de genes em células de mamíferos, marcando um avanço significativo nesta área de pesquisa (Cong et al., 2013). Assim, o sistema CRISPR/Cas9 fornece uma plataforma eficiente e precisa para edição de genes específicos em uma variedade de organismos e áreas de interesse (Hsu, Lander e Zhang, 2014).

O CRISPR/Cas9 oferece várias vantagens significativas que incluem: alta precisão ao modificar genes específicos; facilidade de uso e custos acessíveis se comparados a outras técnicas de edição de genes; rápido *design* do RNA guia, permitindo orientar a Cas9 ao local desejado; versatilidade por ser aplicado em uma ampla variedade de organismos; alta eficiência, garantindo elevada taxa de sucesso na indução de modificações genéticas desejadas; e altamente adaptável, sendo utilizado em diversas aplicações, desde a correção de mutações genéticas até a regulação da expressão gênica (Doudna , 2020; Uddin, Rudin e Sen, 2020).

Sua aplicação terapêutica também é bastante promissora, oferecendo tratamentos personalizados para doenças genéticas específicas. Seja através da edição *ex vivo* de células seguida de transplante de células em um paciente (Frangoul et al., 2021), seja através da edição *in vivo* de células de um paciente através da entrega do sistema CRISPR/Cas9 usando vetores virais (Maeder et al., 2019) ou não virais (Gillmore et al., 2021). Além disso, o CRISPR/Cas9 possui a capacidade não apenas de editar um único gene, mas também de modificar vários genes simultaneamente, como evidenciado em estudos recentes. No contexto do tratamento do vírus da hepatite B (HBV), por exemplo, foi realizado um experimento envolvendo a desativação simultânea de múltiplos genes por meio dessa técnica (Kato et al., 2021).

Desde a sua descoberta em 2012, a técnica de edição de genes CRISPR/Cas9 representa uma promissora solução para uma vasta gama de doenças genéticas conhecidas. Muitas dessas condições carecem de tratamentos eficazes, e grande parte delas é resultado de mutações em um único gene, como ocorre em doenças como mucopolissacaridoses, doença falciforme, β-talassemia, fibrose cística e distrofia muscular, entre outras (Pandey et

al., 2017). Embora a aplicação do CRISPR/Cas9 para doenças monogênicas ainda esteja em estágios de pesquisa e desenvolvimento, as perspectivas terapêuticas são animadoras, representando um passo importante no campo da medicina personalizada. No período de 1998 a 2019, 22 terapias gênicas, incluindo aquelas que empregam a tecnologia CRISPR/Cas9, foram aprovadas para o tratamento de doenças humanas (Ma et al., 2020).

Além disso, as estratégias versáteis e adaptáveis de entrega do sistema CRISPR/Cas9 também são uma das vantagens que essa ferramenta proporciona devido à flexibilidade na escolha do método de entrega. Existem três estratégias desenvolvidas para a edição de genes utilizando o sistema CRISPR/Cas9. Na primeira, baseada em plasmídeo, a proteína Cas9 e o sgRNA são codificados em um mesmo plasmídeo que é introduzido na célula. Esta abordagem garante uma expressão duradoura da Cas9 e do sgRNA, além de evitar múltiplas transfecções. No entanto, o desafio fundamental é introduzir eficientemente o plasmídeo no núcleo das células-alvo. A segunda estratégia consiste na entrega intracelular direta do RNA mensageiro da Cas9 (mRNA) e sgRNA. A célula utiliza o mRNA para produzir temporariamente a Cas9 e o sgRNA para permitir que a edição ocorra. Por fim, a terceira estratégia envolve a entrega direta da proteína Cas9 e do sgRNA. Essa abordagem oferece diversas vantagens, incluindo ação rápida, maior estabilidade e resposta imunológica limitada, tornando-a uma opção altamente eficaz para a edição gênica (Liu et al., 2017).

Devido a todo esse conjunto de vantagens, o sistema CRISPR/Cas9 torna-se uma tecnologia ideal de edição de genes. Seu avanço tem impactado positivamente muitos setores no mundo, sendo sua aplicação mais adequada em comparação com outras tecnologias de edição de genes, como por exemplo as nucleases dedo de zinco (ZFNs, do inglês *zinc finger nucleases*), as meganucleases e as nucleases efetoras semelhantes à ativadores de transcrição (TALENs, do inglês *transcription activator-like effector nucleases*) (Knott e Doudna, 2018). Partindo-se desse avanço, no primeiro artigo original da presente tese de doutorado serão apresentados detalhes sobre o panorama das aplicações na saúde e ensaios clínicos mais recentes do CRISPR/Cas9, especialmente na América do Sul.

Apesar dos progressos notáveis em diversos estudos e na aplicação clínica da edição gênica pelo sistema CRISPR/Cas9, ainda há uma lacuna na sua implementação em grande escala. Um dos principais desafios a serem abordados é a investigação dos potenciais efeitos colaterais do sistema CRISPR/Cas9, algo crucial para garantir sua segurança e eficácia a longo prazo. Entre os desafios enfrentados, um dos pontos críticos a ser considerado é a

imunogenicidade do sistema, pois reações imunológicas podem levar à eliminação das células editadas (Li et al., 2020).

Independentemente do vetor ou da abordagem empregada, a terapia gênica, enfrenta desafios relacionados à resposta imune, tanto contra o vetor, com também contra o transgene. A eficácia dos tratamentos, especialmente *in vivo*, depende da superação de três barreiras imunológicas significativas como ilustrado na figura 2, que são (i) evitar a neutralização do sistema de administrado por anticorpos; (ii) evitar resposta contra o vetor ou seu conteúdo após a entrega; e (iii) evitar resposta imune contra o produto do gene corrigido (Freitas et al., 2022).

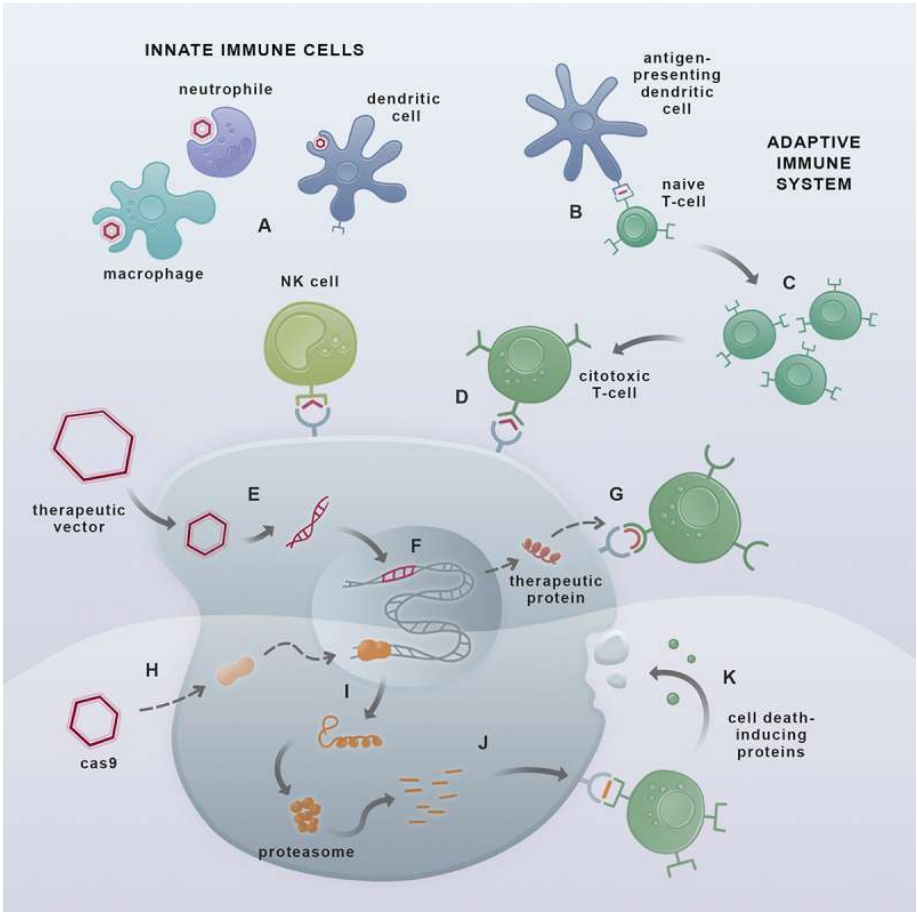

Figura 2: Barreiras imunológicas na terapia gênica e edição de genes. Sob infecção, as defesas primárias são as células do sistema imunológico inato. O conteúdo viral pode ser reconhecido e destruído pelas diferentes células fagocíticas ou outras células, como as células dendríticas ou células *natural killer* (NK) que destroem as células infectadas após interações específicas com receptores (A). A segunda resposta pode ser desencadeada por células apresentadoras de antígenos que conectam os sistemas inato e adaptativo (B). Este contato resulta na supervisão de células T virgens (C) que responde contra o antígeno através de células T efetoras (D). Quando o vetor evita o sistema imune inato, a resposta pode ocorrer mediante o reconhecimento de partes do vetor (E) ou, após a integração do transgene no genoma do hospedeiro (F) sob o reconhecimento do produto transgênico como não próprio (G). A intensidade desta resposta pode depender da existência parcial do produto gênico a

ser inserido. Por fim, a abordagem de edição gênica (H) apresenta um alvo imunológico adicional: a própria proteína de edição. Após promover a edição do gene, a proteína segue a via de degradação (I), resultando em pequenos peptídeos estranhos (J) que podem ser apresentados para as células T citotóxicas. De qualquer forma, a ativação de células CD8+ leva à produção de citocinas pró-inflamatórias, resultando na morte celular (K) **(**Freitas et al., 2022).

Dado que a interação com o sistema imunológico pode impactar os resultados da terapia gênica diversos estudos estão investigando sua interação com o sistema imunológico humano, especialmente com o uso do sistema CRISPR/Cas9. O objetivo é desenvolver soluções que minimizem preocupações de segurança e combatam respostas indesejadas do organismo, visando alcançar uma eficácia a longo prazo na terapia de edição de genes. É essencial reconhecer que o sistema imunológico enfrenta desafios em distinguir quando essas moléculas são utilizadas em benefício do paciente (Freitas et al., 2022).

Para contornar essas questões, estratégias variadas são adotadas, dependendo do tipo de vetor utilizado e de sua interação com o sistema imunológico, tais como: (i) métodos que ocultam o vetor e/ou o produto do sistema imunológico e (ii) métodos que ocultam o sistema imunológico do produto vetor/transgene, ilustradas na figura 3 (Freitas et al., 2022).

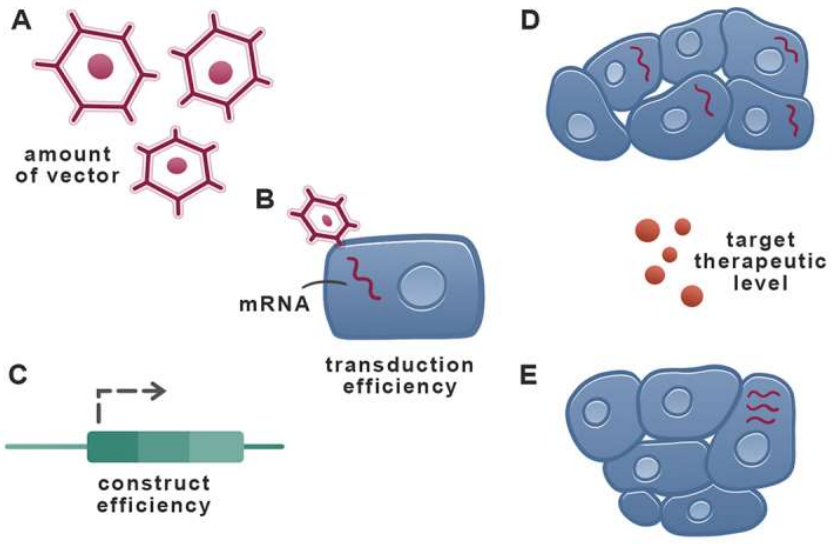

Figura 3: Diferentes estratégias que podem ser utilizadas de forma independente ou combinadas. Idealmente, a quantidade de vetor pode ser controlada para diminuir a resposta imune (A). Isto pode ser compensado por vetores com maior eficiência de transdução (B) e/ou construções com maior expressão de transgene (C). O resultado desejado é um grande número de células que expressam o transgene (D), em oposição a algumas células de alta expressão que podem ser mais facilmente detectadas por uma resposta imune contra a proteína terapêutica (E) (Freitas et al., 2022).

Aproximadamente 40% da população humana é colonizada por *S. aureus* e 20% das crianças em idade escolar são colonizadas por *S. pyogenes*, com anticorpos e células T contra ambas as bactérias ocorrendo em altas porcentagens em adultos. A abundância de *S. aureus* e *S. pyogenes* na população humana, bem como respostas imunes adaptativas humoral e mediadas por células a ambas as espécies, levanta a possibilidade de que os humanos também possam ter imunidade adaptativa pré-existente à Cas9 derivada destas bactérias (Charlesworth et al., 2019). Considerando a alta prevalência de infecção por *S. pyogenes*, Wagner et al. (2019) e Charlesworth et al. (2019), levantaram a hipótese de que a SpCas9 poderia provocar uma resposta imune adaptativa em humanos, visto que a maioria das aplicações terapêuticas visa expressar temporariamente a nuclease Cas9 ou entregar a proteína diretamente nas células-alvo. A presença de respostas imunes adaptativas pré-existentes em humanos, portanto, pode dificultar o uso seguro e eficaz do sistema CRISPR/Cas9 ou causar toxicidade significativa ao tratar pacientes com esta tecnologia. Assim, se os humanos possuem uma resposta imune adaptativa pré-existente à Cas9, então terapias baseadas com a utilização da Cas9 podem sofrer problemas, como por exemplo, uma resposta do linfócito T citotóxico contra a Cas9, irá resultar na destruição de células que apresentem peptídeos Cas9, eliminando assim as células editadas e tornando a terapia ineficaz (Charlesworth et al., 2019; Wagner et al.,2019; Ferdosi et al.; 2019)

As respostas imunes específicas não garantem reações adversas nos pacientes, mas estudos pré-clínicos sugerem riscos. A entrega de SpCas9 em camundongos mostrou edição bem-sucedida do gene *Pten*, apesar de respostas inflamatórias. No entanto, a imunização contra SaCas9 levou a apoptose de hepatócitos e falha na edição (Wang et al., 2015; Li et al., 2020) (Figura 4). Em outro estudo, a expressão de Cas9 em músculos de camundongos resultou em infiltração de linfócitos, indicando uma reação imunológica (Chew et al., 2016). Ajina et al. (2019) demonstraram que tumores que expressam SpCas9 foram rejeitados por células T específicas em camundongos imunocompetentes, mas não em imunodeficientes, sugerindo que níveis mais altos de expressão da Cas9 podem desencadear respostas imunes prejudiciais. Estes resultados têm implicações sérias para terapias que exigem expressão persistente de Cas9 *in vivo* ou dosagens repetidas para serem eficazes clinicamente.

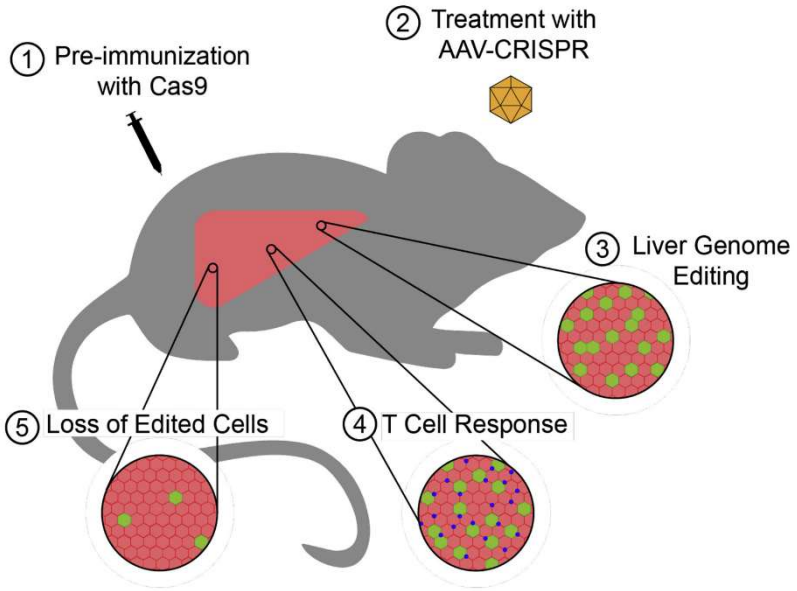

Figura 4: Esquema da imunidade pré-existente para Cas9. Em camundongos imunizados com proteína Cas9 antes do tratamento com AAV-CRISPR/Cas9, a edição é seguida por uma resposta de células T que resulta na perda das células editadas (Gough e Gersbach, 2020).

No estudo conduzido por Charlesworth et al. em 2019, foi investigada a presença de imunidade adaptativa em humanos em relação aos dois ortólogos de Cas9 mais amplamente utilizados, SaCas9 e SpCas9. Utilizando a técnica de ELISA, os pesquisadores identificaram a presença de anticorpos contra SaCas9 e SpCas9 em 78% e 58% da população estudada, respectivamente. Além disso, ao analisar um grupo de 18 doadores em relação às células T reativas ao antígeno Cas9, descobriram que 78% e 68% desses doadores possuíam células T reativas contra SaCas9 e SpCas9, respectivamente.

Em outro estudo relacionado conduzido por Simhadri et al. em 2018, anticorpos contra Cas9 também foram detectados usando a técnica de ELISA em uma amostra de 200 doadores. Os resultados revelaram que 10% da população avaliada dos Estados Unidos possuía anticorpos anti-SaCas9, enquanto apenas 2,5% possuíam anticorpos anti-SpCas9. Vários estudos detectaram imunidade pré-existente contra SpCas9 e SaCas9 na maioria dos indivíduos saudáveis testados, com porcentagens que variam de 57% a 95%. (Tabela 1). Essas descobertas destacam a variabilidade na resposta imunológica das pessoas aos ortólogos de Cas9, um aspecto crucial a considerar em pesquisas e aplicações envolvendo essa tecnologia.

Tabela 1. Estudos que relataram a presença de resposta imune às proteínas efetoras CRISPR em indivíduos saudáveis.

Tipo da Cas	Organismo	Indivíduos que apresentam resposta imune adaptativa pré-existente (%)		População total do estudo	Referência
		Anticorpo	Célula T		
Cas9	*S. pyogenes*	2.5%	N/A	200	Simhadri et al. (2018)
Cas9	*S. aureus*	10%	N/A		
Cas9	*S. pyogenes*	58%	67%	125 (Anticorpo), 18 (Célula T)	Charlesworth et al. (2019)
Cas9	*S. aureus*	78%	78%		
Cas9	*S. pyogenes*	N/A	95%	45	
Cas9	*S. aureus*	N/A	100%	6	Wagner et al. (2019)
Cas12a	*Acidaminococcus* sp.	N/A	100%	6	
Cas9	*S. pyogenes*	5% (soro)	83%	143 (Anticorpo), 12 (Célula T)	Ferdosi et al. (2019)
Cas9	*S. pyogenes*	0% (soro)	66.7%	3	Stadtmauer et al. (2020) *clinical trial
Cas13d	*Ruminococcus flavefaciens*	89%	96%/100% ($CD8^+/CD4^+$)	19 (Anticorpo), 24 (Célula T)	
Cas9	*S. pyogenes*	95%	96%/92% ($CD8^+/CD4^+$)		Tang et al. (2022)
Cas9	*S. aureus*	95%	96%/88% ($CD8^+/CD4^+$)		
Cas9	*S. aureus*	4.8%	70%	123 (Anticorpo), 10 (Célula T)	Shen et al. (2022)
Cas9	*S. pyogenes*	100% (soro) / 15% (líquido vítreo)	N/A	13	Toral et al. (2022)
Cas9	*S. aureus*	100% (soro) / 15% (líquido vítreo)			

Fonte: Adaptado de (Ewaisha and Anderson, 2023).

Até o momento, os estudos descritos na tabela 1, enfatizam a importância de considerar fatores específicos dos pacientes para personalizar os tratamentos, principalmente pelo avanço da ferramenta para as aplicações clínicas. Estas personalizações incluem o estado imunológico, respostas imunológicas prévias, histórico de tratamento e risco imunológico. Porém, essa necessidade de verificar se o paciente tem ou não imunidade à Cas9, aos componentes CRISPR e aos vetores virais torna o uso da ferramenta mais desafiador e complexo.

Ainda, estudos pré-clínicos mostraram que a utilização do sistema CRISPR para tratamentos, pode induzir reações imunes que variam de acordo com o método de administração e o histórico da doença (Chew, 2018). Assim, o sucesso da utilização do sistema CRISPR/Cas9 em camundongos informa, mas não garante, a segurança clínica. Devido a isso, precisamos encontrar maneiras de prever e resolver problemas relacionados à imunogenicidade do CRISPR/Cas9 em pacientes humanos.

1.2. Mucopolissacaridoses

As doenças de armazenamento lisossômico (LSDs) representam um conjunto de mais de 70 doenças raras, cada uma causada por mutações em um único gene. Essas mutações resultam na alteração lisossomal e no acúmulo gradual de substratos dentro do lisossomo, o que leva à disfunção e morte celular (Platt et al., 2018). A maioria dos genes causadores codificam enzimas lisossômicas ou proteínas envolvidas na modificação de enzimas lisossômicas ou transporte, mas também podem codificar proteínas da membrana lisossômica. Normalmente se manifestam na infância e em conjunto afetam cerca de 1 em cada 5 mil nascidos vivos. O número real, entretanto, pode ser maior se os casos não diagnosticados e/ou diagnósticos incorretos forem contabilizados, especialmente considerando que há formas de início na idade adulta, as quais são frequentemente diagnosticadas erroneamente. No entanto, as LSDs avaliadas individualmente são raras, com incidências variando de 1 em 50.000 a 1 em 250.000 nascidos vivos (Platt, 2018).

Aproximadamente 70% das LSDs são doenças neurodegenerativas. Além disso, órgãos e tecidos periféricos também são frequentemente afetados, e a maioria são crônicas (Platt, 2018). Dos aproximadamente 1.300 genes envolvidos na função lisossômica, muitos distúrbios monogênicos foram descritos, incluindo 50 deficiências enzimáticas, que podem

ser subclassificadas de acordo com o tipo bioquímico do substrato armazenado (Platt et al., 2018), como: distúrbios do armazenamento de lipídios, mucopolissacaridoses, distúrbio do armazenamento de glicoproteínas, mucolipidoses e cistinose (Marques; Saftig, 2019).

As mucopolissacaridoses (MPS) são um grupo de doenças lisossômicas causadas por deficiências em enzimas envolvidas na quebra de glicosaminoglicanos (GAGs) nos lisossomos das células (Clarke, 2008). Dependendo da deficiência enzimática ocorre o acúmulo de GAGs específicos (sulfato de condroitina, dermatan sulfato, heparan sulfato, queratan sulfato e ácido hialurônico) em diferentes tecidos, órgãos, que resultam em complicações sistêmicas, desde o envolvimento do sistema nervoso central (SNC) até a falência múltipla de órgãos (Giugliani et al., 2012; Kobayashi, 2019; Williams et al., 2019).

O grupo das MPS é categorizado em sete tipos, os quais incluem doenças decorrentes de onze defeitos enzimáticos diferentes, cada um afetando uma única etapa na via de degradação dos GAGs (Giugliani et al., 2012). A maioria dessas condições (MPS I, MPS III A, MPS III B, MPS III C, MPS III D, MPS IV A, MPS IV B, MPS VI, MPS VII e MPS IX) são herdadas de forma autossômica recessiva, enquanto a MPS II é recessiva ligada ao X (Celik et al., 2021). A incidência de todos os onze tipos de MPS é de aproximadamente 1 em 20.000–25.000 nascidos vivos (Zhou et al., 2020). De acordo com Borges et al. (2020), a incidência global a cada 100.000 nascidos vivos para cada tipo de MPS foi de: MPS I (7.10–2.48), MPS III A (2.36–0.41), MPS III B (1.53–0.37), MPS III C (1.57–0.11), MPS III D (0.46–0.05), MPS IV A (2.36–0.25), MPS IV B (1.68–0.46), MPS VI (1.12–0.18), MPS VII (1.14–0.21) e MPS IX (0.44–0.11).

No Brasil, entre os anos 1982 a 2019, 1.652 pacientes brasileiros foram diagnosticados com MPS no Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre. A MPS I foi classificada como a terceira condição mais diagnosticada, com um total de 315 casos (19,07%). Por outro lado, a MPS II foi a mais diagnosticada, com 493 casos (29,84%). Além disso, a MPS II não apenas representa a MPS mais comum, mas também é a segunda doença de depósito lisossomal mais prevalente diagnosticada no Hospital de Clínicas de Porto Alegre (Josahkian et al., 2021).

Analisando os dados de 1994 a 2018, a incidência geral calculada para MPS no Brasil foi de 1,57 em 100 mil nascidos vivos, totalizando 1.164 pacientes com diagnóstico de MPS. A incidência por 100.000 nascidos vivos para cada tipo de MPS foi a seguinte: MPS I (0,29), MPS II (0,48), MPS III A (0,08), MPS III B (0,12), MPS III C (0,07), MPS III D (0,001), MPS IV A (0,15), MPS IV B (0,003), MPS VI (0,35), MPS VII (0,02) e MPS IX (0). Já as

taxas de incidência de MPS para cada região brasileira foram: Nordeste (1,78), Sul (1,66), Sudeste (1,62), Centro-Oeste (1,26) e Norte (0,88). A MPS II foi predominante em todas as regiões brasileiras (Giugliani et al., 2017; Josahkian et al., 2021).

As diferentes MPS estão descritas na Tabela 2, e as MPS dos tipos I e II serão apresentadas em maiores detalhes a seguir.

Tabela 2. Características principais das Mucopolissacaridoses (MPS).

MPS	***Síndrome***	***Gene Associado***	***Enzima Deficiente***	***GAG Acumulados***	***Tratamento***
MPS I	Hurler e Scheie	*IDUA*	α-L-iduronidase	HS, DS	TRE, TCTH
MPS II	Hunter	*IDS*	Iduronato-2-sulfatase	HS, DS	TRE, TCTH
MPS III A	Sanfilipo A	*SGSH*	Heparan-N-sulfatase	HS	Sintomático / Suporte
MPS III B	Sanfilipo B	*NAGLU*	α-N- acetil-glicosaminidase	HS	Sintomático / Suporte
MPS III C	Sanfilipo C	*HGSNAT*	Acetil-CoA: a-glicosaminideo acetiltransferase	HS	Sintomático / Suporte
MPS III D	Sanfilipo D	*GNS*	N-acetilglicosamina-6-sulfatase	HS	Sintomático / Suporte
MPS IV A	Morquio A	*GALNS*	Galactose-6-sulfatase	KS, CS	TRE, TCTH
MPS IV B	Morquio B	*GLB1*	ß-galactosidase	KS, CS	TRE, TCTH
MPS VI	Marateaux-Lamy	*ARSB*	N-acetilgalactosamina 4-sulfatase	DS, CS	TRE, TCTH
MPS VII	Sly	*GUSB*	ß-glucuronidase	HS, DS, CS	TRE, TCTH
MPS IX	Natawiez	*HYAL1*	Hialuronidase	Hialuronan	Sintomático / Suporte

GAGs: glicosaminoglicanos; HS: sulfato de heparan; DS: sulfato de dermatan; KS: sulfato de queratan; CS: sulfato de condroitina. Adaptado de (Celik et al., 2021).

O acúmulo de heparan sulfato (HS) e dermatan sulfato (DS) nos lisossomos desencadeia uma cascata de eventos celulares que leva à disfunção de órgãos. Na apresentação geral as MPS I e MPS II possuem quadro clínico semelhante com manifestações clínicas que variam desde formas atenuadas até formas mais graves da doença. Por razões históricas a MPS I é classificada em três formas, uma forma atenuada conhecida como Síndrome de Scheie (MPS I S) (OMIM 607016), uma forma intermediária conhecida como Síndrome de Hurler-Scheie (MPS I H/S) (OMIM 607015) e uma forma grave conhecida como Síndrome de Hurler (MPS I H) (OMIM 607014) (Figura 5). A MPS II também possui duas formas de manifestações, atenuada e grave, porém é conhecida somente por Síndrome de Hunter (OMIM 309900) (Figura 6) (Giugliani et al., 2010).

Os pacientes que manifestam as formas graves apresentam sinais e sintomas que ocorrem no início da infância em vários órgãos simultaneamente, sendo que frequentemente apresentam comprometimento cognitivo significativo. Os sintomas podem começar no primeiro ano de vida com manifestações de sintomas graves e multissistêmicos, e sem tratamento adequado, pacientes podem vir a óbito na primeira década de vida. Já os pacientes com a doença atenuada manifestam menos sinais e sintomas, sendo que costumam se manifestar mais tarde e geralmente não apresentam comprometimento cognitivo. Em muitos casos, esses pacientes permanecem sem o diagnóstico por anos (Vijay and Wraith, 2005). Pacientes com sintomas mais leves podem atingir a idade adulta, mas também sofrem de síndromes multissistêmicas (Zhou et al., 2020).

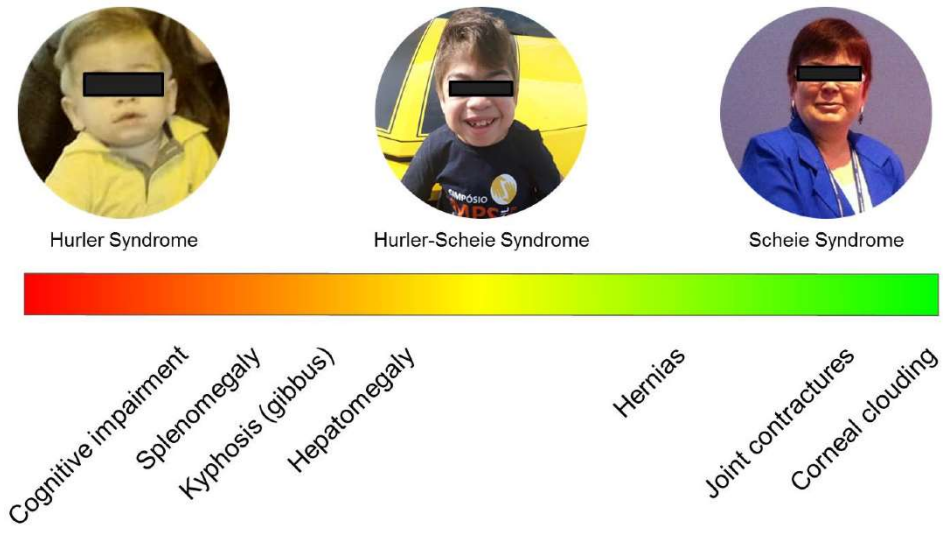

Figura 5: Espectro dos pacientes com MPS I. As manifestações escritas à esquerda são mais encontradas em pacientes com fenótipos graves, enquanto aquelas à direita são frequentemente vistas em pacientes com fenótipos atenuados (Kubaski et al., 2020).

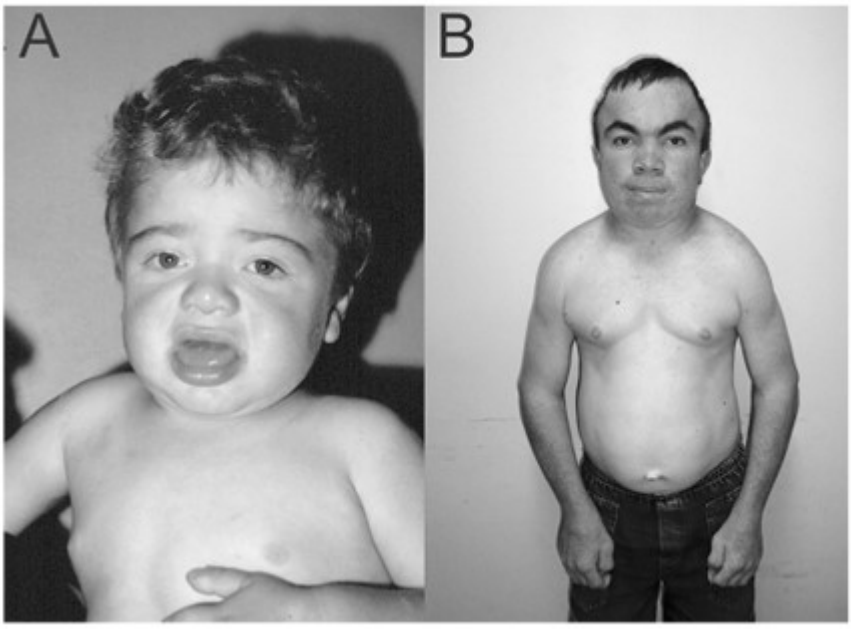

Figura 6: Características da síndrome de Hunter. (A) Criança com 2 anos de idade com fenótipo severo. (B) Adulto com fenótipo atenuado (Giugliani et al., 2014).

Os sintomas observados nas MPS I (Figura 7) e MPS II são bastante similares, sendo perda cognitiva, hepatoesplenomegalia, malformações esqueléticas, anormalidades, complicações cardiorrespiratórias, infecções respiratórias das vias aéreas superiores, perda auditiva e, em muitos casos, degeneração do sistema nervoso central. Cada tipo de MPS tem uma ampla gama de manifestações clínicas, dependendo do GAG envolvido. Cada GAG desempenha seu papel fisiológico e é expresso em tecidos específicos. Por exemplo, MPS I, MPS II, MPS III A-D e MPS VII, com acúmulo predominante de heparan sulfato (HS) tendem a apresentar envolvimento do SNC, enquanto MPS IVA B e MPS VI, com acúmulo predominante de dermatam sulfato (DS) e queratan sulfato (KS) apresentam manifestações esqueléticas (Wood and Bigger, 2022). Devido ao envolvimento de vários órgãos e tecidos, os pacientes frequentemente necessitam de intervenções cirúrgicas com alto índice de complicações (Giugliani et al., 2010).

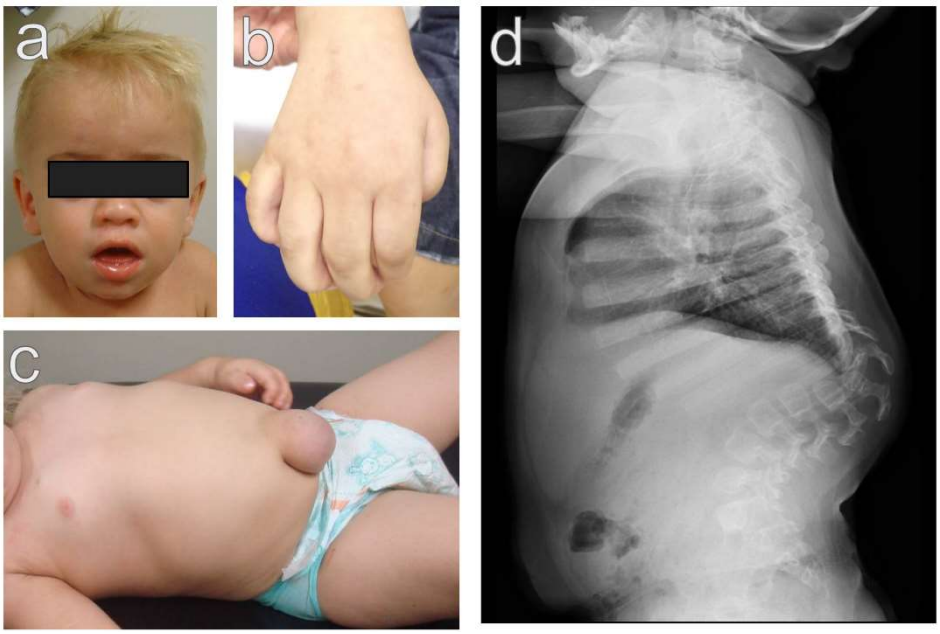

Figura 7: Características da síndrome de Hurler. (a) Características faciais grosseiras. (b) Mãos em garras devido à camptodactilia dos dedos. (c) Hérnia umbilical. (d) Vista lateral da coluna vertebral - cifose toracolombar e costelas largas (Kubaski et al., 2020).

Dentre as manifestações otorrinolaringológicas incluem-se perda auditiva, diminuição da função pulmonar, apneia obstrutiva do sono e obstrução das vias aéreas superiores. Além disso, pacientes com MPS tipo I e tipo II apresentam comprometimento respiratório com disfunção do trato respiratório superior e infecções respiratórias frequentes e recorrentes como sinusite, rinite, otite, bronquite e pneumonia (Giugliani et al., 2010; Tulebayeva et al., 2020), estando presentes em 40% dos pacientes (Muhlebach et al., 2011; Muenzer, 2011). Os subtipos I, II, VI e VII são propensos à obstrução da via aérea por apresentarem gengivas espessas, língua espessada e ingurgitamento de tecidos moles da nasofaringe. O aumento das tonsilas e adenóides causada pelo acúmulo de GAGs nos tecidos linfáticos também contribui para a obstrução (Cimaz e La Torre, 2014). Complicações das vias aéreas podem ser agravadas por secreções excessivamente espessas decorrentes de infecções crônicas ou de repetição (Simonetti et al., 2009). O envolvimento do sistema respiratório no processo patológico da MPS ocorre em 56-63% dos casos. A mortalidade em pacientes com MPS resulta principalmente do comprometimento progressivo dos sistemas cardiovascular e respiratório (Lavery e Hendriksz et al., 2015; Lin et al., 2016).

Nesse sentido é fundamental destacar que pacientes com MPS enfrentam frequentes infecções respiratórias. Distúrbios respiratórios são uma característica presente em todos os tipos de MPS, com manifestações otorrinolaringológicas frequentemente sendo as primeiras a surgirem e tendendo a se agravar com o tempo, como indicado por Berger et al. (2013). Assim, o uso de estratégias baseadas em Cas9 pode apresentar riscos, visto que as Cas9 mais comumente utilizadas provêm de *S. aureus* e *S. pyogenes* e a faringite relacionada ao *S. pyogenes* é uma das doenças mais prevalentes causadas por essa bactéria no mundo. Partindo dessa perspectiva, a tese de doutorado em questão tem como objetivo principal investigar a prevalência de anticorpos anti-*Streptococcus pyogenes* Cas9 em indivíduos saudáveis, bem como em pacientes diagnosticados com Mucopolissacaridose tipo I (MPS I) e Mucopolissacaridose tipo II (MPS II). Esse estudo está detalhado no segundo artigo original desta tese.

As MPS podem frequentemente ser confundidas com outras condições devido à diversidade de sintomas e serem doenças raras e complexas. Portanto, um diagnóstico confiável é indispensável para melhorar a qualidade de vida dos pacientes (Singh et al., 2020). Para um diagnóstico correto é necessário seguir um conjunto de passos, que envolvem uma combinação de abordagens clínicas, testes de laboratório e avaliação genética. Uma atenção especial deve ser dada ao histórico médico dos pacientes e seus familiares, já que as

MPS são doenças hereditárias autossômicas recessivas, com exceção da MPS II (Zhou et al., 2020). A idade de início, ordem cronológica dos sintomas, taxa de progressão e complicações, são informações essenciais de um diagnóstico (Colmenares-Bonilla et al., 2018).

Os GAGs são os principais compostos armazenados que servem como biomarcadores de diagnóstico para as MPS. Pacientes com MPS I e II geralmente apresentam níveis elevados de GAGs devido à incapacidade de degradá-los adequadamente (Kubaski et al., 2020). Portanto, determinar o nível de GAG, bem como a atividade enzimática e a genotipagem são cruciais para o diagnóstico das MPS. Devido às deficiências em enzimas lisossômicas específicas, os GAGs se acumulam em vários tecidos e são parcialmente excretados na urina. Ao detectar a presença de GAGs na urina e identificar seus tipos, torna-se possível conduzir um diagnóstico diferencial das MPS e direcionar o teste bioquímico para analisar as enzimas responsáveis pela degradação dos GAGs excretados. No entanto, o diagnóstico das MPS frequentemente é retardado, uma vez que a maioria dos pacientes parece saudável nos estágios iniciais da doença, e seus níveis totais de GAG na urina podem estar dentro da faixa normal, resultando em falsos negativos (Zhou et al., 2020). As análises de GAGs excretados na urina podem ser feitas por eletroforese ou por cromatografia líquida acoplada à espectrometria de massa em tandem. Além disso, os GAGs podem ser quantificados em amostras de soro, plasma, manchas de sangue seco, células e tecidos.

O diagnóstico definitivo deve ser feito por medida da atividade enzimática. Para MPS I o teste bioquímico inclui a dosagem de Alfa-L-iduronidase. Já para MPS II, é feita a dosagem da atividade da Iduronato-2-Sulfatase e de outra sulfatase para excluir a possibilidade de deficiência múltipla de sulfatases (Kubaski et al., 2020). Além disso, a análise genética é útil para confirmar o diagnóstico ou para identificar portadores assintomáticos e, frequentemente, predizer o fenótipo do paciente, podendo ser um fator determinante na escolha do tratamento (Stapleton et al., 2018). Exames de imagem como radiografias, tomografias computadorizadas (TC) e ressonâncias magnéticas (RM) também são métodos que auxiliam no diagnóstico, especialmente por revelar alterações nos ossos, articulações e órgãos internos, características típicas das MPS (Stapleton et al., 2018).

Atualmente existem duas opções de tratamento para as MPS, a terapia de reposição enzimática (TRE) e o transplante de células-tronco hematopoiéticas (TCTH) (Zhou et al., 2020). A TRE para MPS I é realizada pela administração intravenosa de laronidase, tendo sido aprovada para o tratamento de pacientes nos Estados Unidos e na Europa em 2003, e

no Brasil em 2005. Já a TRE para o tratamento de MPS II é realizada pela administração intravenosa de idursulfase. Em 2006 foi aprovada para o tratamento de pacientes nos Estados Unidos, em 2007 na Europa e em 2008 no Brasil (Giugliani et al., 2010). Já o TCTH, ficou disponível no Brasil, pelo Sistema Único de Saúde (SUS), para as MPS I e II em 2018 (CONITEC, 2018a; b). Essas opções terapêuticas têm sido bem toleradas em pacientes com MPS sem efeitos graves e podem retardar o progresso da doença (Sawamoto et al., 2019). Todavia, ainda que efetivas, nenhuma das técnicas é totalmente eficaz ou curativa (Carvalho et al., 2018). Tratamentos de suporte também são utilizados, incluindo controle da dor, tratamento da insônia, medicamentos anti-inflamatórios, oxigenoterapia, terapia psicomotora e intervenções cirúrgicas. No entanto, a eficiência terapêutica dessas opções é influenciada por vários fatores, como o tipo da MPS, gravidade da doença, novas mutações, idade, entre outros (Celik et al., 2021). Outras opções terapêuticas estão sendo desenvolvidas para melhorar as terapias convencionais, como novas enzimas recombinantes que podem penetrar na barreira hematoencefálica, terapia gênica e terapia de redução de substrato (Kobayashi, 2019). Entretanto, devido a limitação da eficácia e as desvantagens significativas dos tratamentos existentes, a pesquisa continua avançando na busca por alternativas. Entre as alternativas promissoras destaca-se a edição gênica. Esta abordagem oferece esperança para tratamentos mais eficazes e acessíveis das MPS I e II. Até agora, a maior parte das pesquisas sobre edição de genes em MPS tem se concentrado na MPS tipo I. Diversas estratégias, tanto *ex vivo* quanto *in vivo*, estão sendo exploradas, incluindo o uso de vetores virais e não virais para a entrega dos componentes necessários.

Nesse contexto, um artigo elaborado durante o período do doutorado, e que contou com a minha colaboração como co-autora, oferece uma visão aprofundada das características clínicas e moleculares das MPS. Ele explora também o uso de diversas técnicas de edição gênica, métodos de entrega viral e não viral, e apresenta resultados dos ensaios clínicos de fase I/II, especialmente para as mucopolissacaridoses. O documento completo encontra-se anexado ao final da tese.

2. OBJETIVOS

2.1. Objetivo Geral

O presente trabalho busca contribuir para o conhecimento sobre o uso das aplicações terapêuticas utilizando o sistema CRISPR/Cas9 quanto à sua utilização na América Latina e possível resposta imune prévia da população.

2.2. Objetivos Específicos

2.2.1. Traçar o panorama da utilização do sistema CRISPR/Cas9 na América Latina.

2.2.2. Avaliar a prevalência de anticorpos anti-*Streptococcus pyogenes* Cas9 em indivíduos saudáveis e em pacientes com Mucopolissacaridose tipo I (MPS I) e Mucopolissacaridose tipo II (MPS II).

3. RESULTADOS

Os resultados serão apresentados em forma de dois artigos científicos, sendo um publicado e um em processo de submissão a um periódico especializado. As figuras e tabelas principais estão distribuídas ao longo do texto para facilitar a leitura e interpretação. As figuras e tabelas suplementares encontram-se no final do artigo após as referências bibliográficas. Informações complementares estão disponíveis nos anexos.

3.1. Artigo I: CRISPR/Cas patents and health-related publications in South America

(Anais da Academia Brasileira de Ciências)

An Acad Bras Cienc (2023) 95(2): e20220629 DOI 10.1590/0001-3765202320220629
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

HEALTH SCIENCES

CRISPR/Cas patents and health-related publications in South America

LARIANE FRÂNCIO, MARTIELA V. DE FREITAS & URSULA MATTE

Abstract: CRISPR/Cas is being increasingly used for various applications. However, different countries introduce new technologies at different paces and purposes. This study reviews research progress using the CRISPR/Cas system in South America, focusing on health-related applications. The PubMed database was used to identify relevant articles about gene editing with CRISPR/Cas, whereas patents were searched in the Patentscope database. In addition, ClinicalTrials.gov was used to find information on active and recruiting clinical trials. A total of 668 non-duplicated articles (extracted from PubMed) and 225 patents (not all health-related) were found. One hundred ninety-two articles on health-related applications of CRISPR/Cas were analyzed in detail. In 95 out of these, more than 50% of the authors were affiliated with South American institutions. Experimental CRISPR/Cas studies target different diseases, particularly cancer, neurological, and endocrine disorders. Most patents refer to generic applications, but those with clear disease indications are for inborn errors of metabolism, ophthalmological, hematological, and immunological disorders. No clinical trials were found involving Latin American countries. Although research on gene editing in South America is advancing, our data show the low number of national innovations protected by intellectual property in this field.

Key words: gene editing, CRISPR/Cas, CRISPR/Cas9, South America.

INTRODUCTION

The ability to manipulate any genomic sequence by editing genes has created new possibilities for treating genetic diseases, allowing for precise modifications in cell cultures and animal studies (Carvalho et al. 2018). CRISPR/Cas system gene editing is the most promising tool for correcting genetic diseases because of its simplicity and precision, especially compared to other methods, such as TALEN and Zinc Finger (Gonçalves & Paiva 2017, Mills et al. 2020).

As a result of the rapid advance in biotechnology, specifically in gene editing (Ledford 2015, Mills et al. 2020), there is a great promise of applications to develop not only for human health but to improve gene-editing tools for plants, microorganisms, and animals. CRISPR/Cas has been used in human gene editing studies, and it has attracted the attention of scientific and industrial circles. In 2020, Emmanuelle Charpentier and Jennifer Doudna won the Nobel Prize in Chemistry in recognition of their contribution to developing the gene-editing technique using the CRISPR system.

This system consists of a nuclease (Cas9) guided by an RNA (sgRNA, about 20 nucleotides) to the target DNA, resulting in the cleavage of the double strand of DNA at a specific locus (Doudna & Charpentier 2014). This DNA break can be repaired either by non-homologous end-joining (NHEJ) or by homology-directed repair (HDR). The NHEJ occurs predominantly without donor DNA, resulting, in most cases,

in insertions or deletions and the generation of knockout organisms or cells. On the other hand, HDR, predominantly in the presence of donor DNA, generates mainly gene substitutions or additions (Sander & Joung 2014). Thus, the CRISPR/Cas9 technique can remove, replace, or correct non-functional genes (Zhang 2021). This system has continued to evolve in recent years, and many strategies have been developed from basic to applied research, including gene knock-in or knockout (Kherraf et al. 2018), base editing (Gaudelli et al. 2017), and prime editing (Anzalone et al. 2019).

One of the main CRISPR/Cas applications and advantages has been in the generation of disease models and in the development of new treatments for genetic, infectious, and immunological diseases, and cancer, due to its high efficiency and the potential to provide long-term therapy, term after a single treatment (Wu et al. 2020, You et al. 2019, Zhang 2021). In addition, these models can assist in the development of new drug targets. Wu et al. (2020) reviewed the creation of models (cellular and animal) of genetic diseases, preclinical therapies, and clinical trials with the CRISPR/Cas system. In addition, clinical trials involving CRISPR/Cas-based gene editing have been carried out, from applications for correcting disease-causing variants to improving CAR T cell therapy (Li et al. 2020).

Many resources allowed the rapid introduction of the CRISPR system, like specificity, efficiency, precision, and speed. Due to this, the CRISPR/Cas system can be used in less sophisticated laboratories, being considered a low-cost methodology. Since its initial description in late 2012 and early 2013, this technology has gained ground in the scientific scenario. The leading technology researchers found it essential that other laboratories could use this tool, sharing and committing themselves to open science, thus making their original CRISPRs plasmids available. For example, Addgene has distributed over 100,000 CRISPR plasmids to 3,400 laboratories worldwide (LaManna & Barrangou 2018).

In all countries, innovation is essential for economic development and the quality of life for its citizens. Research is fundamental for innovation; therefore, promoting innovation involves encouraging and supporting scientific research. Despite the advantages and facilities of approaches using CRISPR/Cas, when comparing developed and developing countries, there are apparent differences in adopting new technologies, regardless of cost, due to structural deficiencies. South American countries have been trying to increase innovation but still face challenges. Argentina, Brazil, Chile, and Colombia are growing strongly in the number of publications (Olavarrieta & Villena 2014).

In the last twenty years, Brazil has intensified efforts to expand, support, and promote science, technology, and innovation activities (Lima et al. 2019). As a result, in 2014, Brazil dominated the record for publications and was responsible for more than two-thirds of all scientific production in South America and leading publications in Latin America (Olavarrieta & Villena 2014, Van Noorden 2014). In addition, in 2019, the country reached the 23rd position, the first in South America, in the global ranking of scientific quality in the Nature Index (2020).

We have previously shown the progress of gene therapy (Linden & Matte 2014) and of gene and cell therapy (Matte & Peluffo 2020) in South America. Here we present an overview of the progress of gene-editing research in South America. We performed a bibliometric analysis in the PubMed database on research progress using the CRISPR/Cas system in South American countries, focusing on health-related applications. Data on patent filing is

also reported, although not limited to health applications. Finally, the current clinical trials ongoing worldwide are also briefly discussed.

MATERIALS AND METHODS

The PubMed database (https://pubmed.ncbi.nlm.nih.gov) was used to identify relevant articles with the keywords "CRISPR", "CRISPR Cas", "CRISPR/Cas", "CRISPR-Cas", "Gene editing", "Genome editing", AND "Argentina", "Brazil", "Bolivia", "Chile", "Colombia", "Ecuador", "French Guyana", "Guyana", "Paraguay", "Peru", "Suriname", "Uruguay", "Venezuela", published from January 2010 to January 2021. The search was carried out in October 2021. Duplicated publications were removed, and the remaining were pre-evaluated by reading their abstracts. Articles that used other gene-editing techniques were not considered, as well as articles that only mentioned the procedure and articles in any language but English. The remaining articles were classified into four groups according to their topic of study: plants, animals, microorganisms, and health.

The latter group included articles on treating and preventing human disease and were read in full and further characterized. Data on authorship, international collaboration, type of experiment, and disease target were collected. We began by analyzing the distribution of authors in South America. For each article, we counted the authors' countries of affiliation. If more than one author was from the same country, that country was counted only once. Then, articles were classified according to collaboration with international groups. Next, we counted how many authors were affiliated with South American institutions and how many were from foreign institutions for each article. Articles with less than 50% of South American authors were classified as international collaboration. In case of a tie in the number of authors, we considered the first or last author for this classification. Next, the articles were classified according to type: experimental or review. Experimental articles were further divided according to the experiment: *in vitro*, *in vivo*, both (*in vitro* and *in vivo*), and others. In addition, we also verified the target disease of each experimental article.

We also searched for CRISPR/Cas technology patents deposited in South American countries. The research was carried out in the Patentscope database, simple search mode. Data collection was carried out in October 2021. We selected "home page" as the search field, and the terms "CRISPR OR Cas9" were used as keywords for the search. In the "languages" and "offices" fields, we selected "all" and all the South American countries available for consultation (Argentina (AR), Brazil (BR), Chile (CL), Colombia (CO), Ecuador (CE), Peru (PE), Uruguay (UY)). The data collected (request number, publication date, patent title, depositor, and deposit country) were recorded in an Excel spreadsheet to enable analysis and observations of the characteristics of each patent. Patents classified as "world" (WO) were excluded. In further analysis, patents with the same abstract and depositor in different South American countries, or even in the same country, were counted as one.

Clinical trials were reviewed on the website ClinicalTrials.gov, a repository of privately and publicly funded clinical studies conducted worldwide and maintained by the United States National Library of Medicine. Search terms were "CRISPR", "CRISPR/Cas", and "CRISPR/Cas9". The collected data (first posted, official title, phase, estimated enrollment, study type, intervention/treatment, status, condition or disease, sponsors and collaborators) were recorded in an Excel. Studies classified as interventional-diagnostic, observational and not using the CRISPR/Cas system were excluded.

RESULTS

The general steps used in this work are shown in Figure 1. They include: (i) data recovery in publications (extracted from PubMed) and patents (extracted from Patentscope) on gene editing using the CRISPR/Cas technique; (ii) excluding all duplicated items; (iii) reading all abstracts of the remaining articles and exclude articles that do not use or only mention CRISPR/Cas and articles not in English; (iv) exclude patents that do not use the CRISPR/Cas gene-editing technique; (v) classify articles and patents in the subject group. Finally, the remaining articles and patents were analyzed in detail.

Articles

A total of 668 relevant and not duplicated articles were obtained from PubMed. Of these, 435 articles were selected after excluding articles that only mentioned the technique, used techniques other than CRISPR/Cas, or were not in English. These 435 articles were divided into four groups according to the subject: Plants (n=52), Animals (n=37), Microorganisms (n=154), and Health (n=192), as seen in Figure 2.

We focused on exploring in detail the 192 articles classified as health-related. First, the articles were classified according to collaboration with international groups. For 97 out of 192 articles, less than 50% of the authors were affiliated with South American institutions, and the article was classified as international collaboration. The country with the highest number of papers in collaboration was the United States, followed by the United Kingdom, Germany, China, The Netherlands, and Spain. To understand how widespread the studies with CRISPR/Cas from South American countries are, we analyzed the distribution of authors. For each paper, we counted the author's countries of affiliation. Figure 3 shows the countries present in the 192 articles. Brazil

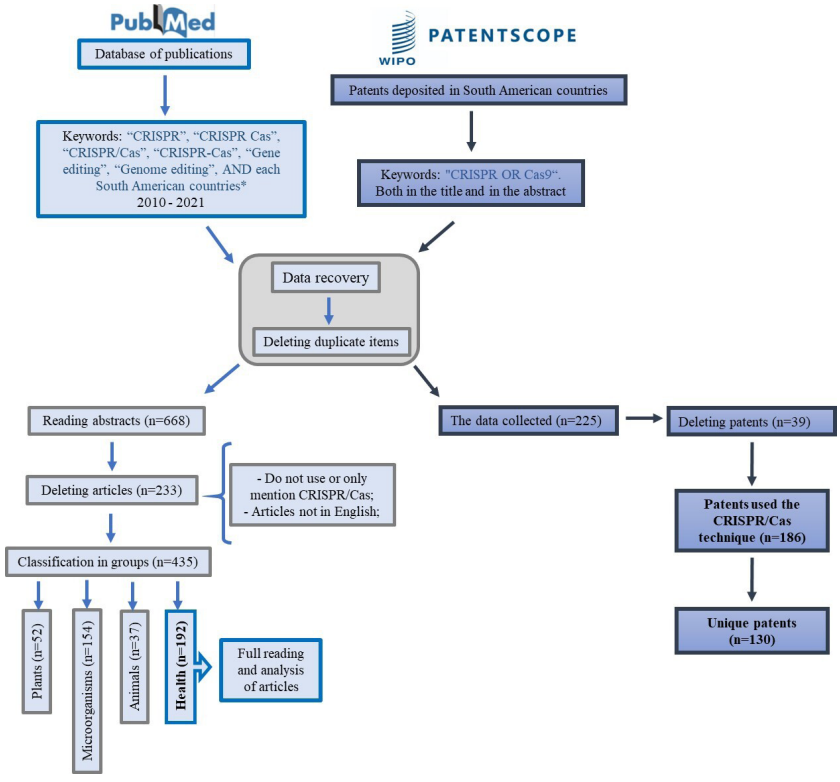

Figure 1. Steps of analysis used in this study. *Argentina, Brazil, Bolivia, Chile, Colombia, Ecuador, French Guyana, Guyana, Paraguay, Peru, Suriname, Uruguay, Venezuela.

was the country with the most publications (n=112), followed by the United States (n= 76), Chile (n=29), Argentina (n=28), and the United Kingdom (n=28). Colombia, with 17 publications, occupied seventh place. Uruguay, Peru, Ecuador, and Paraguay had 8, 5, 2, and 1 publications, respectively. It is important to emphasize that the other countries, "Bolivia," "French Guyana", "Guyana", "Suriname", and "Venezuela", did not return any articles using the CRISPR technique in our search.

All 192 articles were also classified according to the type of article as experimental (n=140) or review (n=52). Figure 4 shows the classification of experimental articles (n=140) regarding the health-related application or topic. In the "others" group, we included articles that did not fit into the other categories, such as ciliopathies, inflammatory bowel disease, cell organization, gene regulation, and Marfan syndrome. Experimental articles (n=140) were further divided according to the type of study into *in vivo* (32), *in vitro* (93), or both (13). Finally, two articles were classified in the "other" category. One article is about opinion polls on CRISPR/Cas9 technology and its application in the future. The other article is about an automated system for predicting large proteins.

Patents

Patent analysis was not restricted to health-related applications. The search for patents deposited in Argentina, Brazil, Chile, Colombia, Ecuador, Peru, and Uruguay at Patentscope returned 225 patents filed until November 2021. All had their summaries read, if available. Patents that did not mention the CRISPR system in their title or abstract were excluded from our analysis (n = 39). The remaining 186 were classified into four groups, according to subject: Plants (n=28), Animals (n=5), Microorganisms (n=11), Disease (n=64) and Generic (n=78) (Figure 5). This last case corresponds to applications to different subjects, such as plants and/or health and/or microorganisms. Brazil was the South American country with the most patents filed (n=128), followed by Argentina (n=23), Colombia (n=14), Chile (n=9), Peru (n=6), Ecuador (n=3), and Uruguay (n=3). After reading patent abstracts,

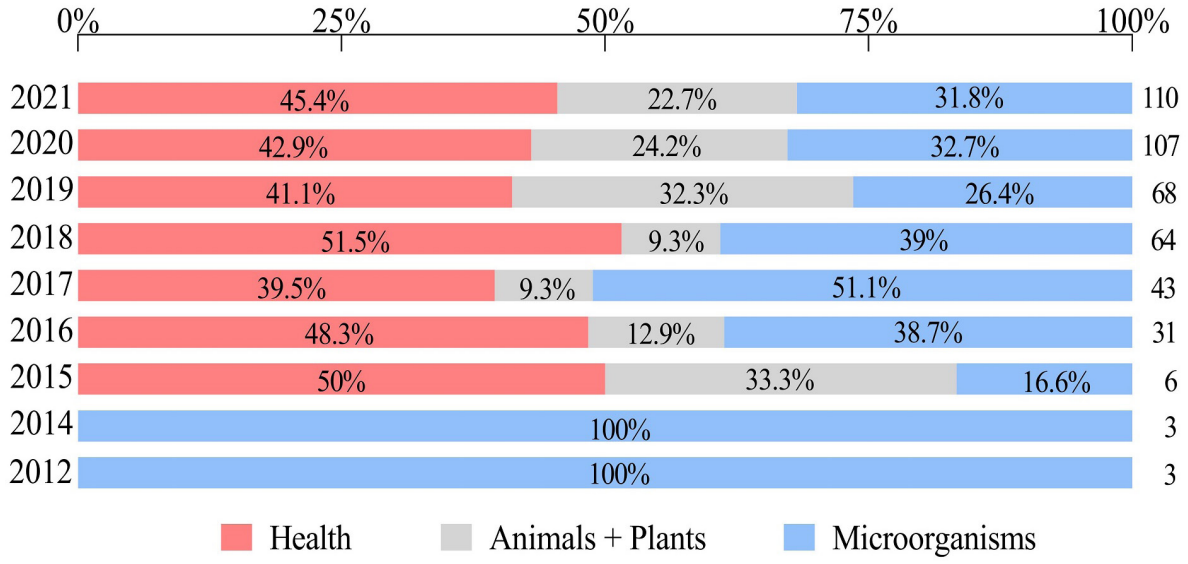

Figure 2. The number of articles per year. Number and type of articles by year of publishing (n=435). Numbers on the right side of the bar correspond to the total number in that year.

duplicated patents filed in different countries were excluded.

The remaining 130 patents were filed by 82 patent depositors, but only two were from Brazilian owners, the only country in South America to own patents. The top 5 patent depositors are the Massachusetts Institute of Technology (MIT) and The Broad Institute, with 15 patents each, followed by the President and Fellows of Harvard College with 14 patents. Pioneer Hi-Bred International comes next with 11 patents, followed by The Institute of Genetics and Developmental Biology and the Chinese Academy of Sciences with six patents (note that some patents are shared among more than one institution).

Disease-related patents (n=40) were also classified according to the target disease (Figure 6). Cancer is the leading topic of experimental research on gene editing conducted in South America, but not in patents. Generic patents correspond to 17.

Clinical trials

Currently, 43 clinical trials with the keyword "CRISPR/Cas9" are registered in the ClinicalTrials.

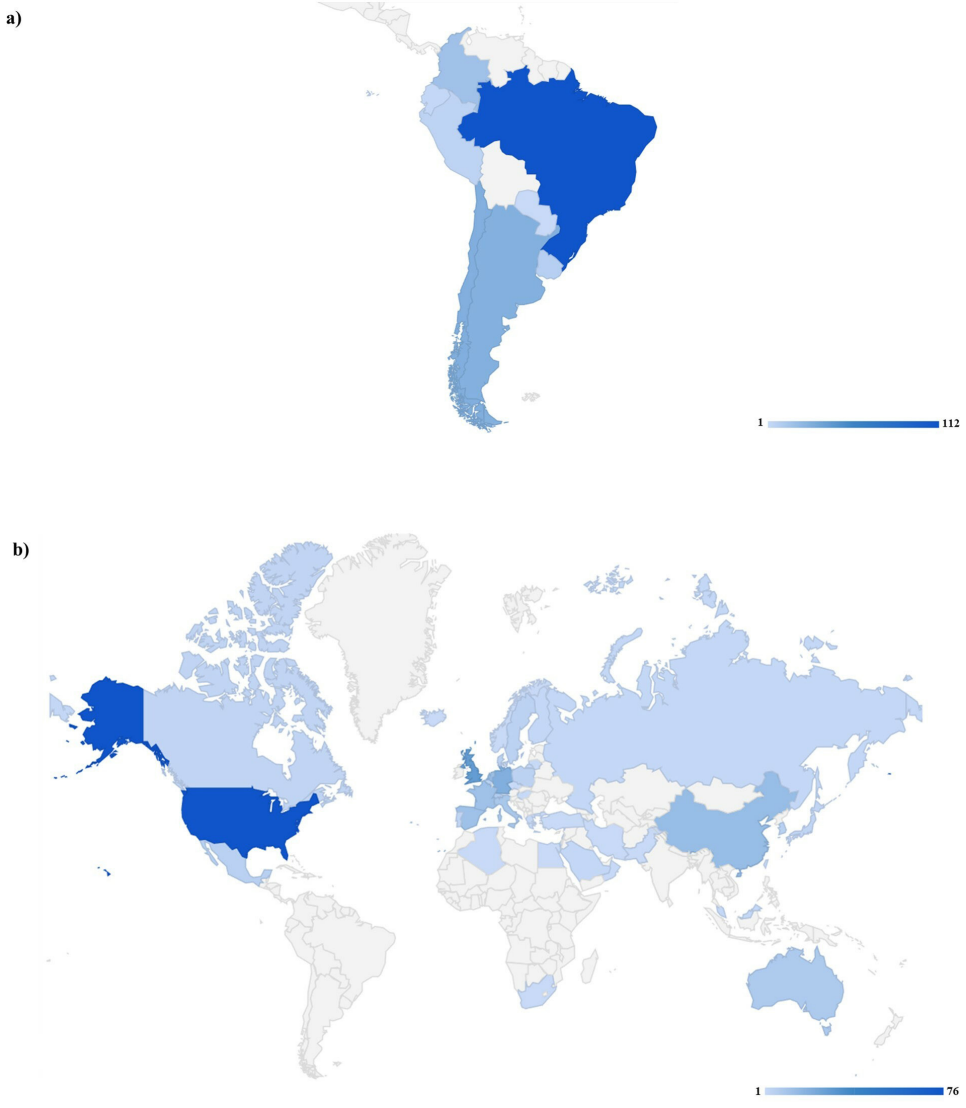

Figure 3. The countries present in articles from the "Health-related" category (n=192). a) South American countries. b) International collaboration. Color intensity shows the number of articles.

Figure 4. Classification of articles and patents by health-related topic. Topics of experimental articles (n=140).

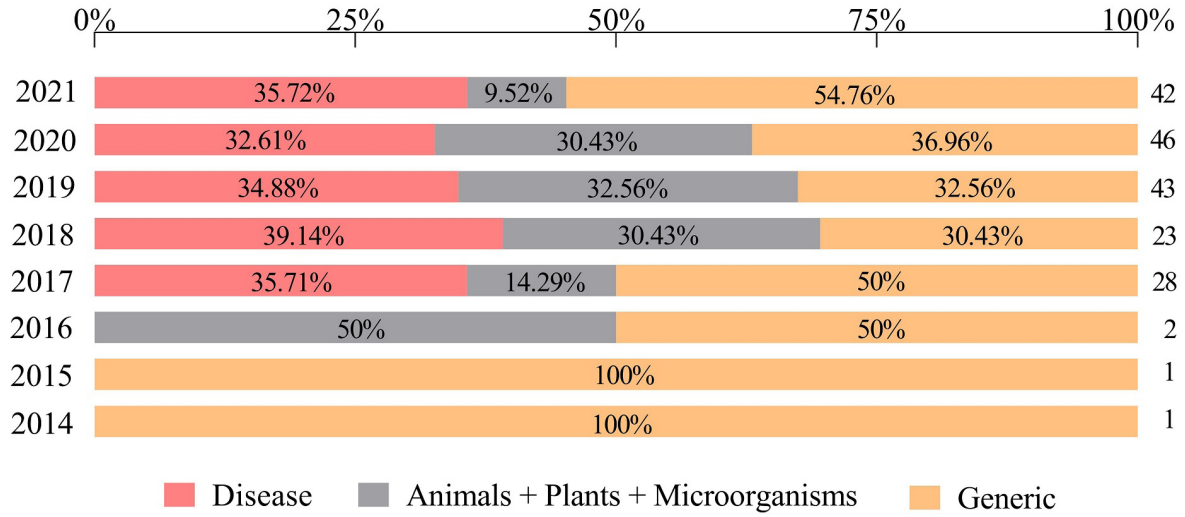

Figure 5. The number of patents per year. Number and type of patents by year of deposit (n=186). Generic = corresponds to applications applied to different subjects, such as plants and/or health and/or microorganisms. Numbers on the right side of the bar correspond to the total number in that year. The same patent will be counted more than once if deposited in two or more countries.

gov clinical database (Supplementary Material - Table SI). There is no clinical trial with the participation of South American countries. China and the United States are the predominant countries conducting clinical trials, with 18 and 19 trials performed with their financial support. Out of the 43 clinical trials, two were suspended due to lack of funding, nine had unknown status, and three were completed. Regarding classification, most clinical trials are on cancer, hematologic, and infectious diseases. Currently, 27 clinical cancer treatment trials with the CRISPR/Cas9 tool are being researched. They target several types of cancer, including skin, gastrointestinal tumors, hematological neoplasms, neurological, gynecological, urological, and lung. Approximately two-thirds of clinical trials performed to date are either phase I or I/II, representing 15,48% of all CRISPR therapy trials. Phase II represents 0,43% of the total, and phases II/III and III represent only 1,72% of all trials.

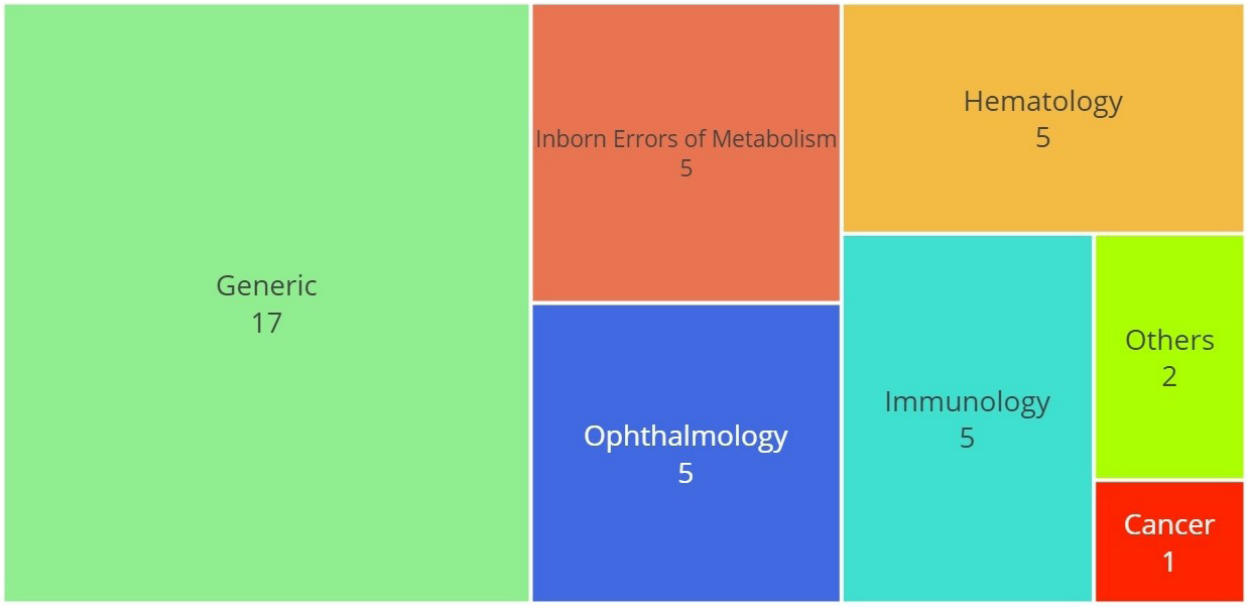

Figure 6. Classification of patents by health-related topic. Topics of the patents in the disease group (n=40). Generic = do not specifically mention which disease.

The first clinical trial, using the CRISPR/Cas9 system, was performed in 2016 to treat lung cancer (NCT02793856). This clinical trial involved editing T-cells to knock out the *PD1* gene in patients with advanced non-small-cell lung cancer. Cells were edited *ex-vivo* and re-infused into the patients, where they were detected in peripheral blood after reinfusion (Lu et al. 2020). Other clinical trials include, for example, lung cancer (NCT02793856), esophageal cancer (NCT03081715), bladder cancer (NCT02863913), and kidney cancer (NCT02867332), and to investigate the safety and efficiency of CAR-T cell therapy mediated by the CRISPR/Cas9 system.

There are also clinical studies related to other diseases, especially hematological diseases such as β-thalassemia and Sickle cell disease (SCD). As inherited blood disorders caused by mutations in the *β*-globin gene (*HBB*), they are considered suitable targets for *ex vivo* therapy in hematopoietic stem cells (HSCs). In 2018, CRISPR Therapeutics and Vertex Pharmaceuticals, in partnership, started a phase 1/2/3 study to evaluate CTX 001 therapy in subjects older than 12 years with β-thalassemia (NCT03655678) and SCD (NCT03745287). The treatment consists of withdrawing and editing patients' CD34+ cells in the *BCL11A* gene before reinjecting the cells in the patients. The modification in the *BCL11A* gene introduces the mutation leading to the persistence of fetal hemoglobin (HbF). Preliminary results in two patients revealed an increase in fetal hemoglobin levels maintained in the bone marrow and peripheral blood cells for more than one year after therapy (Frangoul et al. 2021, Modarai et al. 2021). Vertex Pharmaceuticals has just begun a phase 3 study evaluating CTX 001 in pediatric patients, 2 to 11 years, with both conditions.

Another two clinical trials with satisfactory results were for treating lymphoblastic leukemia (NCT03164135l) and amyloidosis (NCT04601051). For the first, the therapy consisted of the CRISPR/Cas9-mediated disruption of the *CCR5* gene. The results were successful transplant, long-term engraftment, and efficiency of 5,20 to 8,28% in a patient with HIV-1 infection. Lymphoblastic leukemia remission was observed 19 months

after transplant when cells with the modified *CCR5* gene persisted (Xu et al. 2019). For amyloidosis, the *in vivo* therapy consisted of reducing serum TTR concentration. The clinical trial revealed a persistent knockout of *TTR* after a single dose. However, the effects were dose-dependent. For patients who received 0.1 mg/kg, the mean reduction in TTR protein was 52%. While for the group receiving 0.3 mg/kg, the decline was 87% (Gillmore et al. 2021).

There are two clinical studies available for the treatment of ophthalmological diseases: the treatment of Leber Congenital Amaurosis (NCT03872479) and Refractory Viral Keratitis (NCT04560790). In the clinical trial for Leber congenital amaurosis (LCA), a rare genetic eye disorder, is the first retinal gene therapy clinical that consists of EDIT-101 administered via subretinal injection to eliminate the mutation on the CEP290 gene.

DISCUSSION

Among CRISPR/Cas9 applications, one of the most promising is its use for gene therapy. This system presents additional advantages over conventional gene therapy. First, correcting point mutations within the endogenous gene allows for better regulation of gene expression. Second, when gene adding is preferred, CRISPR/Cas9 can target the exogenous gene to genomic safe harbors, regions in which the transgene is not expected to disrupt the function of cellular genes. Finally, it can also be used for knocking out overexpressed genes, as in tumor-related oncogenes (Kelly et al. 2021, Uddin et al. 2020, Zhang et al. 2021). Gene editing has been a great tool in cancer research, with very promising advances (Chen et al. 2018) from the insertion of tumor suppressor genes to immunotherapy. One of its applications has been in screening cancer targets due to its efficiency when compared with the screening approach via blocking gene expression by RNAi (Guo et al. 2022). Another application is to increase the efficiency and safety of CAR-T cell therapy (Eyquem et al. 2017).

As the results of clinical trials show, this strategy is moving towards clinical application, as seen with other types of gene therapy (Ginn et al. 2018). In this scenario, it is strikingly that no clinical trial is associated with a South American country, either in developing or recruiting patients. However, this situation is not specific to gene editing. Most clinical trials for pharmaceutical products in South America are coordinated and sponsored by international companies (da Silva et al. 2018).

In Brazil, as in other countries, a large part of technological innovation comes from universities (De Sandes-Guimaraes et al. 2020). However, in developing countries, there is still a lack of the ability to transform the innovation generated in the university into a final product that can reach the consumer market (Lima et al. 2019). This technological gap is due to factors such as the low number of innovative companies (Melo et al. 2017) and bureaucratic barriers to industry-university partnerships (Turchi & Morais 2017). This can be seen in the low number of gene editing patents registered by South American companies in the Patentscope database.

On the other hand, the scientific contribution of South American countries to the research on CRISPR/Cas9 seems promising. Most publications with or without international collaboration are from Brazil, and the most significant number of patent filings. Most Brazilian papers belong to groups from São Paulo, Rio de Janeiro, and Rio Grande do Sul (data not shown). The regional concentration of resources, financing, and human capital is a characteristic of large developing countries. In Brazil, the most striking example is São Paulo, whose infrastructure and

financing capacity are far superior to the rest of the country (Pereira & Plonski 2010). These three states also host the most significant number of biotechnology companies with applications in human health in the country (Alves et al. 2017). However, the participation in the scientific production of Argentina, Chile, and Colombia is also fundamental since, together with Brazil, they contribute more than 90% of the total production of South America (Carvajal-Tapia & Carvajal-Rodríguez 2018).

According to Picanço-Castro et al. (2020), the low number of patents compared to articles may result from the negative perspective of biotechnology and pharmaceutical companies due to the results of the first gene therapy treatments. They showed that in the early 2000s, the rate of patents was higher than that of publications, with a decline in subsequent years. However, despite the adverse events, several advances have been made in gene therapy, and a return to increasing patenting can be observed. Regarding the worldwide patent landscape related to the CRISPR/Cas9 technology, according to Egelie et al. (2016), there were 93 patent grants and 1363 published patents, ranging from CRISPR/Cas9 components to delivery systems and applications. Therefore, this patent landscape is constantly changing.

Patent filings with the CRISPR technique have increased worldwide; it can be said due to the broad applicability of this technique in several areas: health, plants, biotechnology, and industry. The United States, China, and Europe are the countries that have the most patents and collaborations on articles using this technology (Grobler et al. 2021, Nxumalo et al. 2021). These observations corroborate our data showing the United States, followed by the United Kingdom, Germany, and China, with the highest number of contributions in articles from South American countries.

There are many applications to explore the possibility of patenting with CRISPR technology. Advances in diagnosing pathogens, for example, can be beneficial, especially for developing countries. Mainly due to the scenario we still face with the COVID-19 pandemic, some groups have developed diagnostic methods for SARS-CoV-2 using different Cas (Grobler et al. 2021). In our search, out of 7 publications aiming at pathogen detection, four were focused on SARS-CoV-2. It is also essential that universities and companies foster patent filing to benefit from their applications on an industrial scale.

Cancer is the type of disease most studied for gene editing (Nxumalo et al. 2021) and neurological disorders are second. These observations corroborate the data obtained by Picanço-Castro et al. (2020), which shows cancer as the most studied type of disease worldwide, followed by neurological disorders. Yet, surprisingly, the number of patents for CRISPR cancer-related applications is comparatively lower. Maybe this is due to the advances in non-gene editing cancer therapeutics and the more tailored use of CRISPR/Cas for inherited diseases, even though clinical trials for cancer surpass those for monogenic disease, both by gene editing and gene therapy in general (Ginn et al. 2018).

Analysis of scientific publication and patent filing data, albeit limited, provides essential information and insights for understanding scientific and technological advances. However, it has its limitations. It is important to note that the articles returned in our study do not represent the whole research field in South America, only articles published in journals indexed in PubMed, since this study focused on publications with health-related applications. The search retrieved 435 articles and almost half of them (243) were on microorganisms, animals, or plants. It is worth noticing that these

areas are probably underrepresented as the database used is focused on medical sciences. Even so, the number of publications on plants and animals only confirms South America's strength in agricultural research. The search was not performed in other databases, such as Lilacs, which may result in some bias towards PubMed-indexed publications. Also, non-English journals are underrepresented. Information on ongoing clinical trials was retrieved only from ClinicalTrials.gov, which may not contain all trials performed in South America. According to Silva et al. (2018), in South America, there are only two national registries: The Brazilian Clinical Trials Registry (ReBec) and The Peruvian Clinical Trials Registry (REPEC). However, the search for trials on CRISPR in these databases returned no results.

Our results show that, despite being a relatively easy and low-cost technology for genetic manipulation, CRISPR/Cas is not widely adopted in South America. Even though there are experimental publications, especially in Brazil, most production is performed with international cooperation (and often overseas). More importantly, when considering the intellectual property related to this technology, South American countries are completely surpassed by US and Chinese companies. As a result, the application of this technology in terms of clinical trials is absent in these countries. This reflects the importance of investing in science and fostering partnerships between academic researchers and private companies.

Acknowledgments

We would like to thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) for financial support and for the fellowships provided to LF (Process no.: 149092/2019-9) and UM (Process no.: 312714/2018-1) and FIPE/HCPA.

SUPPLEMENTARY MATERIAL

Table SI.

REFERENCES

ALVES N, VARGAS MA & BRITTO J. 2017. Empresas de biotecnologia e biociências no Brasil: um panorama. São Paulo. Blucher 4: 1069-1088.

ANZALONE AV ET AL. 2019. Search-and-replace genome editing without double-strand breaks or donor DNA. Nature 576: 149-157.

CARVAJAL-TAPIA AE & CARVAJAL-RODRÍGUEZ E. 2018. Status of scientific production in Medicine in South America. 1996-2016. Rev Fac Med 66: 595-600.

CARVALHO TG, SCHUH R, PASQUALIM G, PELLENZ FM, FILIPPI-CHIELA EC, GIUGLIANI R, BALDO G & MATTE U. 2018. CRISPR-Cas9-mediated gene editing in human MPS I fibroblasts. Gene 678: 33-37.

CHEN P, YOU L & LU Y. 2018. Applications of CRISPR-Cas9 Technology in Translational Research on solid-tumor cancers. The CRISPR J 1: 47-54.

DA SILVA RE, AMATO AA, GUILHEM DB, DE CARVALHO MR & NOVAES MRCG. 2018. International Clinical Trials in Latin American and Caribbean Countries: Research and Development to Meet Local Health Needs. Front Pharmacol 8: 961.

DE SANDES-GUIMARAES LV, RIBEIRO ATVB, AXEL-BERG JH, DE ROSSO MANÇOS G & PLONSKI GA. 2020. The Impact of International Student Mobility Programs on Brazilian Students' Perceptions of Entrepreneurialism. J Stud Int Educ 24: 249-268.

DOUDNA JA & CHARPENTIER E. 2014. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science 346: 1258096.

EGELIE KJ, GRAFF GD, STRAND SP & JOHANSEN B. 2016. The emerging patent landscape of CRISPR–Cas gene editing technology. Nat Biotechnol 34: 1025-1031.

EYQUEM J, MANSILLA-SOTO J, GIAVRIDIS T, VAN DER STEGEN SJ, HAMIEH M, CUNANAN KM, ODAK A, GÖNEN M & SADELAIN M. 2017. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nature 543: 113-117.

FRANGOUL H ET AL. 2021. CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β-Thalassemia. N Engl J Med 384: 252-260.

GAUDELLI NM, KOMOR AC, REES HA, PACKER MS, BADRAN AH, BRYSON DI & LIU DR. 2017. Programmable base editing of

A·T to G·C in genomic DNA without DNA cleavage. Nature 551: 464-471.

GILLMORE JD ET AL. 2021. CRISPR-Cas9 In Vivo Gene Editing for Transthyretin Amyloidosis. N Engl J Med 385: 493-502.

GINN SL, AMAYA AK, ALEXANDER IE, EDELSTEIN M & ABEDI MR. 2018. Gene therapy clinical trials worldwide to 2017: An update. J Gene Med 20: e3015.

GONÇALVES GAR & PAIVA RDMA. 2017. Gene therapy: advances, challenges and perspectives. Einstein São Paulo 15: 369-375.

GROBLER L, SULEMAN E & RAJ DBTG. 2021. Patents and technology transfer in CRISPR technology. Prog Mol Biol Transl Sci 180: 153-182.

GUO N, LIU JB, LI W, MA YS & FU D. 2022. The power and the promise of CRISPR/Cas9 genome editing for clinical application with gene therapy. J Adv Res 40: 135-152.

KELLY JJ, SAEE-MARAND M, NYSTRÖM NN, EVANS MM, CHEN Y, MARTINEZ FM, HAMILTON AM & RONALD JA. 2021. Safe harbor-targeted CRISPR-Cas9 homology-independent targeted integration for multimodality reporter gene-based cell tracking. Sci Adv 7: eabc3791.

KHERRAF ZE, CONNE B, AMIRI-YEKTA A, KENT MC, COUTTON C, ESCOFFIER J, NEF S, ARNOULT C & RAY PF. 2018. Creation of knock out and knock in mice by CRISPR/Cas9 to validate candidate genes for human male infertility, interest, difficulties and feasibility. Mol Cell Endocrinol 468: 70-80.

LAMANNA CM & BARRANGOU R. 2018. Enabling the rise of a CRISPR world. The CRISPR J 1: 205-208.

LEDFORD H. 2015. CRISPR, the disruptor. A powerful gene-editing technology is the biggest game changer to hit biology since PCR. But with its huge potential come pressing concerns. Nature 522: 20-24.

LI H, YANG Y, HONG W, HUANG M, WU M & ZHAO X. 2020. Applications of genome editing technology in the targeted therapy of human diseases: mechanisms, advances and prospects. Signal Transduct Target Ther 5: 1-23.

LIMA LA, OLIVEIRA LP, DA SILVA TENÓRIO LX, PY-DANIEL SS, FERNANDES TL, GHESTI GF & DA SILVA ML. 2019. Desenvolvimento tecnológico e a maturidade das pesquisas no âmbito das instituições de pesquisa científica e tecnológica (ICT) no Brasil. Cadernos de Prospecção 12: 31.

LINDEN R & MATTE U. 2014. A snapshot of gene therapy in Latin America. Genet Mol Biol 37(Suppl 1): 294-298.

LU Y ET AL. 2020. Safety and feasibility of CRISPR-edited T cells in patients with refractory non-small-cell lung cancer. Nat Med 26: 732-740.

MATTE U & PELUFFO H. 2020. Spotlight edition on South America. Gene Ther 27: 1.

MELO TM, CORREA AL, CARVALHO EG & POSSAS ML. 2017. Competitividade e gap tecnológico–uma análise comparativa entre Brasil e países europeus selecionados. Rev Bras Inov 16: 129-156.

MILLS EM, BARLOW VL, LUK LY & TSAI YH. 2020. Applying switchable Cas9 variants to in vivo gene editing for therapeutic applications. Cell Biol Toxicol 36: 17-29.

MODARAI SR, KANDA S, BLOH K, OPDENAKER LM & KMIEC EB. 2021. Precise and error-prone CRISPR-directed gene editing activity in human CD34+ cells varies widely among patient samples. Gene Ther 28: 105-113.

NATURE INDEX. 2020 tables: Countries/territories. Available at: https://www.nature.com/nature-index/annual-tables/2020. Accessed on: March 8, 2021.

NXUMALO Z, TAKUNDWA MM & RAJ DBTG. 2021. Patents, ethics, biosafety and regulation using CRISPR technology. Prog Mol Biol Transl Sci 181: 345-365.

OLAVARRIETA S & VILLENA MG. 2014. Innovation and business research in Latin America: An overview. J Bus Res 67: 489-497.

PEREIRA L & PLONSKI GA. 2010. Bridging the technology gap in Brazil: the case of photonics. Int J Manag 27: 243.

PICANÇO-CASTRO V, PEREIRA CG, COVAS DT, PORTO GS, ATHANASSIADOU A & FIGUEIREDO ML. 2020. Emerging patent landscape for non-viral vectors used for gene therapy. Nat Biotechnol 38: 151-157.

SANDER JD & JOUNG JK. 2014. CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotechnol 32: 347-355.

SILVA RED, AMATO AA, GUILHEM DB, CARVALHO MRD & NOVAES MR. 2018. International Clinical Trials in Latin American and Caribbean Countries: Research and Development to Meet Local Health Needs. Front Pharmacol 8: 961.

TURCHI LMO & MORAIS JMDO. 2017. Políticas de apoio à inovação tecnológica no Brasil: avanços recentes, limitações e propostas de ações. Brasília: Inpea, 485 p.

UDDIN F, RUDIN CM & SEN T. 2020. CRISPR gene therapy: applications, limitations, and implications for the future. Front Oncol 10: 1387.

VAN NOORDEN R. 2014. South America: by the numbers. Nature 510: 202-204.

WU SS, LI QC, YIN CQ, XUE W & SONG CQ. 2020. Advances in CRISPR/Cas-based gene therapy in human genetic diseases. Theranostics 10: 4374.

XU L ET AL. 2019. CRISPR-Edited Stem Cells in a Patient with HIV and Acute Lymphocytic Leukemia. N Engl J Med 381: 1240-1247.

YOU L, TONG R, LI M, LIU Y, XUE J & LU Y. 2019. Advancements and Obstacles of CRISPR-Cas9 Technology in Translational Research. Mol Ther Methods Clin Dev 13: 359-370.

ZHANG B. 2021. CRISPR/Cas gene therapy. J Cell Physiol 236: 2459-2481.

ZHANG H ET AL. 2021. Application of the CRISPR/Cas9-based gene editing technique in basic research, diagnosis, and therapy of cancer. Mol Cancer 20: 1-22.

How to cite

FRÂNCIO L, DE FREITAS MV & MATTE U. CRISPR/Cas patents and health-related publications in South America. An Acad Bras Cienc 95: e20220629. DOI 10.1590/0001-3765202320220629.

Manuscript received on July 21, 2022;
accepted for publication on September 9, 2022

LARIANE FRÂNCIO[1,2]
https://orcid.org/0000-0002-4283-3137

MARTIELA V. DE FREITAS[1,2,3]
https://orcid.org/0000-0003-4178-6604

URSULA MATTE[1,2,3,4]
https://orcid.org/0000-0003-4977-6662

[1]Programa de Pós-Graduação em Genética e Biologia Molecular, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9500, 90650-001 Porto Alegre, RS, Brazil

[2]Hospital de Clínicas de Porto Alegre, Laboratório de Células, Tecidos e Genes, Rua Ramiro Barcelos, 2350, 90035-903 Porto Alegre, RS, Brazil

[3]Hospital de Clínicas de Porto Alegre, Núcleo de Bioinformática, Rua Ramiro Barcelos, 2350, 90035-903 Porto Alegre, RS, Brazil

[4]Universidade Federal do Rio Grande do Sul, Instituto de Biociências, Departamento de Genética, Av. Bento Gonçalves, 9500, 90650-001 Porto Alegre, RS, Brazil

Correspondence to: **Ursula Matte**
E-mail: umatte@hcpa.edu.br

Author contributions

LF and MVF conceived the study, analyzed the data, and prepared figures, LF, MVF and UM wrote the manuscript, and UM revised the final version of this paper; all authors read and approved the final version.

SUPPLEMENTARY MATERIAL

Table I. Clinical trials with CRISPR/Cas9 from 2016-2022.

Disease Type	ID	Title	Status	Phase	Sponsor
Hematological	NCT04774536	Transplantation of CRISPRCas9 Corrected Hematopoietic Stem Cells (CRISPR_SCD001) in Patients With Severe Sickle Cell Disease	Not yet recruiting	1, 2	University of California / Mark Walters
	NCT03655678	A Phase 1/2/3 Study of the Safety and Efficacy of a Single Dose of Autologous CRISPR-Cas9 Modified CD34+ Human Hematopoietic Stem and Progenitor Cells (hHSPCs) in Subjects With Transfusion-Dependent β-Thalassemia	Active, not recruiting	2, 3	Vertex Pharmaceuticals Incorporated|CRISPR Therapeutics
	NCT03745287	A Phase 1/2/3 Study to Evaluate the Safety and Efficacy of a Single Dose of Autologous CRISPR-Cas9 Modified CD34+ Human Hematopoietic Stem and Progenitor Cells (CTX001) in Subjects With Severe Sickle Cell Disease	Active, not recruiting	2, 3	Vertex Pharmaceuticals Incorporated|CRISPR Therapeutics
	NCT05356195	A Phase 3 Study to Evaluate the Safety and Efficacy of a Single Dose of CTX001 in Pediatric Subjects With Transfusion-Dependent β-Thalassemia	Recruiting	3	Vertex Pharmaceuticals Incorporated|CRISPR Therapeutics
	NCT05329649	A Phase 3 Study to Evaluate the Safety and Efficacy of a Single Dose of CTX001 in Pediatric Subjects With Severe Sickle Cell Disease	Recruiting	3	Vertex Pharmaceuticals Incorporated / CRISPR Therapeutics
	NCT03728322	A Safety and Efficacy Study of a Single Center, Open-label, Single Arm About the Gene Correction of HBB in Patient-specific iHSCs Using CRISPR/Cas9 That Intervent Subjests With β-thalassemia Mutations	Unknown	2	Allife Medical Science and Technology Co., Ltd.
	NCT04925206	A Multicenter, Open Label Phase 1 Study to Evaluate the Safety and Efficacy of a Single Dose of Autologous CRISPR-Cas9 Modified CD34+ Human Hematopoietic Stem and Progenitor Cells (hHSPCs) in Subjects With Transfusion Dependent β-Thalassaemia	Active, not recruiting	1	EdiGene (GuangZhou) Inc.
	NCT04819841	A Phase I/II Study of GPH101 in Autologous CD34+ Hematopoietic Stem Cells to Convert HbS to HbA for Treating Severe Sickle Cell Disease	Recruiting	1, 2	Graphite Bio, Inc.
	NCT03164135	Safety and Feasibility Study of Allotransplantation of CRISPR/Cas9 CCR5 Gene Modified CD34+ Hematopoietic Stem/Progenitor Cells in HIV-infected Subjects With Hematological Malignances	Unknown	Not Applicable	Affiliated Hospital to Academy of Military Medical Sciences / Peking University / Capital Medical University
Infectious	NCT05144386	A Phase 1/2a, Sequential Cohort, Single Ascending Dose Study of the Safety, Tolerability, Biodistribution, and Pharmacodynamics of EBT 101 in Aviremic HIV-1 Infected Adults on Stable Antiretroviral Therapy	Recruiting	1	Excision BioTherapeutics
	NCT04990557	CRISPR/Cas9-modified Human T Cell (PD-1and ACE2 Knockout Engineered T Cells) for Inducing Long-term Immunity in COVID-19 Patients	Not yet recruiting	1, 2	Mahmoud Ramadan mohamed Elkazzaz - Faculty of science Damietta university
Ophthalmological	NCT04560790	CRISPR/Cas9 mRNA Instantaneous Gene Editing Therapy Assisted Corneal Transplantation in the Treatment of Refractory Viral Keratitis	Active, not recruiting	1, 2	Shanghai BDgene Co., Ltd. / Eye & ENT Hospital of Fudan University
	NCT03872479	Open-Label, Single Ascending Dose Study to Evaluate the Safety, Tolerability, and Efficacy of EDIT-101 in Adult and Pediatric Participants With Leber Congenital Amaurosis Type 10 (LCA10), With Centrosomal Protein 290 (CEP290)-Related Retinal Degeneration Caused by a Compound Heterozygous or Homozygous Mutation Involving c.2991+1655A>G in Intron 26 (IVS26) of the CEP290 Gene ("LCA10-IVS26")	Recruiting	1, 2	Editas Medicine, Inc.
Metabolic	NCT04601051	Phase 1 Two-Part (Open-label, Single Ascending Dose (Part 1) and Open-label, Single Dose Expansion (Part 2)) Study to Evaluate Safety, Tolerability, Pharmacokinetics, and Pharmacodynamics of NTLA-2001 in Patients With Hereditary Transthyretin Amyloidosis With Polyneuropathy (ATTRv-PN) and Patients With Transthyretin Amyloidosis-Related Cardiomyopathy (ATTR-CM)	Recruiting	1	Intellia Therapeutics
Cardiac	NCT05120830	Phase 1/2 Study to Evaluate Safety, Tolerability, Pharmacokinetics, and Pharmacodynamics of NTLA-2002 in Adults With Hereditary Angioedema (HAE)	Recruiting	1, 2	Intellia Therapeutics

Table I. Continuation.

Cancer	NCT03057912	A Safety and Efficacy Study of Transcription Activator-like Effector Nucleases and Clustered Regularly Interspaced Short Palindromic Repeat/Cas9 in the Treatment of HPV-related Cervical Intraepithelial Neoplasia	Unknown	1	First Affiliated Hospital - Sun Yat-Sen University / Jingchu - University of Technology
	NCT04426669	A Phase I/II Trial in Patients With Metastatic Gastrointestinal Epithelial Cancer Administering Tumor-Infiltrating Lymphocytes in Which the Gene Encoding CISH Was Inactivated Using the CRISPR/Cas9 System	Recruiting	1, 2	Intima Bioscience, Inc. / Masonic Cancer Center, University of Minnesota
	NCT03399448	Phase 1 Trial of Autologous T Cells Engineered to Express NY-ESO-1 TCR and CRISPR Gene Edited to Eliminate Endogenous TCR and PD-1 (NYCE T Cells)	Terminated	1	University of Pennsylvania / Parker Institute for Cancer Immunotherapy / Tmunity Therapeutics
	NCT03545815	Phase I Study to Evaluate Treatment of CRISPR-Cas9 Mediated PD-1 and TCR Gene-knocked Out Chimeric Antigen Receptor (CAR) T Cells in Patients With Mesothelin Positive Multiple Solid Tumors.	Recruiting	1	Chinese PLA General Hospital
	NCT04037566	A Safety Study of Autologous T Cells Engineered to Target CD19 and CRISPR Gene Edited to Eliminate Endogenous HPK1 (XYF19 CAR-T Cells) for Relapsed or Refractory Haematopoietic Malignancies.	Recruiting	1	Xijing Hospital / Xi'An Yufan Biotechnology Co.,Ltd
	NCT04244656	A Phase 1 Dose Escalation and Cohort Expansion Study of the Safety and Efficacy of Anti-BCMA Allogeneic CRISPR-Cas9-Engineered T Cells (CTX120) in Subjects With Relapsed or Refractory Multiple Myeloma	Recruiting	1	CRISPR Therapeutics AG
	NCT04438083	A Phase 1 Dose Escalation and Cohort Expansion Study of the Safety and Efficacy of Allogeneic CRISPR-Cas9-Engineered T Cells (CTX130) in Subjects With Advanced, Relapsed or Refractory Renal Cell Carcinoma With Clear Cell Differentiation	Recruiting	1	CRISPR Therapeutics AG
	NCT04502446	A Phase 1, Open-Label, Multicenter, Dose Escalation and Cohort Expansion Study of the Safety and Efficacy of Anti-CD70 Allogeneic CRISPR-Cas9-Engineered T Cells (CTX130) in Subjects With Relapsed or Refractory T or B Cell Malignancies	Recruiting	1	CRISPR Therapeutics AG
	NCT04035434	A Phase 1 Dose Escalation and Cohort Expansion Study of the Safety and Efficacy of Allogeneic CRISPR-Cas9-Engineered T Cells (CTX110) in Subjects With Relapsed or Refractory B-Cell Malignancies (CARBON)	Recruiting	1	CRISPR Therapeutics AG
	NCT04637763	A Phase 1, Multicenter, Open-Label Study of CB-010, a CRISPR-Edited Allogeneic Anti-CD19 CAR-T Cell Therapy in Patients With Relapsed/Refractory B Cell Non-Hodgkin Lymphoma (ANTLER)	Recruiting	1	Caribou Biosciences, Inc.
	NCT04557436	Phase 1, Open Label Study of CRISPR-CAR Genome Edited T Cells (PBLTT52CAR19) in Relapsed /Refractory B Cell Acute Lymphoblastic Leukaemia	Recruiting	1	Great Ormond Street Hospital for Children NHS Foundation Trust / University College, London
	NCT03747965	Phase I Study of CRISPR-Cas9 Mediated PD-1 Gene-knocked Out Mesothelin-directed CAR-T Cells With the Conditioning Regimen of Paclitaxel and Cyclophosphamide in Mesothelin Positive Multiple Solid Tumors	Unknown	1	Chinese PLA General Hospital
	NCT03166878	Phase I/II Study to Determine the Safety, Tolerability, Biological Activity and Efficacy of Universal CRISPR-Cas9 Gene-Editing CAR-T Cells Targeting CD19(UCART019) in Patients With Relapsed or Refractory CD19+ Leukemia and Lymphoma	Unknown	1, 2	Chinese PLA General Hospital
	NCT03081715	Safety and Activity of Programmed Cell Death-1 Knockout Engineered T Cells in Patients With Previously Treated Advanced Esophageal Squamous Cell Carcinoma: An Open-label, Single-arm Phase 1 Study	Completed	1	Hangzhou Cancer Hospital / Anhui Kedgene Biotechnology Co.,Ltd
	NCT03398967	Phase I/II Study to Evaluate Treatment of Relapsed or Refractory Leukemia and Lymphoma With Universal CRISPR-Cas9 Gene-Editing CAR-T Cells Targeting CD19 and CD20 or CD22	Unknown	1, 2	Chinese PLA General Hospital
	NCT04976218	Phase I Study of EGFR Targeted TGFβR-KO CAR T Cells in the Treatment of Previously Treated Advanced EGFR-positive Solid Tumors	Recruiting	1	Chinese PLA General Hospital
	NCT02793856	A Phase I Clinical Trial of PD-1 Knockout Engineered T Cells Treating Patients With Advanced Non-small Cell Lung Cancer	Completed	1	Sichuan University / Chengdu MedGenCell, Co., Ltd.
	NCT04767308	A Single-center, Single-arm Exploratory Clinical Trial to Evaluate the Safety and Efficacy of Fully Human Anti-CD5 Chimeric Antigen Receptor T Cells (CT125A Cells) for the Treatment of Relapsed/Refractory CD5+ Hematopoietic Malignancies	Not yet recruiting	1	Huazhong University of Science and Technology / Shanghai IASO Biotechnology Co., Ltd
	NCT04417764	Safety and Effect Assessment of TACE in Combination With Autologous PD-1 Knockout Engineered T Cells by Percutaneous Infusion in the Paitents With Advanced Hepatocellular Carcinoma.	Unknown	1	Central South University
	NCT02863913	A Dose-escalation Phase I Trial of PD-1 Knockout Engineered T Cells for the Treatment of Muscle-invasive Bladder Cancer	Withdrawn (No funding)	1	Peking University
	NCT02867332	A Dose-escalation Phase I Trial of PD-1 Knockout Engineered T Cells for the Treatment of Metastatic Renal Cell Carcinom	Withdrawn (No funding)	1	Peking University
	NCT03044743	A Phase I/II Trial of PD-1 Knockout EBV-CTLs for Advanced Stage EBV Associated Malignancies	Unknown	1, 2	
	NCT05037669	Phase I Trial of Programmed Allogeneic CRISPR-edited T Cells (PACE) Gene Edited to Eliminate Endogenous TCR, HLA-class I and HLA-class II and Engineered to Express Anti-CD19 Chimeric Antigen Receptor (PACE CART19) in Patients With Relapsed Or Refractory CD19+ Leukemia and Lymphoma	Withdrawn (No available investigational product)	1	University of Pennsylvania
	NCT04244656	A Phase 1 Dose Escalation and Cohort Expansion Study of the Safety and Efficacy of Anti-BCMA Allogeneic CRISPR-Cas9-Engineered T Cells (CTX120) in Subjects With Relapsed or Refractory Multiple Myeloma	Recruiting	1	CRISPR Therapeutics AG
	NCT03398967	Phase I/II Study to Evaluate Treatment of Relapsed or Refractory Leukemia and Lymphoma With Universal CRISPR-Cas9 Gene-Editing CAR-T Cells Targeting CD19 and CD20 or CD22	Unknown	1, 2	Chinese PLA General Hospital
	NCT05066165	Phase 1/2a, Single Dose Study Investigating NTLA-5001 in Subjects With Acute Myeloid Leukem	Recruiting	1, 2	Intellia Therapeutics
	NCT05397184	Phase 1 Study of Base Edited CAR7 T Cells to Treat T Cell Malignancies (TvT CAR7)	Recruiting	1	Great Ormond Street Hospital for Children NHS Foundation Trust / UCL Great Ormond Street Institute of Child Health / Medical Research Council
Endocrine disease	NCT05210530	An Open-Label, First-In-Human Study Evaluating the Safety and Tolerability of VCTX210A Combination Product in Subjects With Type 1 Diabetes Mellitus (T1D)	Recruiting	1	CRISPR Therapeutics AG / ViaCyte

3.2. Artigo II: Prevalence of *Streptococcus pyogenes*-derived Cas9 antibodies in healthy donors and in patients with mucopolysaccharidosis type I and II

Artigo em submissão – The CRISPR Journal

4. DISCUSSÃO

A terapia gênica tem sido destacada como uma abordagem promissora no tratamento de doenças que não podem ser tratadas por métodos convencionais (Kumar et al., 2016). No entanto, seu êxito foi previamente desafiado por resultados imprevisíveis. Vários ensaios ocorreram na década de 1990, mas o avanço clínico estagnou com a morte de um paciente devido a uma resposta imune letal induzida por vetor viral durante um ensaio de segurança para deficiência de ornitina-transcarbamilase (Marshall, 1999). Em outros estudos, vários participantes que sofriam da condição de imunodeficiência combinada grave ligada ao X (SCID-X1), desenvolveram leucemia de células T em resposta à terapia (Check, 2002; Kaiser, 2003; Hacein-Bey-Abina et al., 2010 Gaspar et al., 2011). Ambos os incidentes estavam relacionados a métodos de administração terapêutica inadequados (Lino et al., 2018).

A partir da segunda metade da década passada houve uma retomada dos ensaios clínicos de terapia gênica, com muitos deles tornando-se uma realidade clínica. Desde 2016, produtos de terapia gênica foram aprovados tanto pela Agência Europeia de Medicamentos (EMA) quanto pela *Food and Drug Administration* (FDA) dos EUA (High and Roncarolo, 2019). Atualmente, há mais de 800 programas de terapia celular e gênica em desenvolvimento clínico, incluindo tratamentos para doenças como distrofia muscular de Duchenne e doença de Huntington. Diante desse cenário, é bastante provável que novas terapias sejam desenvolvidas. Contudo, as tecnologias de edição de genes abriram caminho para um novo paradigma (High and Roncarolo, 2019).

O sistema CRISPR/Cas9 destaca-se como uma escolha superior em relação a outras tecnologias de edição de genes por oferecer diversas vantagens significativas. Ao contrário das ferramentas ZFN ou TALEN que requerem modificações da enzima para cada sequência alvo e que devem ser sintetizadas separadamente para cada caso, a proteína Cas9 é universal. Ela pode ser facilmente ajustada para reconhecer novos locais por meio da alteração das sequências do sgRNA correspondentes aos locais desejados. Além disso, em comparação com ZFNs e TALENs, o CRISPR/Cas9 requer menos mão de obra e é mais econômico (Bhattacharya et al., 2015; Cox, Platt e Zhang, 2015; Komor et al., 2016; Yang et al., 2017).

Desde 2012, um gatilho tecnológico foi iniciado com a publicação de vários estudos sobre a ferramenta CRISPR/Cas9. Houve mais de 25.000 trabalhos acadêmicos publicados sobre o tema e mais de 20.000 patentes registradas em todo o mundo (Jefferson et al., 2021).

De acordo com o estudo de Jefferson e colaboradores em 2021, os Estados Unidos e a China lideram os investimentos em pesquisa na tecnologia CRISPR/Cas9. Identificar esses países é essencial para promover colaborações e parcerias mais eficientes. Isso evita duplicações de esforços, reduz custos intelectuais desnecessários e minimiza riscos relacionados à propriedade intelectual.

O estudo de Brinegar et al. (2017) examinou os investimentos em 17 *startups* de edição gênica no período de 2009 a 2015. Mais de 60 investidores apoiaram essas empresas, a maioria delas focada em saúde e biotecnologia. Nos Estados Unidos, de 2013 a 2017, mais de US$1 bilhão foram investidos nessas *startups*, com parcerias estabelecidas por grandes empresas como Google Ventures, Novartis e Bayer. Embora o Reino Unido seja líder em biotecnologia, enfrenta desafios de financiamento devido a regulamentações rigorosas. Enquanto isso, a China está ativamente envolvida em investimentos, com apoio financeiro do governo (Brinegar et al., 2017).

É importante notar que, como mostramos neste estudo, dos 192 artigos com foco em saúde, 97 artigos possuíam menos de 50% dos autores afiliados a instituições sul-americanas. Os Estados Unidos lideraram as colaborações, seguidos pelo Reino Unido, Alemanha, China, Holanda e Espanha. Ao analisar a distribuição de autores, o Brasil liderou com 112 publicações, seguido pelos Estados Unidos, Chile, Argentina e Reino Unido. A Colômbia, com 17 publicações, ocupou o sétimo lugar. Uruguai, Peru, Equador e Paraguai apresentaram 8, 5, 2 e 1 publicação, respectivamente. A falta de publicações de outros países latino-americanos, em especial da América Central, foi observada – ressaltando que o México não foi incluído na análise. Os resultados revelam um número limitado de inovações nacionais protegidas por propriedade intelectual na edição de genes na América do Sul, indicando a necessidade urgente de investimentos para impulsionar o desenvolvimento científico e tecnológico na região (Frâncio, Freitas e Matte, 2023).

Empresas e universidades devem intensificar seus investimentos em pesquisa e propriedade intelectual para desenvolver terapias CRISPR/Cas9 que sejam inovadoras, seguras, eficazes e acessíveis a um amplo número de pacientes. Esses investimentos não apenas impulsionam o progresso científico, mas também promovem transformação significativa no tratamento das doenças genéticas, podendo beneficiar a população, e, ao mesmo tempo, melhorar a qualidade de vida (Subica, 2023).

Desde 2013 o impacto do sistema CRISPR/Cas9 nos laboratórios foi inquestionável, como demonstrado pelos numerosos estudos que o utilizam, bem como pela sua rápida incorporação na criação de modelos animais geneticamente modificados, como camundongos (Li et al., 2020). A tecnologia CRISPR/Cas9 oferece um meio flexível e fácil de desenvolver modelos de doenças para explorar as causas genéticas e avaliar as possíveis estratégias terapêuticas (Papasavva, Kleanthous e Lederer, 2019). Também foram gerados modelos de doenças em animais de porte grande, incluindo ovelhas (Fan et al., 2018), coelhos (Xu et al., 2018), porcos (Yan et al., 2018) e macacos (Yang, Li e Li, 2019.). Além disso, tem sido utilizado na produção de componentes para a própria tecnologia (como reagentes, equipamentos, kits de ferramentas, vetores e entre outros). Mas, o que torna o CRISPR/Cas9 verdadeiramente revolucionário? É altamente preciso, rápido, fácil de implementar e acessível financeiramente, utilizando componentes amplamente disponíveis (Jefferson et al., 2021).

Embora seja comum que muitas tecnologias sigam um ciclo caracterizado por uma empolgação inicial seguida por desilusão antes de uma compreensão realista e produtiva do seu valor, esse padrão não se aplica a todas as inovações. Além disso, a duração e a intensidade de cada fase podem variar consideravelmente de uma tecnologia para outra. É importante notar que, em relação ao CRISPR/Cas9 esse ciclo parece ainda estar na fase ascendente, indicando uma resposta positiva à sua adoção (Musunuru, 2017; Bhushan, Chattopadhyay e Pratap, 2018).

Contudo, uma dúvida persiste: podemos esperar que essa tecnologia tenha um impacto significativo na saúde humana? As pesquisas realizadas até o momento forneceram evidências sólidas sobre como esse sistema de edição gênica contribui para o desenvolvimento de estratégias terapêuticas para diversas doenças humanas, tais como distrofia muscular de Duchenne (Long et al., 2014), fibrose cística (Crane et al., 2015), câncer (Aubrey et al., 2015), doenças cardiovasculares (Tessadori et al., 2018) e outras condições.

A estratégia de entrega mais avançada de edição CRISPR/Cas9 em ambiente clínico envolve a edição *ex vivo*, seguida pela reintrodução das células editadas no doador. O ambiente controlado e o controle de qualidade em relação à administração *in vivo* são as razões pela qual os pipelines *ex vivo* estão entre os primeiros a progredir para a clínica.

Em 2016, a China conduziu o primeiro ensaio clínico *ex vivo* (NCT02793856) utilizando a técnica CRISPR/Cas9 em pacientes com câncer de pulmão (Cyranoski, 2016). O alvo foi o gene *PD-1* em células T editadas, que foram reintroduzidas nos pacientes. O objetivo principal era avaliar a segurança e os efeitos secundários, e não a eficácia. Em 2020, os pesquisadores relataram que o tratamento era seguro para administração e apresentava pequenos efeitos colaterais, como febre, erupção na pele e fadiga. Detectaram também a presença de células T editadas no sangue periférico de todos os pacientes que receberam infusões, indicando a viabilidade e segurança do método, sendo que os pacientes tinham células T editadas dois meses após a infusão, embora em níveis baixos. Pacientes com níveis mais elevados de células editadas tiveram menor progressão da doença. (Lu et al., 2020).

A *CRISPR Therapeutics* anunciou em 2019, sucesso no tratamento do primeiro paciente com doença falciforme com o CTX001 (NCT03745287). A terapia consiste na interrupção do gene *BCL11A* pelo sistema CRISPR/Cas9 em células-tronco isoladas de pacientes com hemoglobinopatias, resultando em um aumento na produção de hemoglobina fetal (The Lancet Haematology, 2019). Neste mesmo ano, os primeiros pacientes com β-talassemia (NCT03655678) foram tratados com a mesma técnica, utilizando HSCs editadas por CRISPR/Cas9, seguido de transplante autólogo. A *CRISPR Therapeutics* e a *Vertex Pharmaceuticals* são as empresas responsáveis por conduzir estes estudos. Até o momento, os pacientes apresentaram níveis de hemoglobina dentro da normalidade ou quase normais. Os pacientes com β-talassemia não necessitam mais de transfusões, enquanto os pacientes com doença falciforme não necessitam mais de transfusões e não apresentam crises de dor incapacitantes. Os testes realizados após um ou mais anos do tratamento indicam a presença contínua de células editadas. Em relação aos efeitos adversos, apenas um paciente apresentou reações imunológicas graves durante o tratamento (Frangoul et al., 2021).

O primeiro ensaio clínico (NCT03872479) que implementa a terapia de edição gênica CRISPR/Cas9 diretamente no corpo humano, desenvolvido pela *Editas Medicine*, utilizou vetores adeno-associados (AAVs) para tratar amaurose congênita de Leber (LCA10) (Ledford, 2020). Terapias gênicas já aprovadas pela ANVISA, como o Luxturna (Russell et al., 2018) e Zolgensma (Waldrop and Kolb, 2019), utilizam esses mesmos vetores virais como método de entrega. Esses vírus são frequentemente escolhidos devido à capacidade de entrega sem inserção no genoma do hospedeiro (Lino et al., 2018; Chuang et al., 2021). Porém, apesar de serem pouco imunogênicos, a resposta imune adaptativa aos AAVs, resulta

em anticorpos neutralizantes e linfócitos T citotóxicos, que pode comprometer a eficácia do tratamento e impor riscos à segurança. Além das respostas imunes adaptativas, esses vetores desencadeiam uma resposta imune inata. Mesmo não sendo considerada clinicamente perigosa, minimizar a indução imune inata permanece um objetivo para maximizar a segurança do paciente e limitar a resposta imune adaptativa (Lochrie et al., 2006; Wang et al., 2011; Tse et al., 2017; Dauletbekov et al., 2019; Xiong et al., 2019). Como alternativa, a utilização de vetores não virais, como ribonucleoproteínas purificadas (RNPs) (Chen et al., 2019) e nanopartículas lipídicas (LNPs) (Lee et al., 2017) tornam-se promissoras.

O desenvolvimento de terapias gênicas é um empreendimento complexo, envolvendo cuidadosas considerações de estratégias que abrangem necessidades médicas não atendidas, abordagens de edição de genes, disponibilidade de modelos de doenças humanas e diversas outras variáveis necessárias para a criação de um medicamento seguro e eficaz. Ao desenvolver produtos de edição gênica, é essencial avaliar a relação entre risco e benefício, envolvendo a análise do risco associado à edição em comparação com os benefícios terapêuticos esperados do tratamento. Essa análise criteriosa é essencial para evitar retrocessos em abordagens promissoras, demandando resultados de testes pré-clínicos, dados farmacocinéticos, avaliação de genotoxicidade e consideração de potenciais efeitos imunogênicos. Além disso, é necessário ter um plano detalhado da fase inicial, seguindo as boas práticas clínicas e as boas práticas de fabricação para garantir a qualidade adequada do produto final (Cornu et al., 2017).

A implementação bem-sucedida do sistema CRISPR/Cas9 na prática clínica enfrenta diversos desafios, incluindo preocupações principalmente relacionadas à segurança e à entrega dessa tecnologia. Avanços estão sendo demonstrados em vários estudos com o intuito aprimorar a especificidade e aumentar a sensibilidade desta ferramenta (Carneiro, 2022 [Dissertação]). Além disso, questões sobre a imunogenicidade da proteína Cas9, derivada de bactérias, precisam ser resolvidas nas aplicações clínicas (Freitas, 2022 [Tese]). Igualmente, considerações importantes relacionadas à população-alvo que receberá o tratamento tornam-se importantes. É sugerido que a terapia seja personalizada conforme a imunidade individual, respostas imunológicas anteriores e histórico de tratamentos (Ewaisha e Anderson, 2023). Ensaios em pacientes imunocomprometidos mostraram reações mínimas ao sistema CRISPR/Cas9 (Xu et al., 2019; Lu et al., 2020; Stadtmauer et al., 2020; Uddin et al., 2020; Gillmore et al., 2021), enquanto para pacientes imunocompetentes, é importante

identificar marcadores de imunotoxicidade baseados no MHC e na proteína Cas9 utilizada (Ferdosi et al., 2018). Estratégias para evitar respostas imunes incluem identificar epítopos imunodominantes, otimizar a especificidade e minimizar a reatividade cruzada (Tang et al., 2022), implementação do sistema CRISPR/Cas para edição no início da vida e órgãos imunoprivilegiados (Rasul et al., 2022). Respostas imunes pré-existentes aos componentes CRISPR, especialmente em relação à reatividade cruzada entre diferentes tipos de CRISPR, e aos vetores virais também podem ser avaliadas individualmente, a fim de garantir a eficácia e a segurança durante a aplicação clínica.

Nosso estudo levanta questões sobre a interação entre a terapia gênica e o sistema imunológico, desafios e implicações da utilização da tecnologia CRISPR/Cas9 em diferentes grupos populacionais. Será que existe evasão da memória imunológica pela infecção por *S. pyogenes*? E por *S. aureus*? Seria possível perder a imunidade a SpCas9?

Compreender a resposta imunológica humana frente ao *S. pyogenes* e *S. aureus* não apenas desempenha um papel crucial na superação dos desafios da edição gênica, mas é essencial para o desenvolvimento eficaz de vacinas, visando prevenir a morbidade e mortalidade associadas às infecções causadas por essas bactérias. Até o momento, não há vacina licenciada contra nenhum desses patógenos. A respeito da imunidade ao *S. aureus*, não existem evidências clínicas sobre a existência de memória imunológica protetora. Algumas hipóteses sugerem que a exposição natural ao *S. aureus* pode induzir uma resposta de memória ao longo da vida, porém essa resposta não seria suficiente para prevenir a reinfecção, potencialmente comprometendo futuras estratégias de vacinação (Li et al., 2015). Já outro estudo sugere que infecções recorrentes por *S. aureus* não conferem imunidade protetora contra infecções subsequentes (Teymournejad e Montgomery, 2021). A dificuldade em desenvolver uma imunidade eficaz contra *S. aureus* pode ser, em parte, atribuída à sua capacidade de modular as respostas imunes inatas e adaptativas, promovendo a tolerância ou evasão imunológica (Li et al., 2015).

No caso do Streptococcus do grupo A (Strep A), observa-se uma imunidade natural, sendo provável que vários mecanismos imunológicos contribuam para a proteção contra infecções em adultos. Porém, os ensaios convencionais para avaliar a resposta imune ao Strep A, usados comumente para confirmar infecções recentes, envolvem medir os anticorpos neutralizantes anti-SLO e anti-DNAse B. Embora a maioria dos adultos tenha alguma atividade neutralizante contra essas proteínas, indicando exposição prévia ao Strep

A, esses anticorpos não garantem proteção específica. A elevação nos títulos destes anticorpos pode indicar uma infecção recente, mas é importante notar que várias cepas não produzem SLO devido a variações em sua região promotora (Frost et al., 2023). Além disso, a natureza da resposta imune ao *S. aureus* e ao *S. pyogenes* é influenciada por diversos fatores, incluindo o local da infecção, a virulência bacteriana e a genética do hospedeiro (Li et al., 2015; Teymournejad e Montgomery, 2021; Frost et al., 2023).

Nosso trabalho inclui questionamentos sobre a resposta imunológica em diferentes faixas etárias, visto que o grupo de pacientes é predominantemente composto por crianças, em contraste ao grupo controle, formado por adultos. Portanto, se houver uma variabilidade na resposta imune entre crianças e adultos, isso deve ser considerado em relação à eficácia e a segurança das terapias gênicas para doenças pediátricas.

Os estudos conduzidos por Charlesworth et al. (2019) e Simhadri et al. (2018) incluíram indivíduos saudáveis com idade superior a 18 anos, descrevendo resultados positivos quanto à presença de anticorpos anti-Cas9, mas sem relação entre a positividade de anticorpos e a idade. Já no estudo conduzido por Toral et al. (2022), a coorte analisada apresenta uma faixa etária mais avançada em comparação com as pesquisas mencionadas anteriormente, registrando uma média de aproximadamente 66 anos de idade. Esses pesquisadores destacam a hipótese de que a idade mais avançada possa estar associada a um aumento no risco ou a uma maior exposição ao longo da vida aos patógenos *S. aureus* ou *S. pyogenes*.

Idealmente, deveríamos comparar os pacientes com MPS I e II com controles da mesma faixa etária. Por um lado, porém, poucos pacientes com essas condições chegam à vida adulta devido à natureza da doença. Além disso, estima-se que o público alvo para terapia seria o pediátrico, que apresenta as formas mais graves da doença. Sendo assim, o desafio seria obter amostras pareadas de controles na mesma faixa etária. Outra possibilidade seria realizar mais ensaios com diferentes grupos, desde idade a outra condição médica, por exemplo, MPS IV, uma condição associada a um baixo comprometimento de infecções respiratórias. Ou a análise de pacientes com condições como fibrose cística, caracterizada por infecções respiratórias recorrentes. Até o momento, não existem estudos que demonstrem uma relação direta entre infecções respiratórias e um aumento na probabilidade de respostas imunes à proteína Cas9. Porém, essa relação necessita de investigação, visto que as duas variantes mais comumente utilizadas da Cas9 são derivadas de patógenos

humanos, os quais estão associados principalmente a doenças que afetam o trato respiratório superior. Além disso, essa investigação poderia fornecer *insights* sobre como as características específicas de diferentes condições médicas podem modular a resposta imunológica à terapia gênica e como o sistema imunológico reage em contextos de maior vulnerabilidade a infecções respiratórias.

Além disso, a ampliação do escopo para pacientes com outras doenças introduz a questão da generalização ou especificidade da resposta imune. Será que diferentes condições médicas induzem padrões distintos de formação de anticorpos anti-Cas9? A resposta imunológica à terapia é uniforme ou altamente contextualizada pela natureza da doença? Os estudos sobre a resposta imune ao *S. pyogenes* até o momento tem sido realizados apenas em populações normais, sem incluir pacientes com enfermidades específicas. No entanto, essas questões, embora desafiadoras, são importantes para o avanço seguro e eficaz da edição de genes. Sua compreensão permitirá refinamentos nas abordagens terapêuticas e uma adaptação mais precisa às necessidades individuais dos pacientes. Ao mesmo tempo, esse tipo de pesquisa pode demonstrar os limites e as potencialidades da tecnologia CRISPR/Cas9 em diferentes cenários clínicos.

Por fim, estudos clínicos devem ser conduzidos para avaliar a segurança em pacientes humanos, prestando atenção especial aos efeitos colaterais e à resposta imunológica. A pesquisa e o monitoramento contínuo são essenciais para aprimorar a segurança e eficácia das terapias com CRISPR/Cas9, focando não apenas na técnica em si, mas também nas implicações éticas e nos efeitos a longo prazo das intervenções nos pacientes (Cornu et al., 2017; Ewaisha e Anderson, 2023).

O propósito inicial deste projeto de tese era avaliar a prevalência de anticorpos anti-*Streptococcus pyogenes* Cas9 e entender como esses anticorpos afetam a eficácia da edição gênica pelo sistema CRISPR/Cas9. A parte inicial do projeto envolvia a análise da prevalência de anti-*Streptococcus pyogenes* Cas9 em indivíduos saudáveis e pacientes com MPS I e MPS II, que foi realizado.

A segunda parte deste projeto propunha avaliar e compreender se os anticorpos anti-*Streptococcus pyogenes* Cas9 afetam o tratamento com o sistema CRISPR/Cas9 e explorar as potencialidades e alternativas da tecnologia de edição de genes. A primeira fase consistia na infecção controlada de camundongos normais com a bactéria *Streptococcus pyogenes*, seguida por um protocolo de tratamento específico. O objetivo primordial dessa etapa seria

conferir imunidade aos camundongos após uma infecção, possibilitando a observação dos efeitos da resposta imunológica na presença da bactéria. Num segundo momento, seria conduzida uma análise para verificar se os camundongos normais foram, de fato, imunizados.

A terceira etapa do projeto envolvia a aplicação da técnica de edição gênica CRISPR/Cas9 nos camundongos previamente imunizados. Além disso, um grupo de camundongos normais não imunizados previamente seria utilizado como grupo controle. Este passo teria como objetivo comparar a eficiência edição gênica utilizando o sistema CRISPR/Cas9 em animais imunizados e não imunizados.

Questões fundamentais seriam exploradas, tais como a relação entre imunização prévia e eficiência da edição de genes pelo sistema CRISPR/Cas9. Isso proporciona *insights* promissores sobre a interação entre esses dois componentes cruciais. Além disso, poderia ser analisado o que acontece com as células editadas quando há presença da resposta imune, elas são excluídas? Permanecem viáveis?

Este projeto teria um potencial impacto significativo na pesquisa, destacando-se pela abordagem integrada de imunização contra *Streptococcus pyogenes* e pela aplicação da tecnologia CRISPR/Cas9. Infelizmente, as circunstâncias adversas e restrições impostas pela pandemia se tornaram um impeditivo significativo. Assim, a relação entre imunização prévia e a eficiência da edição de genes pelo sistema CRISPR/Cas9 foram aspectos que infelizmente não puderam ser plenamente explorados nesta tese.

5. CONCLUSÕES

Neste trabalho, pudemos constatar que pacientes brasileiros com MPS tipo I e tipo II mostraram a maior prevalência de anticorpos contra SpCas9 em comparação com população saudável, um achado importante que poderá ser utilizado para futuras abordagens terapêuticas em pacientes. Observamos uma taxa mais alta de positividade de anticorpos anti-SpCas9 nos grupos de pacientes com MPS em comparação com o grupo de doadores saudáveis, embora fossem mais jovens que os controles, porém não houve relação entre a idade e o sexo com a prevalência de anticorpos contra SpCas9. Embora os resultados não beneficiem diretamente os participantes, entender se esses pacientes tiveram exposição prévia a essa bactéria auxiliará no desenvolvimento de novas abordagens para essa estratégia de tratamento no futuro.

Além disso, através de uma revisão, pudemos constatar que as pesquisas utilizando o sistema CRISPR/Cas com foco em aplicações relacionadas à saúde na América do Sul, estão avançando, porém, ainda permanece baixo o número de inovações nacionais protegidas por propriedade intelectual nesse campo.

6. REFERÊNCIAS

Anders C, Niewoehner O, Duerst A and Jinek M (2014) Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. Nature, 513 (7519), 569 - 573.

Ajina R, Zamalin D, Zuo A, Moussa M, Catalfamo M, Jablonski SA and Weiner LM (2019) SpCas9-expression by tumor cells can cause T cell-dependent tumor rejection in immunocompetent mice. Oncoimmunology, 8 (5), e1577127.

Aubrey BJ, Kelly GL, Kueh AJ, Brennan MS, O'Connor L, Milla L, Wilcox S, Tai L, Strasser A and Herold MJ (2015) An inducible lentiviral guide RNA platform enables the identification of tumor-essential genes and tumor-promoting mutations in vivo. Cell reports, 10 (8), 1422–1432.

Berger KI et al. (2013) Respiratory and sleep disorders in mucopolysaccharidosis. Journal of inherited metabolic disease, 36, 201 - 210.

Bhattacharya D, Marfo CA, Li D, Lane M and Khokha MK (2015) CRISPR/Cas9: An inexpensive, efficient loss of function tool to screen human disease genes in Xenopus. Developmental biology, 408 (2), 196 - 204.

Bhushan K, Chattopadhyay A and Pratap D (2018) The evolution of CRISPR/Cas9 and their cousins: hope or hype?. Biotechnology letters, 40 (3), 465 - 477.

Borges P, Pasqualim G, Giugliani R, Vairo F and Matte U (2020) Estimated prevalence of mucopolysaccharidoses from population-based exomes and genomes. Orphanet Journal of Rare Diseases, 15 (1), 1 - 9.

Brinegar K, Yetisen AK, Choi S, Vallillo E, Ruiz-Esparza GU, Prabhakar AM, Khademhosseini A and Yun SH (2017) The commercialization of genome-editing technologies. Critical reviews in biotechnology, 37 (7), 924 - 932.

Carneiro PB (2022) Avaliação in silico de sítios de inserção em Genomic Safe Harbors para uso do sistema CRISPR/Cas9 em diferentes populações humanas. Dissertação (Mestrado em Genética e Biologia Molecular) – Instituto de Biociências, Universidade Federal do Rio Grande do Sul. Porto Alegre.

Carvalho TG et al. (2018) CRISPR-Cas9-mediated gene editing in human MPS I fibroblasts. Gene, 678, 33 - 37.

Celik B, Tomatsu SC, Tomatsu S and Khan SA (2021) Epidemiology of mucopolysaccharidoses update. Diagnostics, 11 (2), 273.

Charlesworth CT et al. (2019) Identification of preexisting adaptive immunity to Cas9 proteins in humans. Nature medicine, 25 (2), 249 - 254.

Check E (2002) A tragic setback. Nature, 420, 116 - 118.

Chen G, Abdeen AA, Wang Y, Shahi PK, Robertson S, Xie R, Suzuki M, Pattnaik BR, Saha K and Gong S (2019) A biodegradable nanocapsule delivers a Cas9 ribonucleoprotein complex for in vivo genome editing. Nature nanotechnology, 14 (10), 974 - 980.

Chew WL, Tabebordbar M, Cheng JK, Mali P, Wu EY, Ng AH, Zhu K, Wagers AJ and Church GM (2016) A multifunctional AAV-CRISPR-Cas9 and its host response. Nature methods, 13 (10), 868 - 874.

Chew WL (2018) Immunity to CRISPR Cas9 and Cas12a therapeutics. Wiley interdisciplinary reviews. Systems biology and medicine, 10 (1), 10.1002/wsbm.1408.

Chuang YF, Phipps AJ, Lin FL, Hecht V, Hewitt AW, Wang PY and Liu GS (2021) Approach for in vivo delivery of CRISPR/Cas system: a recent update and future prospect. Cellular and molecular life sciences: CMLS, 78 (6), 2683 - 2708.

Cimaz R and La Torre F (2014) Mucopolysaccharidoses. Current rheumatology reports, 16, 1 - 9.

Clarke LA (2008) The mucopolysaccharidoses: a success of molecular medicine. Expert reviews in molecular medicine, 10, e1.

Colmenares-Bonilla D, Colin-Gonzalez C, Gonzalez-Segoviano A, Garcia EE, Vela-Huerta MM and Lopez-Gomez FG (2018) Diagnosis of mucopolysaccharidosis based on history and clinical features: evidence from the Bajio region of Mexico. Cureus, 10 (11).

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA and Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. Science (New York, N.Y.), 339 (6121), 819 - 823.

CONITEC (2018a) Comissão Nacional de Incorporação de Tecnologias no Sistema Único de Saúde. Protocolo Clínico e Diretrizes Terapêuticas da Mucopolissacaridose tipo I. Disponível em: http://antigo-conitec.saude.gov.br/images/Relatorios/2018/Relatorio_PCDT_MPS_TipoI.pdf

CONITEC (2018b) Comissão Nacional de Incorporação de Tecnologias no Sistema Único de Saúde. Protocolo Clínico e Diretrizes Terapêuticas da Mucopolissacaridose tipo II. Disponível em: http://antigo-conitec.saude.gov.br/images/Protocolos/PCDT_MPS-II.pdf

Cox DB, Platt RJ and Zhang F (2015) Therapeutic genome editing: prospects and challenges. Nature medicine, 21 (2), 121 - 131.

Cornu TI, Mussolino C and Cathomen T (2017) Refining strategies to translate genome editing to the clinic. Nature Medicine, 23 (4), 415 - 423.

Crane AM et al. (2015) Targeted correction and restored function of the *CFTR* gene in cystic fibrosis induced pluripotent stem cells. Stem cell reports, 4 (4), 569 - 577.

Cyranoski D (2016) CRISPR gene-editing tested in a person for the first time. Nature, 539 (7630), 479.

Dai WJ, Zhu LY, Yan ZY, Xu Y, Wang QL and Lu XJ (2016) CRISPR-Cas9 for in vivo Gene Therapy: Promise and Hurdles. Molecular therapy. Nucleic acids, 5 (8), e349.

Dauletbekov DL, Pfromm JK, Fritz AK and Fischer MD (2019) Innate Immune Response Following AAV Administration. Advances in experimental medicine and biology, 1185, 165 - 168.

Doudna JA (2020) The promise and challenge of therapeutic genome editing. Nature, 578 (7794), 229 - 236.

Ewaisha R and Anderson KS (2023) Immunogenicity of CRISPR therapeutics-Critical considerations for clinical translation. Frontiers in bioengineering and biotechnology, 11, 1138596.

Fan Z et al. (2018) A sheep model of cystic fibrosis generated by CRISPR/Cas9 disruption of the CFTR gene. JCI insight, 3 (19), e123529.

Ferdosi SR, Ewaisha R, Moghadam F, Krishna S, Park JG, Ebrahimkhani MR, Kiani S and Anderson KS (2019) Multifunctional CRISPR-Cas9 with engineered immunosilenced human T cell epitopes. Nature communications, 10 (1), 1842.

Frâncio L, Freitas MV and Matte U (2023) CRISPR/Cas patents and health-related publications in South America. Anais da Academia Brasileira de Ciências, 95, e20220629.

Frangoul H et al. (2021) CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β-Thalassemia. The New England journal of medicine, 384 (3), 252 - 260.

Freitas MV, Frâncio L, Haleva L and Matte U (2022) Protection is not always a good thing: The immune system's impact on gene therapy. Genetics and molecular biology, 45 (3 Suppl 1), e20220046.

Freitas MV (2022) OTIMIZANDO A EDIÇÃO GÊNICA: DO SISTEMA IMUNE AOS *OFF-TARGETS*. Tese (Doutorado em Genética e Biologia Molecular) – Instituto de Biociências, Universidade Federal do Rio Grande do Sul. Porto Alegre.

Frost H, Excler JL, Sriskandan S and Fulurija A (2023) Correlates of immunity to Group A Streptococcus: a pathway to vaccine development. NPJ vaccines, 8 (1), 1.

Gasiunas G, Barrangou R, Horvath P and Siksnys V (2012) Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. Proceedings of the National Academy of Sciences of the United States of America, 109 (39), E2579 - E2586.

Gaspar HB, et al. (2011) Long-term persistence of a polyclonal T cell repertoire after gene therapy for X-linked severe combined immunodeficiency. Science translational medicine, 3 (97), 97ra79.

Gillmore JD et al. (2021) CRISPR-Cas9 In Vivo Gene Editing for Transthyretin Amyloidosis. The New England journal of medicine, 385 (6), 493 - 502.

Giugliani R et al. (2010) Mucopolysaccharidosis I, II, and VI: Brief review and guidelines for treatment. Genetics and molecular biology, 33, 589 - 604.

Giugliani R et al. (2012) Emerging treatment options for the mucopolysaccharidoses. Research and Reports in Endocrine Disorders, 53 - 64.

Giugliani R et al. (2014) Guidelines for diagnosis and treatment of Hunter Syndrome for clinicians in Latin America. Genetics and molecular biology, 37, 315 - 329.

Giugliani R, Federhen A, Michelin-Tirelli K, Riegel M and Burin M (2017) Relative frequency and estimated minimal frequency of Lysosomal Storage Diseases in Brazil: Report from a Reference Laboratory. Genetics and molecular biology, 40, 31 - 39.

Gough V and Gersbach CA (2020) Immunity to Cas9 as an Obstacle to Persistent Genome Editing. Molecular therapy: the journal of the American Society of Gene Therapy, 28 (6), 1389 - 1391.

Hacein-Bey-Abina S et al. (2010) Efficacy of gene therapy for X-linked severe combined immunodeficiency. The New England journal of medicine, 363 (4), 355 - 364.

High KA and Roncarolo MG (2019) Gene Therapy. The New England journal of medicine, 381 (5), 455 - 464.

Hsu PD., Lander ES and Zhang F (2014) Development and applications of CRISPR-Cas9 for genome engineering. Cell, 157 (6), 1262 - 1278.

Ishino Y, Shinagawa H, Makino K, Amemura M and Nakata A (1987) Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. Journal of bacteriology, 169 (12), 5429 - 5433.

Jansen R, Embden JDV, Gaastra W and Schouls LM (2002) Identification of genes that are associated with DNA repeats in prokaryotes. Molecular microbiology, 43 (6), 1565 - 1575.

Jefferson OA, Lang S, Williams K, Koellhofer D, Ballagh A, Warren B, Schellberg B, Sharma R and Jefferson R (2021) Mapping CRISPR-Cas9 public and commercial innovation using The Lens institutional toolkit. Transgenic research, 30 (4), 585 - 599.

Jiang F, Taylor DW, Chen JS, Kornfeld JE, Zhou K, Thompson AJ, Nogales E and Doudna JA (2016) Structures of a CRISPR-Cas9 R-loop complex primed for DNA cleavage. Science (New York, N.Y.), 351 (6275), 867 - 871.

Jiang F and Doudna JA (2017) CRISPR-Cas9 Structures and Mechanisms. Annual review of biophysics, 46, 505 - 529.

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA and Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science (New York, N.Y.), 337 (6096), 816 - 821.

Jinek M et al. (2014) Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. Science (New York, N.Y.), 343 (6176), 1247997.

Josahkian JA et al. (2021) Updated birth prevalence and relative frequency of mucopolysaccharidoses across Brazilian regions. Genetics and Molecular Biology, 44.

Kaiser J (2003) Gene therapy. Seeking the cause of induced leukemias in X-SCID trial. Science (New York, N.Y.), 299 (5606), 495.

Kato Y, Tabata H, Sato K, Nakamura M, Saito I and Nakanishi T (2021) Adenovirus Vectors Expressing Eight Multiplex Guide RNAs of CRISPR/Cas9 Efficiently Disrupted Diverse Hepatitis B Virus Gene Derived from Heterogeneous Patient. International journal of molecular sciences, 22 (19), 10570.

Knott GJ and Doudna JA (2018) CRISPR-Cas guides the future of genetic engineering. Science (New York, N.Y.), 361 (6405), 866 - 869.

Kobayashi H (2019) Recent trends in mucopolysaccharidosis research. Journal of Human Genetics, 64 (2), 127 - 137.

Komor AC, Kim YB, Packer MS, Zuris JA and Liu DR (2016) Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature, 533 (7603), 420 - 424.

Kubaski F, de Oliveira Poswar F, Michelin-Tirelli K, Matte U, Horovitz DD, Barth AL, Baldo G, Vairo F, Giugliani R (2020) Mucopolysaccharidosis Type I. Diagnostics. 10 (3), 161.

Kumar SR, Markusic DM, Biswas M, High KA and Herzog RW (2016) Clinical development of gene therapy: results and lessons from recent successes. Molecular Therapy-Methods & Clinical Development, 3.

Lavery C and Hendriksz C (2015) Mortality in patients with morquio syndrome a. JIMD reports, 15, 59 - 66.

Ledford H (2020) CRISPR treatment inserted directly into the body for first time. Nature, 579 (7798), 185.

Lee K et al. (2017) Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA in vivo induces homology-directed DNA repair. Nature biomedical engineering, 1, 889 - 901.

Li Z, Peres AG, Damian AC and Madrenas J (2015) Immunomodulation and Disease Tolerance to *Staphylococcus aureus*. Pathogens (Basel, Switzerland), 4 (4), 793 - 815.

Li H, Yang Y, Hong W, Huang M, Wu M and Zhao X (2020) Applications of genome editing technology in the targeted therapy of human diseases: mechanisms, advances and prospects. Signal transduction and targeted therapy, 5 (1), 1.

Li A et al. (2020) AAV-CRISPR Gene Editing Is Negated by Pre-existing Immunity to Cas9. Molecular therapy: the journal of the American Society of Gene Therapy, 28 (6), 1432 - 1441.

Lin HY et al. (2016) Causes of death and clinical characteristics of 34 patients with Mucopolysaccharidosis II in Taiwan from 1995-2012. Orphanet journal of rare diseases, 11 (1), 85.

Lino CA, Harper JC, Carney JP and Timlin JA (2018) Delivering CRISPR: a review of the challenges and approaches. Drug delivery, 25 (1), 1234 - 1257.

Liu C, Zhang L, Liu H and Cheng K (2017) Delivery strategies of the CRISPR-Cas9 gene-editing system for therapeutic applications. Journal of controlled release: official journal of the Controlled Release Society, 266, 17–26.

Liu M, Rehman S, Tang X, Gu K, Fan Q, Chen D and Ma W (2019) Methodologies for Improving HDR Efficiency. Frontiers in genetics, 9, 691.

Liu Y, Zou RS, He S, Nihongaki Y, Li X, Razavi S, Wu B and Ha T (2020) Very fast CRISPR on demand. Science (New York, N.Y.), 368 (6496), 1265 - 1269.

Lochrie MA, Tatsuno GP, Christie B, McDonnell JW, Zhou S, Surosky R, Pierce GF and Colosi P (2006) Mutations on the external surfaces of adeno-associated virus type 2 capsids that affect transduction and neutralization. Journal of virology, 80 (2), 821 - 834.

Long C, McAnally JR, Shelton JM, Mireault AA, Bassel-Duby R and Olson EN (2014) Prevention of muscular dystrophy in mice by CRISPR/Cas9-mediated editing of germline DNA. Science (New York, N.Y.), 345 (6201), 1184 - 1188.

Lu Y et al. (2020) Safety and feasibility of CRISPR-edited T cells in patients with refractory non-small-cell lung cancer. Nature medicine, 26 (5), 732 - 740.

Ma CC, Wang ZL, Xu T, He ZY and Wei YQ (2020) The approved gene therapy drugs worldwide: from 1998 to 2019. Biotechnology advances, 40, 107502.

Maeder ML et al. (2019) Development of a gene-editing approach to restore vision loss in Leber congenital amaurosis type 10. Nature medicine, 25 (2), 229 - 233.

Makarova KS et al. (2011) Evolution and classification of the CRISPR-Cas systems. Nature reviews. Microbiology, 9 (6), 467 - 477.

Marques ARA and Saftig P (2019) Lysosomal storage disorders - challenges, concepts and avenues for therapy: beyond rare diseases. Journal of cell science, 132(2), jcs221739.
Marshall E. (1999). Gene therapy death prompts review of adenovirus vector. Science (New York, N.Y.), 286 (5448), 2244 - 2245.

Mojica FJ, Díez-Villaseñor C, Soria E, and Juez G (2000) Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. Molecular microbiology, 36 (1), 244 - 246.

Mojica FJ, Díez-Villaseñor C, García-Martínez J and Soria E (2005) Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. Journal of molecular evolution, 60 (2), 174 - 182.

Muenzer J (2011) Overview of the mucopolysaccharidoses. Rheumatology, 50 (suppl_5), v4 - v12.

Muhlebach MS, Wooten W and Muenzer J (2011) Respiratory manifestations in mucopolysaccharidoses. Paediatric respiratory reviews, 12 (2), 133 - 138.

Musunuru K (2017) The Hope and Hype of CRISPR-Cas9 Genome Editing: A Review. JAMA cardiology, 2 (8), 914 - 919.

Nishimasu H, Ran FA, Hsu PD, Konermann S, Shehata SI, Dohmae N, Ishitani R, Zhang F and Nureki O (2014) Crystal structure of Cas9 in complex with guide RNA and target DNA. Cell, 156 (5), 935 - 949.

Pandey VK, Tripathi A, Bhushan R, Akhtar A and Dubey PK (2017) Application of CRISPR/Cas9 genome editing in genetic disorders: a systematic review up to date. J Genet Syndr Gene Ther, 8 (2), 1 - 10.

Papasavva P, Kleanthous M and Lederer CW (2019) Rare Opportunities: CRISPR/Cas-Based Therapy Development for Rare Genetic Diseases. Molecular diagnosis & therapy, 23 (2), 201 - 222.

Platt FM (2018) Emptying the stores: lysosomal diseases and therapeutic strategies. Nature reviews. Drug discovery, 17 (2), 133 - 150.

Platt FM, d'Azzo A, Davidson BL, Neufeld EF and Tifft CJ (2018) Lysosomal storage diseases. Nature Reviews Disease Primers, 4 (1), 27.

Ran FA et al. (2015) In vivo genome editing using *Staphylococcus aureus* Cas9. Nature, 520 (7546), 186 - 191.

Rasul MF et al. (2022) Strategies to overcome the main challenges of the use of CRISPR/Cas9 as a replacement for cancer therapy. Molecular cancer, 21 (1), 64.

Russell S, Bennett J, Maguire AM and High KA (2018) Voretigene neparvovec-rzyl for the treatment of biallelic RPE65 mutation–associated retinal dystrophy. Expert Opinion on Orphan Drugs, 6 (8), 457 - 464.

Sawamoto K, Stapleton M, Alméciga-Díaz CJ, Espejo-Mojica AJ, Losada JC, Suarez DA and Tomatsu S (2019) Therapeutic options for mucopolysaccharidoses: current and emerging treatments. Drugs, 79 (10), 1103 - 1134.

Simhadri VL, McGill J, McMahon S, Wang J, Jiang H and Sauna ZE (2018) Prevalence of Pre-existing Antibodies to CRISPR-Associated Nuclease Cas9 in the USA Population. Molecular therapy. Methods & clinical development, 10, 105 - 112.

Simonetti, G. D., Casaulta, C., Vuissoz, J. M., Bianchetti, M. G., & Nuoffer, J. M. (2009). Recurrent unexplained episodes of facial cyanosis and shortness of breath in Hunter disease. The Journal of pediatrics, 155 (1), 144 - 144.e1.

Singh R, Chopra S, Graham C, Langer M, Ng R, Ullal AJ and Pamula VK (2020) Emerging approaches for fluorescence-based newborn screening of mucopolysaccharidoses. Diagnostics, 10 (5), 294.

Sharma G, Sharma AR, Bhattacharya M, Lee SS and Chakraborty C (2021) CRISPR-Cas9: A Preclinical and Clinical Perspective for the Treatment of Human Diseases. Molecular Therapy: The Journal of the American Society of Gene Therapy, 29 (2), 571 - 586.

Shen X et al. (2022) Reduction of Pre-Existing Adaptive Immune Responses Against SaCas9 in Humans Using Epitope Mapping and Identification. The CRISPR journal, 5 (3), 445 - 456.

Stadtmauer EA et al. (2020) CRISPR-engineered T cells in patients with refractory cancer. Science (New York, N.Y.), 367 (6481), eaba7365.

Stapleton M, Arunkumar N, Kubaski F, Mason RW, Tadao O and Tomatsu S (2018) Clinical presentation and diagnosis of mucopolysaccharidoses. Molecular genetics and metabolism, 125 (1-2), 4 - 17.

Subica AM (2023) CRISPR in Public Health: The Health Equity Implications and Role of Community in Gene-Editing Research and Applications. American journal of public health, 113 (8), 874 - 882.

Tang XE, Tan SX, Hoon S and Yeo GW (2022) Pre-existing adaptive immunity to the RNA-editing enzyme Cas13d in humans. Nature medicine, 28 (7), 1372 - 1376.

Tessadori F, Roessler HI, Savelberg SMC, Chocron S, Kamel SM, Duran KJ, van HaelstMM, van Haaften G and Bakkers J (2018) Effective CRISPR/Cas9-based nucleotide editing in zebrafish to model human genetic cardiovascular disorders. Disease models & mechanisms, 11 (10), dmm035469.

Teymournejad O and Montgomery CP (2021) Evasion of Immunological Memory by *S. aureus* Infection: Implications for Vaccine Design. Frontiers in immunology, 12, 633672.

The Lancet Haematology (2019) CRISPR-Cas9 gene editing for patients with haemoglobinopathies. The Lancet. Haematology, 6 (9), e438.

Toral MA, Charlesworth CT, Ng B, Chemudupati T, Homma S, Nakauchi H, Bassuk AG, Porteus MH and Mahajan VB (2022) Investigation of Cas9 antibodies in the human eye. Nature communications, 13 (1), 1053.

Tse LV., Klinc KA, Madigan VJ, Castellanos Rivera RM, Wells LF, Havlik LP, Smith JK, Agbandje-McKenna M and Asokan A (2017) Structure-guided evolution of antigenically distinct adeno-associated virus variants for immune evasion. Proceedings of the National Academy of Sciences of the United States of America, 114 (24), E4812 - E4821.

Tulebayeva A, Sharipova M and Boranbayeva R (2020) Respiratory Dysfunction in Children and Adolescents with Mucopolysaccharidosis Types I, II, IVA, and VI. Diagnostics (Basel, Switzerland), 10 (2), 63.

Uddin F, Rudin CM and Sen T (2020) CRISPR Gene Therapy: Applications, Limitations, and Implications for the Future. Frontiers in oncology, 10, 1387.

Vijay S and Wraith JE (2005) Clinical presentation and follow-up of patients with the attenuated phenotype of mucopolysaccharidosis type I. Acta Pædiatrica, 94 (7), 872 - 877.

Wagner DL et al. (2019) High prevalence of *Streptococcus pyogenes* Cas9-reactive T cells within the adult human population. Nature medicine, 25 (2), 242 - 248.

Waldrop MA and Kolb SJ (2019) Current Treatment Options in Neurology-SMA Therapeutics. Current treatment options in neurology, 21 (6), 25.

Wang L, Calcedo R, Bell P, Lin J, Grant RL, Siegel DL and Wilson JM (2011) Impact of pre-existing immunity on gene transfer to nonhuman primate liver with adeno-associated virus 8 vectors. Human gene therapy, 22 (11), 1389 - 1401.

Wang D et al. (2015) Adenovirus-Mediated Somatic Genome Editing of Pten by CRISPR/Cas9 in Mouse Liver in Spite of Cas9-Specific Immune Responses. Human gene therapy, 26 (7), 432 - 442.

Williams N, Willet J, Clark D and Ketteridge D (2019) Tarsal tunnel syndrome in the mucopolysaccharidoses: A case series and literature review. JIMD reports, 46 (1), 16 - 22.

Wood SR and Bigger BW (2022) Delivering gene therapy for mucopolysaccharide diseases. Frontiers in Molecular Biosciences, 9, 965089.

Xiong W, Wu DM, Xue Y, Wang SK, Chung MJ, Ji X, Rana P, Zhao SR, Mai S and Cepko CL (2019) AAV cis-regulatory sequences are correlated with ocular toxicity. Proceedings of the National Academy of Sciences of the United States of America, 116 (12), 5785 - 5794.

Xu Y, Wang Y, Song Y, Deng J, Chen M, Ouyang H, Lai L and Li Z (2018) Generation and Phenotype Identification of PAX4 Gene Knockout Rabbit by CRISPR/Cas9 System. G3 (Bethesda, Md.), 8 (8), 2833 - 2840.

Xu L et al. (2019) CRISPR-Edited Stem Cells in a Patient with HIV and Acute Lymphocytic Leukemia. The New England journal of medicine, 381 (13), 1240 - 1247.

Yan S et al. (2018) A Huntingtin Knockin Pig Model Recapitulates Features of Selective Neurodegeneration in Huntington's Disease. Cell, 173 (4), 989 - 1002.e13.

Yang H, Wu JJ, Tang T, Liu KD and Dai C (2017) CRISPR/Cas9-mediated genome editing efficiently creates specific mutations at multiple loci using one sgRNA in Brassica napus. Scientific reports, 7 (1), 7489.

Yang W, Li S and Li XJ (2019) A CRISPR monkey model unravels a unique function of PINK1 in primate brains. Molecular neurodegeneration, 14 (1), 17.

Yang H, Ren S, Yu S, Pan H, Li T, Ge S, Zhang J and Xia N (2020) Methods Favoring Homology-Directed Repair Choice in Response to CRISPR/Cas9 Induced-Double Strand Breaks. International journal of molecular sciences, 21 (18), 6461.

Zhou J, Lin J, Leung WT and Wang L (2020) A basic understanding of mucopolysaccharidosis: Incidence, clinical features, diagnosis, and management. Intractable & Rare Diseases Research, 9 (1), 1 - 9.

7. ANEXOS

7.1. Publicações como co-autora

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

- Artigo 1: Freitas, Martiela V. et al. Protection is not always a good thing: The immune system's impact on gene therapy. Genetics and Molecular Biology, v. 45, 2022.

- Artigo 2: Fachel, F. et al. Gene editing strategies to treat lysosomal disorders: The example of mucopolysaccharidoses. Advanced Drug Delivery Reviews, 114616. 2022.

Genetics and Molecular Biology, 45, 3(suppl 1), e20220046 (2022)

DOI: https://doi.org/10.1590/1678-4685-GMB-2022-0046

Review
Young Brazilian Geneticists - Special Issue

Protection is not always a good thing: The immune system's impact on gene therapy

Martiela Vaz de Freitas[1,2,4], Lariane Frâncio[1,2], Laura Haleva[3] and Ursula da Silveira Matte[1,2,4,5]

[1]*Hospital de Clínicas de Porto Alegre, Centro de Pesquisa Experimental, Laboratório Células Tecidos e Genes, Porto Alegre, RS, Brazil.*
[2]*Universidade Federal do Rio Grande do Sul, Programa de Pós-Graduação em Genética e Biologia Molecular, Porto Alegre, RS, Brazil.*
[3]*Universidade Federal do Rio Grande do Sul, Instituto de Biociências, Porto Alegre, RS, Brazil.*
[4]*Hospital de Clínicas de Porto Alegre, Núcleo de Bioinformática Centro de Pesquisa Experimental, Porto Alegre, RS, Brazil.*
[5]*Universidade Federal do Rio Grande do Sul, Departamento de Genética, Porto Alegre, RS, Brazil.*

Abstract

There are many clinical trials underway for the development of gene therapies, and some have resulted in gene therapy products being commercially approved already. Significant progress was made to develop safer and more effective strategies to deliver and regulate genetic products. An unsolved aspect is the immune system, which can affect the efficiency of gene therapy in different ways. Here we present an overview of approved gene therapy products and the immune response elicited by gene delivery systems. These include responses against the vector or its content after delivery and against the product of the corrected gene. Strategies to overcome the hurdles include hiding the vector or/and the transgene product from the immune system and hiding the immune system from the vector/transgene product. Combining different strategies, such as patient screening and intelligent vector design, gene therapy is set to make a difference in the life of patients with severe genetic diseases.

Keywords: Gene delivery, gene therapy, immune response.

Received: February 17, 2022; Accepted: May 18, 2022.

Gene therapy

According to the MedlinePlus (2019) definition, gene therapy is a treatment that involves introducing genetic material into cells to compensate for the function of abnormal genes or to produce a therapeutic protein. Therapeutic gene therapy is performed only in somatic cells, as germline gene therapy is not approved. In addition, all protocols must assure that the transgene will not pass onto the patient's offspring. The delivery of the therapeutic genetic material can be performed *ex vivo* by systemic delivery or not (also called *in situ*) or *in vivo*.

In *ex vivo* therapies, the cells to be corrected are harvested, the gene transfer is performed in the laboratory, and cells are reinfused into the patient after correction. It is the preferred method for targeting bone marrow-derived cells in cancer treatment, including CAR-T cells (He *et al.,* 2020; Sterner and Sterner, 2021).

Of course, this is not an option for many target cells. In such cases, *in vivo* or *in situ* methods must be used. The first is the systemic administration of a vector carrying the therapeutic genetic material. And is used for systemic genetic diseases (Soofiyani *et al.,* 2013), and the therapeutic genetic material tends to be uptaken by the liver (Jacobs *et al.,* 2012).

Send correspondence to Ursula da Silveira Matte. Hospital de Clínicas de Porto Alegre, Centro de Pesquisa Experimental, Laboratório Células Tecidos e Genes, Rua Ramiro Barcelos, 2350, 90035-90, Porto Alegre, RS, Brazil. E-mail: umatte@hcpa.edu.br.

In contrast, the latter is a particular type in which the therapy administration occurs directly into the target cells or tissue. It is a method used to treat some types of cancer, such as melanoma or brain tumors, and genetic diseases like muscular dystrophy (Hromic-Jahjefendic and Lundstrom, 2020; Banerjee *et al.,* 2021; Elangkovan and Dickson, 2021).

In addition to traditional gene therapy, gene editing is a novel tool for correcting gene-caused diseases. Gene editing relies on three main strategies, as shown in Figure 1, that aim to modify the cell's DNA, promoting a local and definitive correction of the targeted cells. A similar approach, gene addition, seeks to insert an extra sequence coding for the desired proteins on the cell's DNA.

All methods described above require strategies to transfer the genetic material to target cells. Although many pre-clinical protocols use non-viral methods such as naked DNA, liposomes, or nanoemulsions (Ramamoorth and Narvekar, 2015), most clinical trials rely on viral vectors taking advantage of viruses' natural ability to use the host's cell machinery to express their genetic content. Figure 2 shows the main types of viral vectors used in clinical trials, whereas Table 1 presents their characteristics.

Gene therapy has gained momentum for treating different diseases worldwide with several clinical trials underway (Figure 3) and has made its way to the market with over ten approved products (Table 2). However, it is interesting to note that even though the definition of gene therapy refers

to "genetic material", RNA-based therapies (Table 3) are not considered gene therapy products by most regulatory agencies, including the FDA (USA), EMA (Europe) and ANVISA (Brazil). Several RNA-based products have been commercially approved worldwide. It is important to note that RNA-based therapies refer to products directed to decrease or abolish protein translation and consist of oligonucleotides that interact with mRNA. On the other hand, "traditional gene therapy" aims to provide de novo protein synthesis, thus leading to gene addition or replacement.

The *ex vivo* approved gene therapies

Indicated for treating severe combined immunodeficiency with no suitable stem cell donor, Strimvelis (GSK2696273) is the first *ex vivo* autologous gene therapy approved by EMA in 2016 (EMA, 2016a). The treatment consists of autologous $CD34^+$ enriched cells transduced with a retroviral vector encoding the adenosine deaminase (ADA) cDNA sequence. The patient's hematopoietic stem cells (HSCs) are extracted and purified until only CD34 expressing cells remain. They are then cultured and transduced with a gamma-retrovirus containing the human adenosine deaminase gene. At the end of the process, the cells are re-infused into the patient. The treatment was developed by GlaxoSmithKline (GSK) and had fever as the most common side effect.

Zalmoxis is a gene therapy for hematological malignancies, constituted of T lymphocytes genetically modified to express a shortened variant of the human low-affinity nerve growth factor receptor (LNGFR) and herpes simplex I virus thymidine kinase (HSV-TK Mut2) as a suicide gene (EMA, 2016b). It is an additional therapy for haploidentical hematopoietic stem cell transplantation (HSCT)

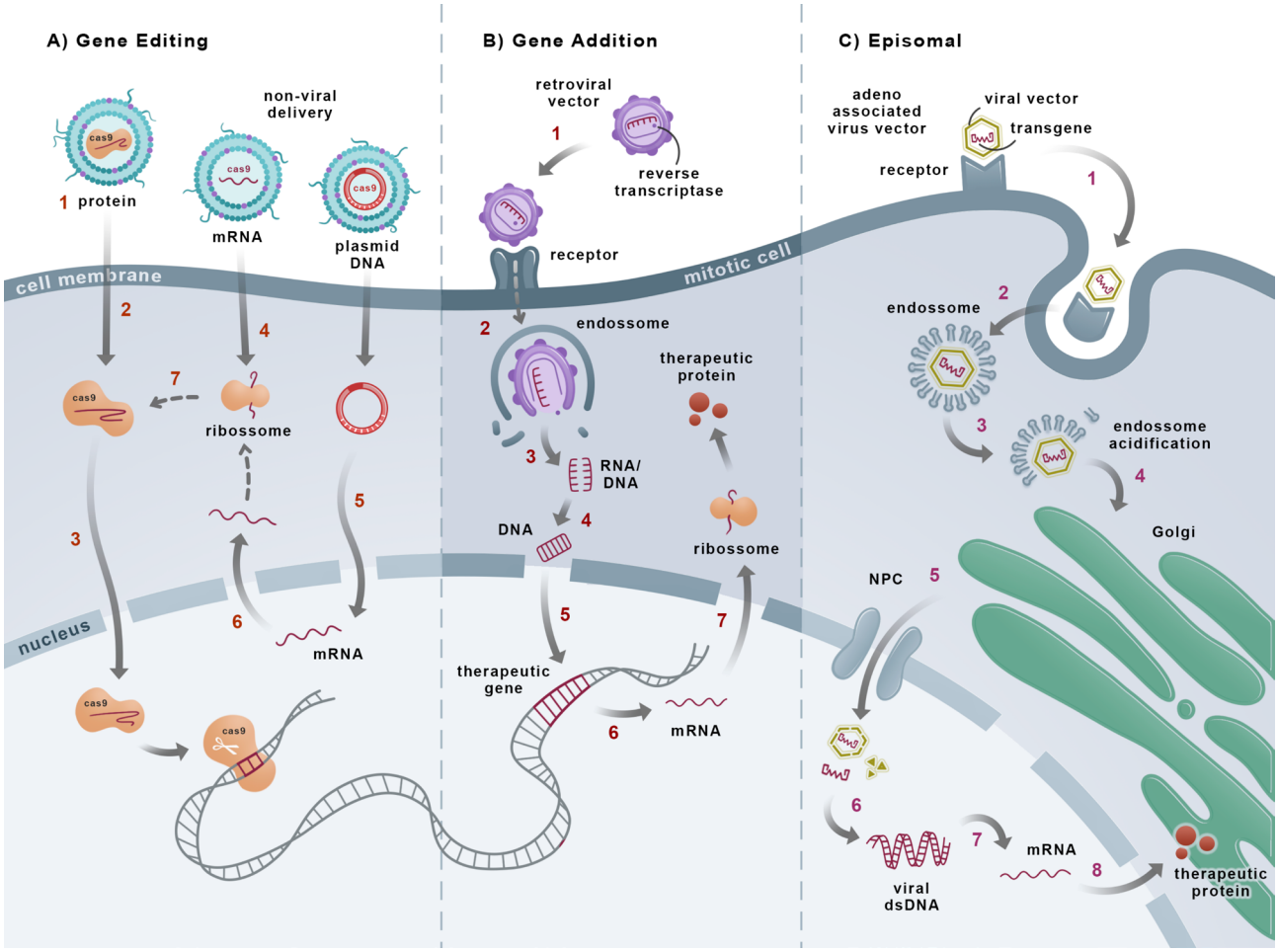

Figure 1 - Gene therapy strategies. **A**) Gene editing: CRISPR/Cas9 may be delivered as plasmid DNA, mRNA, or protein (1). In this example, the non-viral vector binds to the cellular membrane. After endocytosis into the cell, the particles escape from the endo/lysosome (2). Protein delivery is instantaneous and transient, results in the most immediate onset of gene editing, and avoids the concern of permanently integrating CRISPR genes into the host genome (3). Transferred mRNA must be released into the cytosol to enable mRNA translation to protein (4). Plasmid DNA needs to be translocated into the nucleus. The target cell's native transcription mechanism must be recruited to transcribe the gene into mRNA (5) and transport the mRNA into the cytoplasm (6). There, it will be translated into the protein, which must be transported back into the nucleus and modify the cell DNA (7). **B**) Gene addition: 1. In this example, the retroviral vector binds to the receptors on the cell surface and enters the cytoplasm through endocytosis (2). Once the endosome releases its content (3), the ssDNA is converted to dsDNA (4). It then gains access to the cell nucleus once the cell is in mitosis (5). The gene is inserted into the host cell DNA and transcribed into mRNA (6). In the cytoplasm, the ribosome translates it to the therapeutic protein (7). **C**) Episomal: In this example, the adeno-associated viral vector binds to receptors at the cell surface (1), and endocytosis occurs (2). The acidification process inside the endosome leads to vector liberation (3), and the Golgi-mediated capsid transport begins (4). After, the viral vector enters the nucleus through the nuclear pore complex (NPC) (5). The ssDNA is released from the vector and converted into dsDNA (6). Then, the episomal foreign DNA is transcripted into mRNA (7) and translated into the therapeutic protein (8).

of adult patients with high-risk hematological malignancies (EMA, 2016b). Infusion of genetically modified donor T cells into HSCT (T cell depletion) transplant recipients can easily restore immunity to protect against infections. Additionally, it may also challenge cancer cells. However, it might target the host's normal cells, leading to Graft Versus Host Disease (GVHD). In this case, a rescue mechanism using the suicide gene can be triggered using Ganciclovir (Belete, 2021). This drug induces the death of HSV-TK expressing T-cells, controlling GVHD. Zalmoxis was withdrawn after noticing that the product did not affect disease-free survival (EMA, 2019).

Kymriah (tisagenlecleucel) started to be developed by the University of Pennsylvania and was finished by Novartis to treat patients under 25 years old with B-cell acute lymphoblastic leukemia (ALL) or refractory Diffuse large B-cell lymphoma (DLBCL). Kymriah became the first treatment using gene therapy approved in the United States in August 2017 (FDA, 2021a). As with Strimvelis, the treatment is personalized to each patient. Purified T cells extracted from the patient's blood are modified to make a chimeric cell surface receptor (CAR-T) infused into the patient with the primary goal of targeting the CD19 protein common in B cells (Brandt *et al.,* 2020). The most common side effects of Kymriah are cytokine release syndrome (CRS) and the decrease of platelets, hemoglobin, or white blood cells. Among patients with DLBCL, the side effects might occur in 3 of 10 patients (EMA, 2018a).

Another gene therapy for B cells indicated to lymphoma that failed on conventional treatment is Yescarta (axicabtagene ciloleucel). Kite Pharma submitted it for FDA approval as a treatment for non-Hodgkin lymphoma in 2017. This gene therapy also focuses on genetically modifying immune cells from patients to make them able to act against the tumor cells. The drug mechanism is very similar to Kymriah. The engineered CAR-T cells bind to the tumor B-cells' CD19 protein, leading to activation of T-cell proliferation and, after, systemic cytokines release such as interleukin (IL)-15 and other chemokines, resulting in cell apoptosis (EMA, 2018b; Kite Pharma, 2017; FDA, 2021b) . Some of the adverse events related to Yescarta involve mainly CRS, correlated with the high levels of IFN-γ, noticed until 12 hours after the infusion. Although the principal symptom is fever, it may include hypoxemia and damage to the heart and kidneys. Neurological events developing up to two weeks following vector administration have also been described (National Library of Medicine [NLM], NCT02348216, 2015; NLM, NCT03391466).

Zynteglo (betibeglogene autotemcel) gene therapy involves modifying the patient's stem cells with a viral vector made on parts of the human immunodeficiency virus (HIV), modified not to cause AIDS. The virus contains normal copies of the beta-globin coding gene (βA^{-T87Q}-globin gene) that are integrated into the cell. These cells are then reinfused into the patient, producing HbAT87Q hemoglobin at levels that considerably reduce the requirement for transfusions (NLM, NCT01745120). First designated as an orphan drug to treat beta-thalassemia, a blood condition that requires monthly blood transfusions in individuals aged 12 and above, in the US in 2015 and for medical purposes in the EU in 2019, Zynteglo has been suspended as a precaution in Europe in February 2021. The allegation was that the viral vector used in this therapy might be responsible for the two cases of acute myeloid leukemia in a clinical trial for treating sickle cell disease. However, the EMA's human medicines committee (CHMP) has validated the conclusions of a review, published in July 2021, made by the Pharmacovigilance Risk Assessment Committee (PRAC) that found no evidence of vector in Zynteglo caused AML in those patients (PRAC, 2021; EMA, 2021). Although approved in the EU, it is not approved in the US yet.

The *in vivo* and *in situ* approved gene therapies

Gendicine (recombinant human p53adenovirus [Ad5RSV-p53]) was the first approved gene therapy globally. It was developed by Shenzhen SiBiono GeneTech, in China and approved by the CFDA in 2003 as a treatment for head and neck squamous cell carcinoma with any *KRAS* mutation. The vector enters the tumor cells through receptor-mediated endocytosis and overexpresses p53, stimulating the apoptotic pathways in the cell. The therapeutic product was shown to induce an elevated level of tumor-suppressing gene expression (Zhang *et al.,* 2018).

Oncorine (recombinant Human Adenovirus Type 5 Injection) was also developed to treat head and neck cancer. However, it consists of a modified adenovirus produced by Shanghai Sunway Biotech and was the first oncolytic virus to be approved as a treatment by SFDA in 2005. This treatment was designed to disable a viral defense mechanism that interacts with the p53 gene, commonly dysregulated in cancer cells (Liang, 2018). The mechanism has not been fully understood since the adenovirus used in this therapy targets cells regardless of their p53 status (Garber, 2006). A similar situation, in which viral replication seemed restricted to p53-defective tumor cells, was described by O'Shea *et al.* (2004). In this case, the authors showed that the absence of E1B-55K results in the induction but not in the activation of p53 during adenoviral replication. Authors suggest that RNA export may be compromised in tumor cells, which would explain the effects of ONYX-015. However, it has been shown to kill the tumor cells preferentially, with better results than chemotherapy only (Jhawar *et al.,* 2017).

Glybera (alipogene tiparvovec) is a gene therapy treatment developed by uniQure biopharma BV for lipoprotein lipase deficiency (LPLD) {OMIM: 238600}. This rare recessive disease causes severe pancreatitis due to mutations in the *LPL* gene. The first approval came from the European Medicines Agency (EMA) in July 2012 (EMA, 2017a,b). The drug, administered as injections in the leg muscles, contains the *LPL* gene encoded by an adeno-associated virus vector and soon has been called the "million-dollar drug" (Bulcha *et al.,* 2021) due to the price of the therapy. Glybera has been used in only one patient, reimbursed by the German government after lots of bureaucracy. This resulted in uniQure withdrawing from the European Union two years after the approval. However, the drug has been supplied at a nominal price for patients approved for treatment until the expiration date on October 25, 2017, by the Italian marketing partner Chiesi Farmaceutici (Senior, 2017).

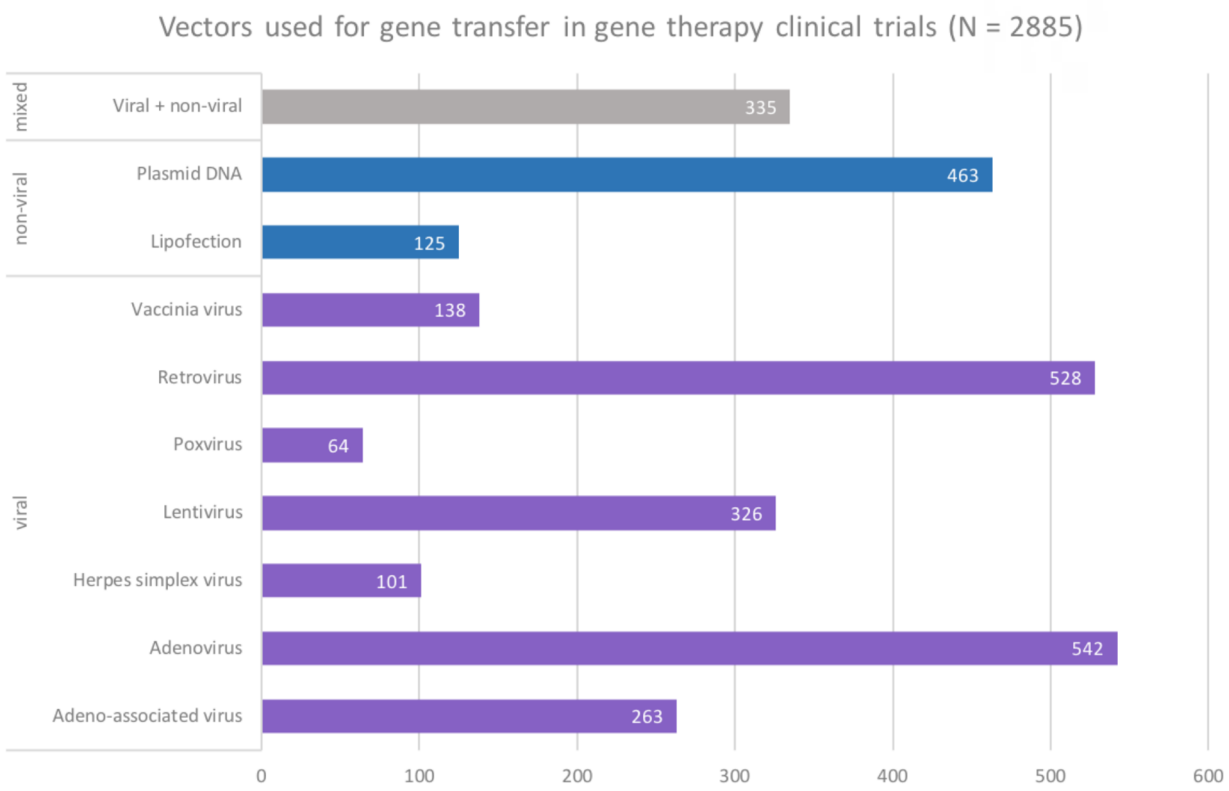

Figure 2 - Types of vectors used in clinical trials, according to (Ginn *et al.*, 2018).

Table 1 - Characteristics of the leading viral vectors used in clinical trials.

Vector	Strategy	Packing Capacity	Infection	Integration	Tranduction Efficiency	Expression	Immunogenic potential
Adenovirus	*in vivo*	8 kb	Dividing and non-dividing cells	Episomal	High	Transient	High
Retrovirus	*ex vivo*	8 kb	Dividing cells	Integrative	Moderate	Stable	Moderate
Lentivirus	*ex vivo*	8 kb	Dividing and non-dividing cells	Integrative	Moderate	Stable	Moderate
Adeno-associated virus	*in vivo*	4.5 kb	Dividing and non-dividing cells	Episomal	Moderate	Transient/Stable	Low

Developed in Russia in 2010, Neovasculgen (Cambiogenplasmid) is an encoding plasmid DNA for VEGF165, controlled by a cytomegalovirus (CMV) promoter. Composed of a transcription start site, the encoding VEGF165 isoform, a polyadenylation signal, a splicing signal, and an SV40 transcription terminator, Neovasculgen aims to treat atherosclerotic peripheral arterial disease (Bondar *et al.,* 2015). The drug's intramuscular delivery increases the ankle-brachial index, blood flow velocity, and pain-free walking distance. As a result, it was proposed as a viable treatment option for moderate to severe claudication or limping caused by persistent lower limb ischemia and approved by EMA (Deev *et al.,* 2015).

In 2007, Rexin-G (Mx-dnG1), a tumor-targeted retrovector bearing a cytocidal cyclin G1 construct, received approval from the Philippines FDA to treat all chemotherapy-resistant solid tumors. However, the approval for orphan drug designation came earlier in the United States. The FDA granted Rexin-G market protection for pancreatic cancer in 2003. Later, in 2008, the drug received orphan drug designation for osteosarcoma and soft tissue sarcoma (Orphan Drug Designations, 2008a,b; Chawla *et al.,* 2010; Gordon and Hall, 2010). Still, this drug does not figure as FDA-approved gene therapy.

Imlygic (talimogene laherparepvec), a genetically engineered herpes virus, was developed to be delivered by injection directly in inoperable melanoma. The virus is two genes absent and is modified to have the human *GM-CSF* gene. These modifications stimulate the immune system against the patient's tumor cells. The drug developed by Amgen was the first oncolytic immunotherapy approved by the FDA in October 2015 (FDA, 2015a,b).

Spark Therapeutics has developed and commercialized Voretigene Neparvovec-rzyl (AAV2-hRPE65v2), called Luxturna. It is the first gene therapy for inherited conditions authorized by the FDA in the United States on December 19, 2017, and by EMA on November 23, 2018 (FDA, 2018; EMA, 2018). Luxturna is an orphan medication used intraocularly to treat hereditary retinal degeneration caused by biallelic *RPE65* mutations (Ramlogan-Steel *et al.,* 2019). The clinical phenotypes of Leber congenital amaurosis type 2 (LCA2) {OMIM:204100} and retinitis pigmentosa type 20 (RP20) {OMIM: 613794} are caused by this kind of inherited retinal dystrophies (IRD). Retinitis pigmentosa (RP) is the most prevalent type of IRD. Both LCA2 and RP20 have an autosomal recessive inheritance pattern. The isomerase deficiency in the *RPE65* gene causes retinal pigment epithelium cells to lose their capacity to respond to light (Chung *et al.,* 2018). The therapeutic virus contains copies of the *RPE65* normal gene. The injection of Luxturna into the eye leads to the virus infection of retinal cells, allowing them to produce the missing enzyme and reducing the disease's development. The adeno-associated virus employed in this treatment does not cause any illness in humans (EMA, 2018).

Zolgensma (onasemnogene abeparvovec-xioi) is one of the most recent gene therapies approved by the FDA (May 2019). It is the first gene therapy approved to treat children under two years of age, carriers of spinal muscular atrophy (SMA) {OMIM: 253550}. This is one of the most severe forms of the disease and is a prominent hereditary cause of infant death. A mutation in the *SMN1* gene is the cause of SMA as the SMN protein encoded by this gene is essential for the maintenance and function of specialized nerve cells known as motor neurons. These cells, present in the brain and spinal cord, control muscle movements. In children, the signs and symptoms of the disease may appear at birth or by the age of six months (FDA, 2021c). The therapy consists of a non-replicating recombinant AAV9 with a functional copy of the human *SMN1* gene controlled by the CMV enhancer/chicken-actin-hybrid promoter (CB) to express *SMN1* in SMA patients' motor neurons. The AAV9 capsid's ability to penetrate the blood-brain barrier allows effective CNS delivery by intravenous injection. Additionally, the AAV ITR is modified to create a self-complementary DNA molecule that makes a double-stranded transgene that improves active transcription (Waldrop and Kolb, 2019).

Delytact (teserpaturev/G47Δ) is the gene therapy made by Daiichi Sankyo company conditionally approved by Japan's Ministry of Health, Labour and Welfare (MHLW) to treat malignant glioma in June 2021. The drug is based on genetically modified oncolytic herpes simplex virus (oHSV) type 1. The drug has a triple mutation on the viral genome, implying selective replication in cancer cells. G207 was the first oHSV approved in gene therapies (Herbring *et al.,* 2016). The third generation of this oHSV (G47Δ) results from the deletion of infected cell proteins (ICP)47 that places the late Us11 gene, a PKR inhibitor, under the control of the immediate-early ICP47 promoter. These changes block the protein shut-off (Jahan *et al.,* 2021). Once MHC-I expression is absent due to the ICP47 presence in HSV-infected cells, the human lymphocytes more efficiently recognize the antigens from the tumor and the virus in cancer cells when the ICP47 is deleted on the oncolytic vector (Jahan *et al.,* 2021; Zeng *et al.,* 2021). The conditional approval was received considering the results of the phase II trial (UMIN000015995, 2014) (Daiichi Press Release, 2021).

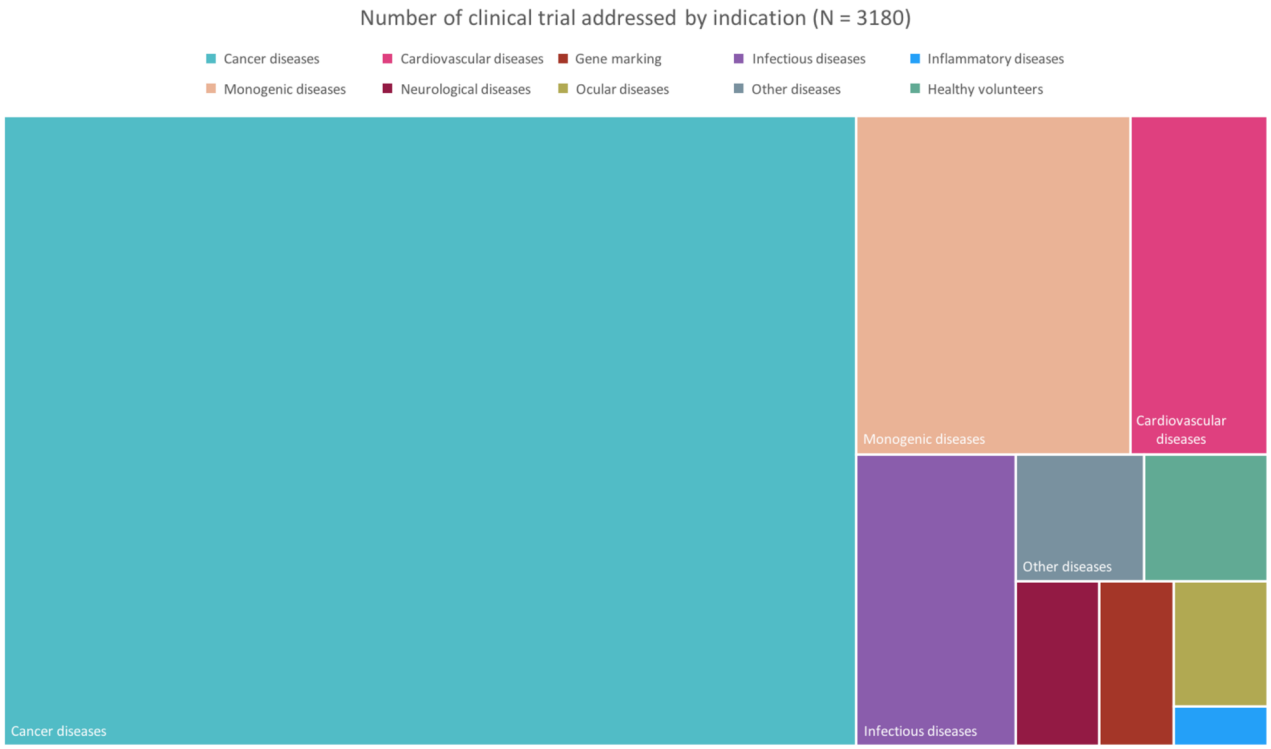

Figure 3 - Application of gene therapy in clinical trials according to Wiley Gene Therapy Clinical Trial Databases (Ginn *et al.*, 2018). Almost 70% of all clinical trials are designed for cancer diseases (light green), while monogenic diseases (coral) account for approximately 11%.

Table 2 - Approved gene therapies for gene addition or gene replacement.

Type	Name	Indication	Method	Immune-related issues*	Situation
in vivo	Gendicine	Head and neck squamous cell carcinoma	Recombinant human serotype-5 adenovirus	Fever, chill, pain at the injection site, fatigue, nausea, and diarrhea (Zhang *et al.,* 2018).	2003 - Approved
	Oncorine	Nasopharyngeal carcinoma	Recombinant human Adenovirus type 5 injection, H101	Fever, local pain at the injection site, and flu-like symptoms. (Ma *et al.,* 2009).	2005 - Approved
	Rexin-G	Metastatic pancreatic cancer	Retroviral vector	Chill, fatigue and headache. No serious drug-related adverse events (AE) were reported. (Chawla *et al.,* 2010)	2007 - Approved
	Neovasculgen	Atherosclerotic Peripheral Arterial Disease (PAD)	Plasmid DNA	Prophylactic treatment with acetylsalicylic acid to decrease risk of cardiovascular ischemic event. No AEs related. (Deev *et al.,* 2015)	2010 - Approved
	Glybera	Lipoprotein Lipase Deficiency (LPLD)	Adeno-associated virus serotype I	Immune response against AAV even with use of immunosuppressants.	2012 - Approved 2014 - Withdrawn
	Imlygic	Melanoma	Genetically manipulated oncolytic herpes simplex virus type 1 (HSV)	Flu-like illness, fevers and chills. Muscle pain (myalgia), painful/swollen joints (arthralgia), limb pain, vasculitis and glomerulonephritis (very rare). Autoimmmune reactions. Plasmocytoma.	2015 - Approved
	Luxturna	Inherited retinal dystrophies	Adeno-associated virus	Acute serious liver injury, acute liver failure, and elevated aminotransferases.	2017 - Approved
	Zolgensma	Treatment of Spinal Muscular Atrophy (Type I)	Non-replicating recombinant AAV9	Acute and chronic GvHD, febrile neutropenia, haemoglobin decreased, platelet count decreased.	2019 - Approved
	Delytact	Residual or recurrent glioblastoma	Oncolytic virus therapy - replication-conditional Herpes simplex virus type 1	Pyrexia, brain oedema, cytopenia, seizure, haemorrhage, infection, normal pressure hydrocephalus, and autoimmune diseases involving the central nervous system.	2021 - Conditional approval in Japan
ex vivo	Strimvelis	Severe combined immunodeficiency	Autologous Hematopoietic stem cells retroviral vector GSK3336223	Anaemia, neutropenia, autoimune haemolytic anaemia, autoimune aplastic anaemia, autoimune thrombocytopenia, autoimune thyroiditis, Guillain-Barré syndrome, rhinitis allergic, asthma, dermatites atopic, eczema.	2016 - Approved
	Zalmoxis	Hematopoietic Stem Cell Transplantation	Allogeneic T cells genetically modified with a retroviral vector	Acute and chronic GvHD, febrile neutropenia, haemoglobin decreased, platelet count decreased, hepatic failure, bronchitis and hepatic failure.	2016 - Approved 2019 - Withdrawn
	Kymriah	B-cell precursor acute lymphoblastic leukemia	Autologous T cells - CAR T cell therapy modified with lentiviral vector	Cytokine Release Syndrome. Neurological toxicities. Infections and Febrile Neutropenia. Prolonged Cytopenias. Hypogammaglobulinemia. Other manifestations included seizures, mutism and aphasia.	2017 - Approved
	Yescarta	Refractory large B-cell lymphoma	Autologous T cells - CAR T cell therapy modified by retroviral transduction	Cytokine Release Syndrome. Neurologic toxicities, both including fatal or life-threatening reactions.	2017 - Approved
	Invossa	Knee osteoarthritis	Allogeneic chondrocytes	Under Phase III for safety and efficacy evaluation.	2017 - Phase III Clinical Trial approved in US

* Information obtained from package insert, unless indicated otherwise.

Table 3 - Approved products for gene silencing.

Type	Name	Indication	Method*	Immune-related issues**	Situation
in vivo	Vitravene (fomivirsen)	Cytomegalovirus retinitis	Gene-silencing antisense therapy (ASO - antisense oligonucleotide)	Uveitis, including iritis and vitritis. Conjunctival and retinal inflammation. Anaemia, asthenia, diarrhea, fever, infection, rash, sepsis. More rare: abnormal liver function, allergic reactions, kidney failure, lymphoma like reaction, neuropathy, neutropenia, pancreatitis, thrombocytopenia.	2002 - Withdrawn (EU) 2006 - Withdrawn (US)
	Macugen (pegaptanib)	Neovascular age-related macular degeneration (AMD)	RNA oligonucleotide	Anterior chamber inflammation, punctate keratitis. Rare cases of anaphylaxis/ anaphylactoid reactions, including angioedema, have been reported.	2004 – Approved (FDA) 2011 – Withdrawn (EU)
	Kynamro (mipomersen sodium)	Homozygous familial hypercholesterolemia (HoFH)	Gene-silencing antisense therapy (ASO)	Elevation of alanine aminotransferase, Hepatic steatosis, influenza-like illness, pyrexia, arthralgia.	2013 – Approved
	Exondys 51 (eterplisen)	Duchenne muscular distrophy	ASO	Contact dermatitis	2016 - Approved
	Spinraza (nusinersen)	Spinal muscular atrophy (SMA)	Gene-silencing antisense therapy (ASO)	Thrombocytopenia and Coagulation Abnormalities, Renal Toxicity, lower respiratory infection and upper respiratory infection.	2016 - Approved
	Tegsedi (inotersen)	Transthyretin-mediated amyloidosis (hATTR)	ASO	Injection site reactions and fever. Thrombocytopenia, glomerulonephritis, renal toxicity, hepatic dysfunction, strokes. Rarer: antineutrophil cytoplasmic autoantibody (ANCA)-positive systemic vasculitis.	2018 - Approved
	Patisiran (onpattro)	Polyneuropathy caused by hereditary transthyretin-mediated amyloidosis (hATTR)	RNA interference	Upper respiratory tract infections and infusion-related reactions.	2018 - Approved
	Vyondis 53 (golodirsen)	Duchenne muscular distrophy	ASO	Hypersensitivity reaction, rash, pyrexia, pruritus, urticaria, dermatitis, skin exfoliation, nasopharyngitis. Renal toxicity.	2019 - Approved
	Waylivra (volanesorsen)	Lipoprotein lipase deficiency; Hypertriglyceridaemia	ASO	Injection site reactions. Allergic reaction including rash and fever. Thrombocytopenia, renal and hepatic toxicity.	2019 - Approved

* As described in the approval document. ** Information obtained from package insert, unless indicated otherwise.

The immune response

As shown above, different strategies are employed in approved gene therapy protocols. However, regardless of the vector or the targeting approach, the immune response against the vector and the transgene are significant challenges that gene therapy products may face. To be effective *in vivo*, gene therapy treatments must often overcome three significant immunological barriers (Wagner *et al.,* 2021), as resumed above and in Figure 4:

i. Avoid antibody neutralization of the delivery system;

ii. Avoid response against the vector or its content after delivery;

iii. Avoid immune response against the product of the corrected gene.

All these issues may be circumvented with different strategies that depend on the type of vector and how they interact with the immune system.

Immune response against the vectors

Non-viral vectors are safer and easier to build but pose significant cell targeting and transfection efficiency challenges. Generally, strategies to reduce liposome and nanoemulsion opsonization use polyethylene glycol (PEG) (Shi *et al.,* 2021). Even naked DNA strategies may present immune activation, either against DNA itself or contamination from bacterial products. Although seldom remembered, bacteria are essential players in gene therapy, as most constructs and plasmids are grown inside bacterial cells. Adequate purification procedures, however, make their participation in immune response unlikely.

Viruses have a natural ability to manipulate foreign genetic content into the host genome and therefore are preferred in terms of efficacy of gene transduction. On the other hand, viral vectors are often related to immune responses. When a pathogen invades the organism, an innate response is triggered to prevent infection. Cytokine production and the recruitment of nonspecific inflammatory cells, such as macrophages, NK cells, and others, are activated by toll-like receptors recognizing pathogenic peptides in the viral capsid.

Adenoviral vectors

Adenoviral vectors are non-enveloped double-stranded DNA vectors with a capacity of packing around 35kb (Talmadge and Cowan, 2020). The AdV vectors may be divided into two regions: early (E) - comprehending E1, E2, E3, and E4 regions, and late (L) - L1, L2, L3, L4, and L5 regions, each one named according to the time of their expression during the virus replication. Adenoviruses are common respiratory viruses that most people have contact with. Zsengellér and colleagues evaluate the central role of the alveolar macrophages during an adenovirus respiratory tract infection. They have shown that 30 minutes after the infection, alveolar macrophages started to express TNF-α and IL-6 in murine models (Zsengellér *et al.,* 2000). In primates, this response may take a little more time, as the systemic production of IL-6 and macrophage activation occurs around 2 hours after the administration in monkeys (Schnell *et al.,* 2001).

The first generation of AdV vectors was known for triggering the immune system. Even the absence of the E1 region, lowering the chances of expression of viral genes, did not completely diminish the viral replication capability. In terms of the immune system, this is enough to start the activation of cytotoxic T cells (CTLs) (Lusky *et al.,* 1998). This strong response has resulted in acute inflammation for several patients and death for a participant of a clinical trial (NLM, NCT00004386, 1999) that used an adenoviral vector for ornithine transcarbamylase deficiency (OTC) {OMIM: 311250} (Lehrman, 1999; Wilson, 2009).

The patient received an administration of $6x10^{11}$ particles/kg of an AdV type 5 preparation on the right hepatic artery. The first adverse reactions were noticed 18 hours after the administration and consisted of jaundice and altered mental status. Systemic inflammatory response syndrome and disseminated blood clots were reported in the subsequent hours. The death occurred 98 hours after the administration from multiple organ system failures. In the post-mortem analysis, it was possible to identify high levels of IL6, IL10, and TNF-a, in comparison with other participants in the study (Raper *et al.,* 2003).

Although the last generation of adenoviral vectors - characterized by the deletion of all viral genes from the vector - shows a favorable adaptive response, it still induces an innate response (Rogers *et al.,* 2011). The activation of innate response is dose-dependent and limited to a certain threshold (Ronzitti *et al.,* 2020). This response can be used for therapeutic purposes. One of the applications is DNX-2401 (Delta-24-RGD; tasadenoturev), a tumor-selective replication-competent oncolytic adenovirus designed as a monotherapy to treat long-term CRs in glioblastoma (Lang *et al.,* 2018). Although the mechanisms are still not fully understood, the therapy has shown anti-glioma efficacy, with tumor regression being observed several months after the administration without viral replication detection, suggesting that the effect is likely due to immune response. (Ferrera-Sal *et al.,* 2021).

Adeno-associated viral vectors

First discovered from laboratory AdV preparations (Atchison *et al.,* 1965), adeno-associated vectors are small non-enveloped viruses with a single strand DNA genome (4.7 Kb) contained in an icosahedral capsid. It was found not to be pathogenic in humans (Rogers *et al.,* 2011) many years later than its first discovery in human tissues (Blacklow *et al.,* 1967). All the research on adeno-associated virus (AAV) to characterize and understand its composition, replication, and transcription process and assembly made it possible for scientists to clone AAV into plasmids.

The AAV genome comprises two genes, rep and cap, flanked by two palindromic inverted terminal repeats (ITR). Rep codes for proteins involved in viral DNA replication, AAV genome packing, and viral genome integration into the host DNA (Wang *et al.,* 2019). Cap genes encode the capsid proteins and can be switched between serotypes (pseudotyping). Additionally, it produces two accessories proteins: AAP (assembly activating protein) and MAAP (membrane assembly activating protein) through alternative open reading frames (Sonntag *et al.,* 2010; Ogden *et al.,* 2019). AAV stays latent in integrated or non-integrated forms after infection until a helper virus offers the required functions for its replication (Ronzitti *et al.,* 2020).

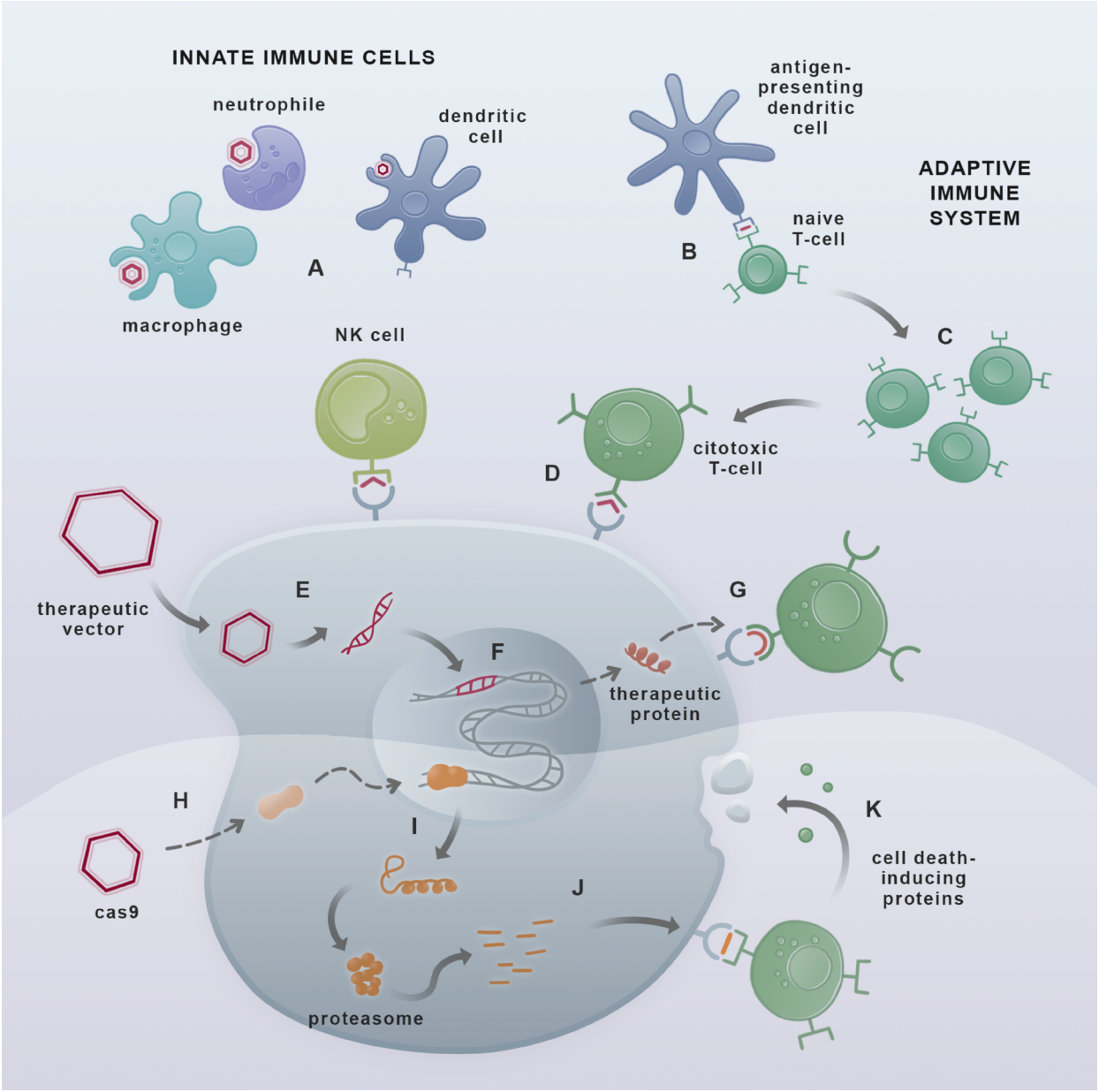

Figure 4 - Immune barriers to gene therapy and gene editing. Under infection, the primary defenses are the cells from the innate immune system. Here the viral content can be recognized and destroyed by the different phagocytic cells or recruiting other cells through specific cytokines such as the dendritic cells or natural killer cells (NK) that destroy infected cells upon specific receptor interactions (**A**). The second layer of response can be triggered by antigen-presenting cells that connect the innate and the adaptive immune systems (**B**). This contact results in the proliferation of naive T cells (**C**) that respond against the antigen through effector T cells (**D**). When the vector evades the innate immune system, the response may occur upon the recognition of parts of the vector (**E**) or, after the integration of the transgene into the host genome (**F**) under the recognition of the transgene product as non-self (**G**). The intensity of this response may depend on the partial existence of the gene product to be inserted. Finally, the gene-editing approach (**H**) presents an additional immune target: the editing protein itself. After promoting the gene edition, the protein follows the degradation pathway (**I**), resulting in small foreign peptides (**J**) that might be presented to cytotoxic T cells. In any case, CD8+ activation leads to the production of proinflammatory cytokines, resulting in cell death (**K**).

AAV seems not to induce the IL-8, IP-10, and RANTES chemokines expression, in contrast to adenoviral vectors (Zaiss *et al.,* 2002), which do so in a dose-dependent manner. Due to this lack of pathogenicity, AAV became the preferred vector for many applications (Kuzmin *et al.,* 2021) and is the base for approved gene therapy drugs cited before: Glybera, Luxturna, and Zolgensma for lipoprotein lipase deficiency (LPLD), inherited retinal disease (IRD), and spinal muscular atrophy (SMA), respectively.

AAV can elicit a cell-mediated action by the immune system even without triggering any innate response. Zhang *et al.* (2000) showed this response occurring *in vitro* when immature dendritic cells from femurs and tibias of 8- to 10-week-old female C57BL/6 mice incorporate the vector; and *in vivo* after adoptive transfer. This characteristic can also be used for oncolytic gene therapy. For example, Liu *et al.* (2001) showed that AAV-mediated transduction of dendritic cells led to increased cytokine production. Using the system

to deliver the HPV-16 E6 antigen gene into the cells, they could induce a class I (MHC-I)-restricted cervical cancer cell killing activity.

Severe adverse events caused by immune responses against AAV have recently been described. Three young males died after receiving the highest dose (3.5×1014 vg/kg) of AAV to treat X-linked Myotubular Myopathy (NLM, NCT03199469, 2018). Later, another boy died after receiving the lowest dose (1.3×10^{14} vg/kg) in the same study. The first side effects were liver dysfunction, followed by progressive cholestatic hepatitis and liver failure. In the mini-dystrophin gene therapy study from Pfizer (PF-06939926), the death of a patient was also related to immune response due to AAV high dose (Agarwal, 2020). These cases show the importance of considering immune responses more deeply in AAV clinical trials.

However, as in the AdV cases, this response may favor gene therapy. This is the case of oncolytic vectors such as Imlygic, which stimulates the immune system against the patient's tumor cells using an attenuated HSV-1 that enhances the preferential tumor-killing property of the virus.

Lentiviruses

Lentiviruses are a subtype of retrovirus capable of infecting nondividing and actively dividing cells. They are composed of single-strand RNA converted to double-strand DNA during their replication process. Retroviruses' general gene composition, also present in lentiviruses, is gag (the precursor to structural proteins), pro (protease enzymes), pol (integrase and reverse transcriptase precursors), and env (precursor to envelope glycoproteins) (Krebs *et al.*, 2021). The main issues linked to these vectors are insertional mutagenesis and genotoxicity (David and Doherty, 2017; Morgan *et al.,* 2021). Indeed, in 2002, in a trial for X-linked severe combined immunodeficiency, four out of ten patients developed leukemia presumably associated with vector integration (Hacein-Bey-Abina *et al.,* 2003).

This vector type, typically derived from HIV1, has primarily replaced retrovirus due to safety concerns and is involved in 10% of all gene therapy clinical trials worldwide (Ginn *et al.*, 2013), both *in vivo* and *ex vivo*. Most commonly, they are derived from primate lentiviruses Human Immunodeficiency Virus Type 1 (HIV-1) (Reiser *et al.,* 1996) and Type 2 (HIV-2) (Arya *et al.,* 1998) and Simian Immunodeficiency Virus (SIV) or non-primate lentiviruses (Schnell *et al.,* 2000), Feline Immunodeficiency Virus (FIV) (Poeschla *et al.,* 1998) and Equine infectious anemia virus (EIAV) (Olsen, 1998).

Brown *et al.* (2007a) were the first to demonstrate innate response in mice following LV delivery. Overall, the multiple interactions of LVs with the innate immune system occur depending on the LV dose, pseudotype, method of production, model strain, or recipient species remains (Annoni *et al.,* 2019). The primary responses are mediated by IFN-α,β production; pDC and cDC activation; and TLR-7 signaling (Nayak and Herzog, 2009).

In vivo therapies are in the pre-clinical phase (Palfi *et al.,* 2018; Link *et al.,* 2020), but the immune response can limit effectiveness and safety. The administration of the vector leads to a humoral and cell-mediated response against the LV capsid that may culminate in the inactivation of the vector, abrogating transduction, or eliminating transduced cells while the system is still exposed to the LV-derived antigens. However, vector re-administration and detailed characterization of anti-LV immune responses after systemic delivery still need to be investigated in animal models. Moreover, allogeneic immune responses can still occur against LVs produced by human-derived cells (Annoni *et al.,* 2019). A crucial point to be observed is the known ability of the LV vectors to transduce APC cells. Transduction of Kupfer cells (liver), macrophages, B cells, and dendritic cells in the spleen has been demonstrated by Van den Driesschee *et al.* (2002).

The parental HIV-1 elicits cell- and antibody-mediated responses in humans. Their immunogenicity indicates that LVs can activate innate and adaptive immunity (Follenzi *et al.,* 2007). One limiting factor on gene therapies that use HIV as a vector for application in patients is the pre-existing immunity to the wild-type virus (Annoni *et al.,* 2013). However, the persistence of the LV-modified T cells has been demonstrated in a clinical trial (NLM, NCT00295477) that administered autologous T cells in HIV-infected patients. This result suggests that pre-existing anti-HIV immunity is not enough to affect the efficiency of gene therapy. The most probable explanation is that even after receiving multiple infusions, the modified T cells did not carry over LV- or VSV.G-derived antigens (Annoni *et al.,* 2019).

Ex vivo therapies using hematopoietic stem cells and progenitors (Tucci *et al.,* 2021) or T cells (Ribeil *et al.,* 2017) are under clinical trials (NLM, NCT01852071; NLM, NCT01515462, 2012) and have shown satisfactory results in the use of HSPCs and LVs. Although recipients are not directly exposed to LVs, as in the i*n vivo* gene therapy, there is some risk of an immune response induced by the carry-over of antigens derived from the vector by the infused cells.

Still, the main issue about LV triggering immune responses is not related to the vector itself but the reactions against the transgene product. The intensity of this response may lead to a clearance of all cells that express the transgene proteins, as explained in the next topic.

Immune response against the transgene

The risk of response against the transgene is dependent on factors such as the type and route of the vector administration and the target tissue. Also, it depends on the host's characteristics, such as disease-specific tissue inflammation and the amount of pre-existing gene product (Shirley *et al.,* 2020). It has been shown that the innate immune system can regulate the transgene expression through the IFNγ and TNFα cytokines, inhibiting transgene expression (Sung *et al.,* 2001).

The response against transgene products might happen with different intensities, depending on host genomic alteration, a process also observed in enzyme replacement therapy (ERT). Knowing if the patient expresses a truncated form of the protein or does not express it is relevant to assessing the intensity of an immune response. In the first case, the immune response against the protein is attenuated since it would be dependent on the neo-antigens derived from the therapeutic protein. In the second case, however, the absence of the natural protein might result in a more intense immune response. In a

nonrandomized study (NLM, NCT00882921) for evaluation of the idursulfase long-term ERT for mucopolysaccharidosis II patients, it was found that 50% of the patients presented idursulfase-specific IgG antibodies (Giugliani *et al.,* 2017).

While a humoral response is expected on ERTs (Lenders and Brand, 2018), in gene therapy, it is most likely developing a cellular response against the transgene product. In phase I/II gene therapy study, patients with Duchenne's muscular dystrophy had a mini-dystrophin gene transferred by AAV (Mendell *et al.,* 2010). The result was a poor protein expression caused by possible pre-existing T cell immunity because of occasional endogenous dystrophin expression in revertant fibers. Again, this issue is not exclusive to gene therapy. Previously, Tremblay *et al*. (1993) showed the induction of immune response against the dystrophin after transplantation of myoblasts into the cardiac tissue of Duchenne patients.

Another issue not directly related to the transgene but that may affect it is the innate response against the promoters-generated RNA from transgenic AAV cassette that results in opposite complementary transcripts, triggering an innate response shown by Shao *et al.* (2018). It was demonstrated by measuring the levels of the transgene, which was increased when a plasmid with the 3′-ITR deletion and decreased with the insertion of a reversed polyA sequence between 5′-ITR and the start codon.

Immune response against the gene-editing proteins

Gene editing presents an additional target to the immune system: the reaction against the proteins used for double-strand DNA break. Although a lot has been done to understand the mechanisms of B-cell response against the vectors that deliver CRISPR/Cas9 into the cells, the T-cell response has been just recently shown.

Wang *et al*. (2015) found T-cell response against Cas9 proteins in immunocompetent mice. An adenovirus vector delivered a *Streptococcus pyogenes*-derived Cas9 system targeting the *Pten* gene, a frequently mutated gene in patients with sporadic cancer and involved in nonalcoholic steatohepatitis (NASH). The authors showed that hepatocytes were lost under humoral and cellular immune response against the AdV vectors between two weeks and four months after the injection. But they also noted an immune response against the Cas9 protein, detected through an ELISA assay for SpCas9 antibodies. In addition, a robust IgG1 antibody formation against Cas9 fourteen days after the administration of the adenovirus was reported.

Chew *et al*. (2016) and Chew (2018) have shown the same response when using AAV or electroporation to overexpress a transgene in the same type of animal. In 2016, the authors tested the functionality of AAV-Cas9-gRNA targeting *Mstn* (AAV9-Cas9-gRNA^{M3+M4}) by intraperitoneal injection on neonatal mice. The ELISA assay has confirmed a Cas9-specific humoral immune response, and Cas9 peptides were mapped using serum from the animals with M13 phage libraries covered with the Cas9 transgene. The T-cell response has also been found in studies that overexpressed SpCas9 in tumors transplanted into immunocompetent mice (Ajina *et al.,* 2019). Moreover, Li *et al*. (2020) demonstrated that the immunizations with SaCas9 in mice a week before the delivery of AVV-liver therapy decreased the long-term survival of the *in vivo* edited hepatocytes. This suggests an immune response against treatments mediated by Cas9 proteins due to memory acquired from previous infections.

Crudele and Chamberlain (2018) shed light on a pre-existent response against the most common human pathogens, *Staphylococcus aureus* and *Streptococcus pyogenes*, known for causing MRSA and strep throat from whom Cas9 protein is derived. Charlesworth *et al*. (2019) showed that our immune system could recognize Cas9 peptides as non-self, and the prevalence of anti-Cas9 response in healthy human adults is 79% anti-Cas9 IgG for SaCas9 and 67% for SpCas9. Simhadri *et al*. (2018), on the other hand, found rates around 10% and 2.5% for anti-SaCas9 e anti-SpCas9 in samples from the US.

Works from Wagner *et al*. (2019) and Ferdosi *et al.* (2019) have found similar results, pointing out that 85% and 5% of the blood donors have anti-SpCas9 antibodies and anti-SpCas9 T cells. While Charlesworth and Wagner's works have shown such a response using the entire recombinant protein through ELISPOT and flow cytometry, Ferdosi *et al*. (2019) used a different approach. Using *in silico* tools, they selected and built a pool of 38 peptides to test using HLA-A*02:01 pentamers associating ELISPOT and flow cytometry, reporting 83% (n =12) of the sample with IFN-γ+ response. Stadtmauer *et al*. (2020) used the same pool of peptides and suggested that 66% of the sample (n=3) are responsive to SpCas9. In addition, they reported the first human clinical trial designed to test the safety and feasibility of CRISPR-Cas9 editing of T cell receptors.

Avoiding the immune system

As interaction with the immune system may hamper gene therapy results, there is a need for countermeasures. Sack and Herzog (2009) divide the alternatives to circumvent the immune response into two categories

i) Methods that hide the vector or/and the transgene product from the immune system;

ii) Methods that hide the immune system from the vector/transgene product.

In the first scenario, it is possible to decrease the vector dose through capsid or transgene modifications or to use hydrodynamic injections. It is also possible to deliver the therapy only on immune-privileged sites such as the eyes, brain, knees, or liver. More sophisticated strategies include preventing the APC expression through tissue-specific promoters or miRNA targeting. Hiding the immune system from vectors can be achieved by suppression or modulation. One of the most common suppression mechanisms is to block cell division. Another is the depletion of specific cell types with antibodies. In immune modulation, it is possible to induce or adoptively transfer regulatory T cells (Tregs) or block the co-stimulation. In any of these processes, a balanced strategy to keep therapeutic levels of target proteins is desired (Figure 5).

Hiding the vector from the immune system

From the vector to the transgene, lots of work has been done on avoiding immune response and improving the outcomes of gene therapy techniques. Lowering adenoviral vector doses was one of the first measures. This strategy

is effective in hepatic gene transfer, including developing immune tolerance to coagulation factor IX (see Mingozzi and High, 2007). However, low doses of AdV may be quickly neutralized by the immune system in such a way that must be compensated by an increase of efficiency for the gene therapy to occur correctly.

The most studied methods to evade the immune system have been the modifications in the viral capsid since the response, toxicity, and clearance result from the interaction between the viral capsid and the host cells (Ahi *et al.,* 2011). With this in mind, covalent modifications have been developed to change the immunodominant epitopes and the capsid components needed for this interaction, as in studies using PEG.

PEG is an FDA-approved substance that allows covalent coupling of proteins, modifying major capsid proteins, fibers, hexons, and pentons. Croyle *et al*. (2002) demonstrated lower levels of immune response specific to AdV and increased transgene expression *in vivo* using PEGylation of E1 depleted AdV vector in liver cells of murine models.

Several capsid modifications to insert specific sequences that improve the binding to adapter molecules have also been reported. For example, human adenovirus serotype 5 (HAd5) infection occurs through interactions between the AdV fiber knob and some surface cell receptors such as Coxsackievirus-adenovirus receptor (CAR) (Excoffon, 2020), heparan sulfate glycosaminoglycan (Smith *et al.,* 2003; Mitra *et al.,* 2021), or sialic acid saccharide (Arnberg *et al.,* 2000). Fiber knob modifications consist of a knob-specific neutralizing antibody complex that retargets the Adv to another receptor (Bradley *et al.,* 2012). This new chimeric receptor confers to the vector the ability to decrease its immunogenicity.

Capsid modifications are also used for AAV through rational design (Bartel *et al.,* 2011). Lochrie *et al.* (2006) demonstrated that mapping the immunodominant epitopes of AAV-2 and their mutagenesis were enough to reduce the neutralization by the murine and human immune cells. The modifications were performed in 64 positions (especially on glycines and alanines) on the external surface of the AAV-2. While the reduced neutralization by the monoclonal antibody (A20) on murine accounted for more minor modifications, for human sera or IVIG (purified human IgG), the neutralization was increased when more mutations were combined.

Capsid modifications are also used for AAV. Rational design of the AAV capsid is one of the approaches (Bartel *et al.,* 2011), and the work from Lochrie *et al*. (2006) have demonstrated that mapping of the immunodominant epitopes of AAV-2 and their mutagenesis were enough to reduce the neutralization by the murine and human immune cells. The modifications were performed in 64 positions (especially on glycines and alanines) on the external surface of the AAV-2. While the reduced neutralization by the monoclonal antibody (A20) on murine accounted for more minor modifications, for human sera or IVIG (purified human IgG), the neutralization was increased when more mutations were combined.

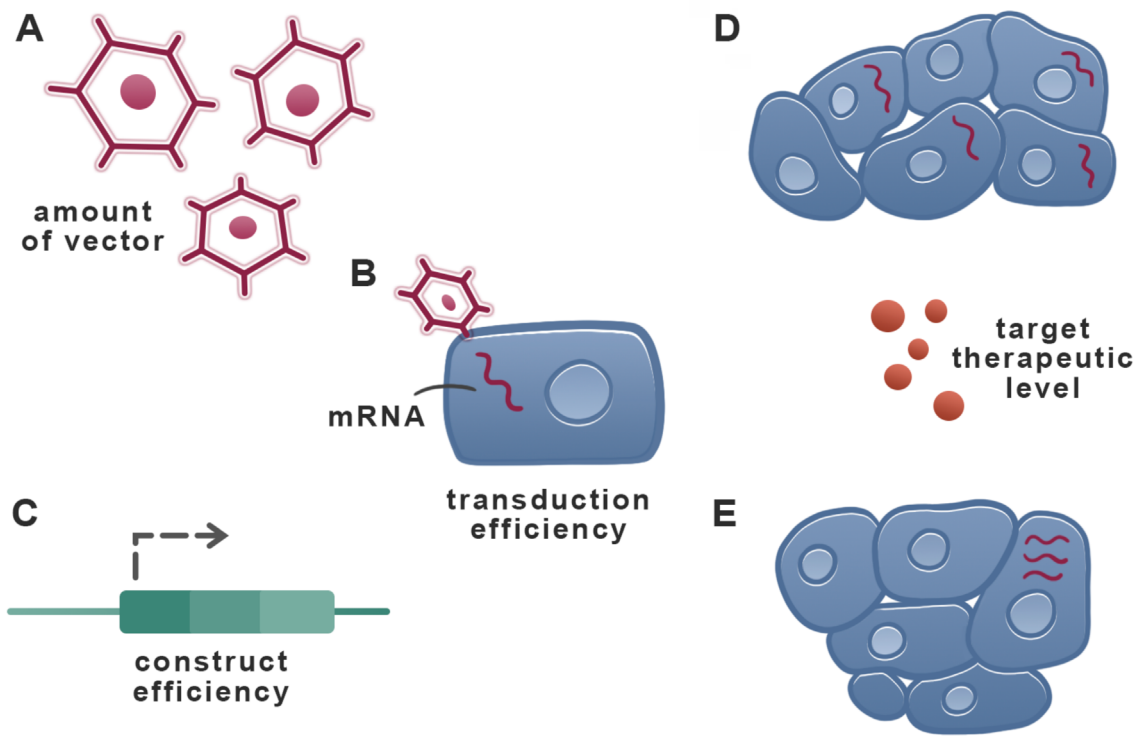

Figure 5 - Different strategies that may be used independently or combined to achieve target therapeutic levels of the transgene, in this case a secreted protein. Ideally, the amount of vector can be controlled in order to decrease the immune response (A). This can be compensated by vectors with higher transduction efficiency (B) and/or constructs with higher transgene expression (C). The desired outcome is a large number of transduced cells expressing the transgene in physiological levels (D) as opposed to a few high-expressing cells that may be more easily detected by an immune response against the therapeutic protein (E).

Brown *et al.* (2007b) have found an interesting solution to avoid an immune response against the transgene and the LVs. They took advantage of the miRNA regulation system, incorporating copies of a mirT targeting miRNA highly expressed in hematopoietic cells with hepatocyte-specific promoters. The strategy prevented the off-target expression of the transgene in hematopoietic-derived cells and resulted in a tissue-specific therapy.

Milani *et al.* (2017) showed that modifying LVs producing cells to inhibit the MHC I complex expression reduces the immunological response against the vectors. This is due to the attachment of cell surface proteins in the LV capsid. Another solution for response against LVs is the development of integration-defective lentiviral vectors (IDLVs) studied as a vaccine platform for antigen delivery. IDLVs are non-replicating, non-integrating vectors that incorporate a mutated integrase protein, preventing genome integration (Gallinaro *et al.,* 2018). Whereas it also prevents insertional mutagenesis, the vector's transduction efficiency *in vitro* and *in vivo* remains high, as shown by Wanisch and Yáñez-Muñoz (2009). Mátrai *et al.* (2011) have also demonstrated the benefits of the induction of active tolerance to the transgene and transgenic-specific Tregs in hepatocyte-targeted IDLV gene transfer due to their low but long-lasting transgene expression.

While a complete non-immunogenic Cas9 protein seems to be a distant reality for gene editing, engineered selective mutations based on the immunogenic peptide studies may be an alternative, as shown by Ferdosi *et al*. (2019). They show that silencing one peptide for HLA-A:02:01 was enough to diminish the Ca9 immunogenicity for the other three HLA-A alleles. Considering that human populations have around 19,000 HLA alleles (Robinson *et al.,* 2015), the above results are promising, as they show a common approach capable of considering the particularities within populations.

Although HLA is the main responsible for defining which peptide will be presented to lymphocytes (Gfeller and Bassani-Sternberg, 2018), different sizes of peptides must be considered to properly represent the immunogenic peptides and the modification of the Cas9 protein. On the other hand, developing new types of Cas9 may be a much more feasible attempt. For instance, the miniature CRISPR-Cas system (Harrington *et al.,* 2018; Xu *et al.,* 2021) is now in its early steps for gene editing. The authors showed that the system, with half the size of a Cas9 and Cas12, is efficient and very specific for gene activation, and it allows not only genome editing but also base editing. Intuitively, one may think that half of the sequence, half of the problems in the immune system and adaptive response. However, nothing has been shown about the capabilities of this Cas9 to stay out of the immune surveillance sight.

As a last resource, in many clinical trials, patients are methodically chosen. Patients with low titers of antibodies against the viral vector selected are less likely to present an immune response against the vector. In addition, patients with residual protein levels should offer a less aggressive immune response against the therapeutic product. For example, the developing therapies for hemophilia A and B, until 2016, recruited for their clinical trial only patients that did not present any inhibitory antibody against the protein administered in replacement therapies (Dolgin, 2016).

Hiding the immune system from the vector

Many drugs used for organ transplantation and autoimmune diseases may be used in gene therapy to modulate the immune response, avoid cell elimination, and promote tolerance. One evidence of such an approach is the use of rapamycin and IL-10 in gene therapy studies of canines with hemophilia (Nayak and Herzog, 2009). Another uses cyclosporine and anti-thymocyte globulin in dogs with Duchenne muscular dystrophy treated with AAV vectors (Wang *et al.,* 2007).

In humans, literature has shown that immune responses may be attenuated after ocular gene therapy using steroid drugs (Chan *et al.,* 2021). However, this strategy is not failure-proof. In hemophilia, the administration of oral immunosuppressors (IS) has failed to prevent immune response against the AAV vector proteins, limiting the efficiency of the therapy in patients (Mingozzi and High, 2013).

Samelson-Jones *et al*. (2020) showed that the moment of T cell-directed IS administration is crucial in determining transgene-product tolerance. Through a nonhuman primate model and using rabbit thymocyte-globulin (ATG), they evaluated the intensity of T cell response against AAV-mediated transfer of human factor IX (FIX). The results showed that anti-FIX antibody production occurred when the ATG was administered concomitantly with the AAV but was not found when the ATG was delayed five weeks after vector administration.

Conclusions

Gene therapy has come a long way from its first days and represents one of the major advances in genetic disease treatment. During its development, more than one well-known or recently discovered biotechnological tool has been studied as a tool for gene therapy: recombinant DNA, RNAi, gene delivery, and CRISPR. The possibility of curing genetic diseases and improving the lifespan of cancer patients are part of gene therapy's promises. However, even being an exciting field, safety always remains a point of concern, with the immune response as an obstacle that must be faced to guarantee the benefits of gene therapy.

Viral vectors were always a point of interest given their gene delivery ability, especially how some of them can circumvent the immune system. Although not discussed in this review, non-viral vectors are a promising tool for gene therapy. However, much improvement is needed, in particular related to transfection efficiency. Despite the variety of non-viral vectors developed in recent years, many still have problems related to stability in a physiological environment, uptake, and endosome evasion, as pointed out by Thapa and Narain (2016).

Studies focused on the transgene also show the importance of improving the understanding of the immune response and immune tolerance mechanisms. Santos *et al.* (2017), highlighted the use of different animal models that suggested Syrian hamsters as models for understanding

oncolytic adenoviruses mechanisms while using mice to obtain detailed immunological analyses. The use of gene-editing technologies poses an additional challenge due to the immune response against the nuclease. In this sense, the development and use of bioinformatics tools to predict and redesign immunogenic epitopes may be helpful.

Finally, combining different strategies, such as patient screening, intelligent vector design, forms of immunosuppression, or inducing tolerance, seems to be the right path for safer and efficient treatment. As new strategies progress, gene therapy makes its way into clinical practice. But the immune system, designed to protect us from foreign nucleic acid molecules, has a hard time understanding when these are used for our benefit.

Acknowledgments

We would like to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support and for the fellowships provided to MF (Process no.: 140002/2018-9), LF (Process no.: 149092/2019-9) and UM (Process no.: 312714/2018-1). We also thank FIPE/HCPA for financial support.

Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

MF and LF substantially contributed to the conception and design of the article and interpretation of the relevant literature; MF, LF, LH, and UM drafted the article or revised it critically for important intellectual content; MF and LH designed and produced the figures. UM published or can otherwise be considered an expert on the topic.

References

Agarwal S (2020) High-dose AAV gene therapy deaths. Nat Biotechnol. 2020 38:910.

Ahi YS, Bangari DS and Mittal SK (2011) Adenoviral vector immunity: Its implications and circumvention strategies. Curr Gene Ther 11:307-320.

Ajina R, Zamalin D, Zuo A, Moussa M, Catalfamo M, Jablonski SA and Weiner LM (2019) SpCas9-expression by tumor cells can cause T cell-dependent tumor rejection in immunocompetent mice. Oncoimmunology 8:e1577127.

Annoni A, Goudy K, Akbarpour M, Naldini L and Roncarolo MG (2013) Immune responses in liver-directed lentiviral gene therapy. Transl Res 161:230-240.

Annoni A, Gregori S, Naldini L and Cantore A (2019) Modulation of immune responses in lentiviral vector-mediated gene transfer. Cell Immunol 342:103802.

Arnberg N, Edlund K, Kidd AH and Wadell G (2000) Adenovirus type 37 uses sialic acid as a cellular receptor. J Virol 74:42-48.

Arya SK, Zamani M and Kundra P (1998) Human immunodeficiency virus type 2 lentivirus vectors for gene transfer: Expression and potential for helper virus-free packaging. Hum Gene Ther 9:1371-1380.

Atchison RW, Casto BC and Hammon WM (1965) Adenovirus-associated defective virus particles. Science 149:754-756.

Banerjee K, Núñez FJ, Haase S, McClellan BL, Faisal SM, Carney SV, Yu J, Alghamri MS, Asad AS, Candia JN *et al.* (2021) Current approaches for glioma gene therapy and virotherapy. Front Mol Neurosci 14:621831.

Bartel M, Schaffer D and Büning H (2011) Enhancing the clinical potential of AAV vectors by capsid engineering to evade pre-existing immunity. Front Microbiol 2:204.

Belete TM (2021) The current status of gene therapy for the treatment of cancer. Biologics 15:67-77.

Blacklow NR, Hoggan MD and Rowe WP (1967) Isolation of adenovirus-associated viruses from man. Proc Natl Acad Sci U S A 58:1410-1415.

Bondar O, Shevchenko V, Martynova A, Salakhieva D, Savina I, Shtyrlin Y and Abdullin T (2015) Intracellular delivery of VEGF165 encoding gene therapeutic using trifunctional copolymers of ethylene oxide and propylene oxide. Eur Polym J 68:680-686.

Bradley RR, Lynch DM, Iampietro MJ, Borducchi EN and Barouch DH (2012) Adenovirus serotype 5 neutralizing antibodies target both hexon and fiber following vaccination and natural infection. J Virol 86:625-629.

Brandt LJB, Barnkob MB, Michaels YS, Heiselberg J and Barington T (2020) Emerging approaches for regulation and control of CAR T cells: A mini review. Front Immunol 11:326.

Brown BD, Cantore A, Annoni A, Sergi LS, Lombardo A, Valle PD, D'Angelo A and Naldini L (2007a) A microRNA-regulated lentiviral vector mediates stable correction of hemophilia B mice. Blood 110:4144-4152.

Brown BD, Sitia G, Annoni A, Hauben E, Sergi LS, Zingale A, Roncarolo MG, Guidotti LG and Naldini L (2007b) *In vivo* administration of lentiviral vectors triggers a type I interferon response that restricts hepatocyte gene transfer and promotes vector clearance. Blood 109:2797-2805.

Bulcha JT, Wang Y, Ma H, Tai PWL and Gao G (2021) Viral vector platforms within the gene therapy landscape. Signal Transduct Target Ther 6:53.

Chan YK, Dick AD, Hall SM, Langmann T, Scribner CL, Mansfield BC and Ocular Gene Therapy Inflammation Working Group (2021) Inflammation in viral vector-mediated ocular gene therapy: A review and report from a workshop hosted by the Foundation Fighting Blindness, 9/2020. Transl Vis Sci Technol 10:3.

Charlesworth CT, Deshpande PS, Dever DP, Camarena J, Lemgart VT, Cromer MK, Vakulskas CA, Collingwood MA, Zhang L, Bode NM *et al.* (2019) Identification of preexisting adaptive immunity to Cas9 proteins in humans. Nat Med 25:249-254.

Chawla SP, Chua VS, Fernandez L, Quon D, Blackwelder WC, Gordon EM and Hall FL (2010) Advanced phase I/II studies of targeted gene delivery *in vivo*: Intravenous Rexin-G for gemcitabine-resistant metastatic pancreatic cancer. Mol Ther 18:435-441.

Chew WL (2018) Immunity to CRISPR Cas9 and Cas12a therapeutics. Wiley Interdiscip Rev Syst Biol Med 10:e1408.

Chew WL, Tabebordbar M, Cheng JKW, Mali P, Wu EY, Ng AHM, Zhu K, Wagers AJ and Church GM (2016) A multifunctional AAV-CRISPR-Cas9 and its host response. Nat Methods 13:868-874.

Chung DC, Bertelsen M, Lorenz B, Pennesi ME, Leroy BP, Hamel CP, Pierce E, Sallum J, Larsen M, Stieger K *et al.* (2018) The natural history of inherited retinal dystrophy due to biallelic mutations in the RPE65 gene. Am J Ophthalmol 199:58-70.

Croyle MA, Chirmule N, Zhang Y and Wilson JM (2002) PEGylation of E1-deleted adenovirus vectors allows significant gene expression on readministration to liver. Hum Gene Ther 13:1887-1900.

Crudele JM and Chamberlain JS (2018) Cas9 immunity creates challenges for CRISPR gene editing therapies. Nat Commun 9:3497.

David RM and Doherty AT (2017) Viral vectors: The road to reducing genotoxicity. toxicological sciences, 155:315–325.

Deev RV, Bozo IY, Mzhavanadze ND, Voronov DA, Gavrilenko AV, Chervyakov YV, Staroverov IN, Kalinin RE, Shvalb PG and Isaev AA (2015) pCMV-vegf165 intramuscular gene transfer is an effective method of treatment for patients with chronic lower limb ischemia. J Cardiovasc Pharmacol Ther 20:473-482.

Dolgin E (2016) Early clinical data raise the bar for hemophilia gene therapies. Nat Biotechnol 34:999-1001.

Elangkovan N and Dickson G (2021) Gene therapy for Duchenne muscular dystrophy. J Neuromuscul Dis 8:S303-S316.

Excoffon KJDA (2020) The coxsackievirus and adenovirus receptor: Virological and biological beauty. FEBS Lett 594:1828-1837.

Ferdosi SR, Ewaisha R, Moghadam F, Krishna S, Park JG, Ebrahimkhani MR, Kiani S and Anderson KS (2019) Multifunctional CRISPR-Cas9 with engineered immunosilenced human T cell epitopes. Nat Commun 10:1842.

Ferrera-Sal M, Moya-Borrego L, Bazan-Peregrino M and Alemany R (2021) Evolving status of clinical immunotherapy with oncolytic adenovirus. Clin Cancer Res 27:2979-2988.

Follenzi A, Santambrogio L and Annoni A (2007) Immune responses to lentiviral vectors. Curr Gene Ther 7:306-315.

Gallinaro A, Borghi M, Bona R, Grasso F, Calzoletti L, Palladino L, Cecchetti S, Vescio MF, Macchia D, Morante V *et al.* (2018) Integrase defective lentiviral vector as a vaccine platform for delivering influenza antigens. Front Immunol 9:171.

Garber K (2006) China approves world's first oncolytic virus therapy for cancer treatment. J Natl Cancer Inst 98:298-300.

Gfeller D and Bassani-Sternberg M (2018) Predicting antigen presentation-what could we learn from a million peptides? Front Immunol 9:1716.

Ginn SL, Alexander IE, Edelstein ML, Abedi MR and Wixon J (2013) Gene therapy clinical trials worldwide to 2012 - an update. J Gene Med 15:65-77.

Ginn SL, Amaya AK, Alexander IE, Edelstein M, & Abedi MR (2018) Gene therapy clinical trials worldwide to 2017: An update. J Gene Med 20:5.

Giugliani R, Harmatz P, Jones SA, Mendelsohn NJ, Vellodi A, Qiu Y, Hendriksz CJ, Vijayaraghavan S, Whiteman DAH and Pano A (2017) Evaluation of impact of anti-idursulfase antibodies during long-term idursulfase enzyme replacement therapy in mucopolysaccharidosis II patients. Mol Genet Metab Rep 12:2-7.

Gordon EM and Hall FL (2010) Rexin-G, a targeted genetic medicine for cancer. Expert Opin Biol Ther 10:819-832.

Hacein-Bey-Abina S, von Kalle C, Schmidt M, Le Deist F, Wulffraat N, McIntyre E, Radford I, Villeval J-L, Fraser CC, Cavazzana-Calvo M and Fischer A (2003) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Engl J Med 348:255-256

Harrington LB, Burstein D, Chen JS, Paez-Espino D, Ma E, Witte IP, Cofsky JC, Kyrpides NC, Banfield JF and Doudna JA (2018) Programmed DNA destruction by miniature CRISPR-Cas14 enzymes. Science 362:839-842.

He X, Feng Z, Ma J, Ling S, Cao Y, Gurung B, Wu Y, Katona BW, O'Dwyer KP, Siegel DL *et al.* (2020) Bispecific and split CAR T cells targeting CD13 and TIM3 eradicate acute myeloid leukemia. Blood 135:713-723.

Herbring V, Bäucker A, Trowitzsch S and Tampé R (2016) A dual inhibition mechanism of herpesviral ICP47 arresting a conformationally thermostable TAP complex. Sci Rep 6:36907.

Hromic-Jahjefendic A and Lundstrom K (2020) Viral vector-based melanoma gene therapy. Biomedicines 8:60.

Jacobs F, Gordts SC, Muthuramu I and De Geest B (2012) The liver as a target organ for gene therapy: State of the art, challenges, and future perspectives. Pharmaceuticals (Basel) 5:1372-1392.

Jahan N, Ghouse SM, Martuza RL and Rabkin SD (2021) In situ cancer vaccination and immunovirotherapy using oncolytic HSV. Viruses 13:1740.

Jhawar SR, Thandoni A, Bommareddy PK, Hassan S, Kohlhapp FJ, Goyal S, Schenkel JM, Silk AW and Zloza A (2017) Oncolytic viruses-natural and genetically engineered cancer immunotherapies. Front Oncol 7:202.

Krebs A-S, Mendonça LM and Zhang P (2021) Structural analysis of retrovirus assembly and maturation. Viruses 14:54.

Kuzmin DA, Shutova MV, Johnston NR, Smith OP, Fedorin VV, Kukushkin YS, van der Loo JCM and Johnstone EC (2021) The clinical landscape for AAV gene therapies. Nat Rev Drug Discov 20:173-174.

Lehrman S (1999) Virus treatment questioned after gene therapy death. Nature 401:517-518.

Lenders M and Brand E (2018) Effects of enzyme replacement therapy and antidrug antibodies in patients with Fabry disease. J Am Soc Nephrol 29:2265-2278.

Li A, Tanner MR, Lee CM, Hurley AE, De Giorgi M, Jarrett KE, Davis TH, Doerfler AM, Bao G, Beeton C *et al.* (2020) AAV-CRISPR gene editing is negated by pre-existing immunity to Cas9. Mol Ther 28:1432-1441.

Liang M (2018) Oncorine, the world first oncolytic virus medicine and its update in China. Curr Cancer Drug Targets 18:171-176.

Link JO, Rhee MS, Tse WC, Zheng J, Somoza JR, Rowe W, Begley R, Chiu A, Mulato A, Hansen D *et al.* (2020) Clinical targeting of HIV capsid protein with a long-acting small molecule. Nature 584:614-618.

Liu Y, Chiriva-Internati M, Grizzi F, Salati E, Roman JJ, Lim S and Hermonat PL (2001) Rapid induction of cytotoxic T-cell response against cervical cancer cells by human papillomavirus type 16 E6 antigen gene delivery into human dendritic cells by an adeno-associated virus vector. Cancer Gene Ther 8:948-957.

Lochrie MA, Tatsuno GP, Christie B, McDonnell JW, Zhou S, Surosky R, Pierce GF and Colosi P (2006) Mutations on the external surfaces of adeno-associated virus type 2 capsids that affect transduction and neutralization. J Virol 80:821-834.

Lusky M, Christ M, Rittner K, Dieterle A, Dreyer D, Mourot B, Schultz H, Stoeckel F, Pavirani A and Mehtali M (1998) In vitro and in vivo biology of recombinant adenovirus vectors with E1, E1/E2A, or E1/E4 deleted. J Virol 72:2022-2032.

Mátrai J, Cantore A, Bartholomae CC, Annoni A, Wang W, Acosta-Sanchez A, Samara-Kuko E, De Waele L, Ma L, Genovese P *et al.* (2011) Hepatocyte-targeted expression by integrase-defective lentiviral vectors induces antigen-specific tolerance in mice with low genotoxic risk. Hepatology 53:1696-1707.

Mendell JR, Campbell K, Rodino-Klapac L, Sahenk Z, Shilling C, Lewis S, Bowles D, Gray S, Li C, Galloway G *et al.* (2010) Dystrophin immunity in Duchenne's muscular dystrophy. N Engl J Med 363:1429-1437.

Milani M, Annoni A, Bartolaccini S, Biffi M, Russo F, Di Tomaso T, Raimondi A, Lengler J, Holmes MC, Scheiflinger F *et al.* (2017) Genome editing for scalable production of alloantigen-free lentiviral vectors for *in vivo* gene therapy. EMBO Mol Med 9:1558-1573.

Mingozzi F and High KA (2007) Immune responses to AAV in clinical trials. Curr Gene Ther 7:316-324.

Mingozzi F and High KA (2013) Immune responses to AAV vectors: Overcoming barriers to successful gene therapy. Blood 122:23-36.

Mitra D, Hasan MH, Bates JT, Bierdeman MA, Ederer DR, Parmar RC, Fassero LA, Liang Q, Qiu H, Tiwari V *et al.* (2021) The degree of polymerization and sulfation patterns in heparan sulfate are critical determinants of cytomegalovirus entry into host cells. PLoS Pathog 17:e1009803.

Morgan MA, Galla M, Grez M, Fehse B and Schambach A (2021) Retroviral gene therapy in Germany with a view on previous experience and future perspectives. Gene Ther 28:494-512.

Nayak S and Herzog RW (2009) Progress and prospects: Immune responses to viral vectors. Gene Ther 17:295-304.

O'Shea CC, Johnson L, Bagus B, Choi S, Nicholas C, Shen A, Boyle L, Pandey K, Soria C, Kunich J *et al.* (2004) Late viral RNA export, rather than p53 inactivation, determines ONYX-015 tumor selectivity. Cancer Cell 6:611-623.

Ogden PJ, Kelsic ED, Sinai S and Church GM (2019) Comprehensive AAV capsid fitness landscape reveals a viral gene and enables machine-guided design. Science 366:1139-1143.

Olsen JC (1998) Gene transfer vectors derived from equine infectious anemia virus. Gene Ther 5:1481-1487.

Palfi S, Gurruchaga JM, Lepetit H, Howard K, Ralph GS, Mason S, Gouello G, Domenech P, Buttery PC, Hantraye P *et al.* (2018) Long-term follow-up of a phase I/II study of ProSavin, a lentiviral vector gene therapy for Parkinson's disease. Hum Gene Ther Clin Dev 29:148-155.

Poeschla EM, Wong-Staal F and Looney DJ (1998) Efficient transduction of nondividing human cells by feline immunodeficiency virus lentiviral vectors. Nat Med 4:354-357.

PRAC (2021) PRAC meeting highlights July 2021: Zynteglo and COVID-19 vaccines. Reactions Weekly 1865:2.

Ramamoorth M and Narvekar A (2015) Non viral vectors in gene therapy - an overview. J Clin Diagn Res 9:GE01-6.

Ramlogan-Steel CA, Murali A, Andrzejewski S, Dhungel B, Steel JC and Layton CJ (2019) Gene therapy and the adeno-associated virus in the treatment of genetic and acquired ophthalmic diseases in humans: Trials, future directions and safety considerations. Clin Exp Ophthalmol 47:521-536.

Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao G-P, Wilson JM and Batshaw ML (2003) Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. Mol Genet Metab 80:148-158.

Reiser J, Harmison G, Kluepfel-Stahl S, Brady RO, Karlsson S and Schubert M (1996) Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles. Proc Natl Acad Sci U S A 93:15266-15271.

Ribeil J-A, Hacein-Bey-Abina SH, Payen E, Magnani A, Semeraro M, Magrin E, Caccavelli L, Neven B, Bourget P, El Nemer W *et al.* (2017) Gene therapy in a patient with sickle cell disease. N Engl J Med 376:848-855.

Robinson J, Halliwell JA, Hayhurst JD, Flicek P, Parham P and Marsh SGE (2015) The IPD and IMGT/HLA database: Allele variant databases. Nucleic Acids Res 43:D423-D431.

Rogers GL, Martino AT, Aslanidi GV, Jayandharan GR, Srivastava A and Herzog RW (2011) Innate immune responses to AAV vectors. Front Microbiol 2:194.

Ronzitti G, Gross D-A and Mingozzi F (2020) Human immune responses to adeno-associated virus (AAV) vectors. Front Immunol 11:670.

Sack BK and Herzog RW (2009) Evading the immune response upon in vivo gene therapy with viral vectors. Curr Opin Mol Ther 11:493-503.

Samelson-Jones BJ, Finn JD, Favaro P, Wright JF and Arruda VR (2020) Timing of intensive immunosuppression impacts risk of transgene antibodies after AAV gene therapy in nonhuman primates. Mol Ther Methods Clin Dev 17:1129-1138.

Santos JM, Havunen R, Siurala M, Cervera-Carrascon V, Tähtinen S, Sorsa S, Anttila M, Karell P, Kanerva A and Hemminki A (2017) Adenoviral production of interleukin-2 at the tumor site removes the need for systemic postconditioning in adoptive cell therapy. Int J Cancer 141:1458-1468.

Schnell MA, Zhang Y, Tazelaar J, Gao G, Yu QC, Qian R, Chen SJ, Varnavski AN, LeClair C, Raper SE *et al.* (2001) Activation of innate immunity in nonhuman primates following intraportal administration of adenoviral vectors. Mol Ther 3:708-722.

Schnell T, Foley P, Wirth M, Munch J and Uberla K (2000) Development of a self-inactivating, minimal lentivirus vector based on simian immunodeficiency virus. Hum Gene Ther 11:439-447.

Senior M (2017) After Glybera's withdrawal, what's next for gene therapy? Nat Biotechnol 35:491-492.

Shao W, Earley LF, Chai Z, Chen X, Sun J, He T, Deng M, Hirsch ML, Ting J, Samulski RJ *et al.* (2018) Double-stranded RNA innate immune response activation from long-term adeno-associated virus vector transduction. JCI Insight 3:e120474.

Shi L, Zhang J, Zhao M, Tang S, Cheng X, Zhang W, Li W, Liu X, Peng H and Wang Q (2021) Effects of polyethylene glycol on the surface of nanoparticles for targeted drug delivery. Nanoscale 13:10748-10764.

Shirley JL, de Jong YP, Terhorst C and Herzog RW (2020) Immune responses to viral gene therapy vectors. Mol Ther 28:709-722.

Simhadri VL, McGill J, McMahon S, Wang J, Jiang H and Sauna ZE (2018) Prevalence of pre-existing antibodies to CRISPR-associated nuclease Cas9 in the USA population. Mol Ther Methods Clin Dev 10:105-112.

Smith KD, Andersen-Nissen E, Hayashi F, Strobe K, Bergman MA, Barrett SLR, Cookson BT and Aderem A (2003) Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. Nat Immunol 4:1247-1253.

Soofiyani SR, Baradaran B, Lotfipour F, Kazemi T and Mohammadnejad L (2013) Gene therapy, early promises, subsequent problems, and recent breakthroughs. Adv Pharm Bull 3:249-255.

Stadtmauer EA, Fraietta JA, Davis MM, Cohen AD, Weber KL, Lancaster E, Mangan PA, Kulikovskaya I, Gupta M, Chen F et al. (2020) CRISPR-engineered T cells in patients with refractory cancer. Science 367:eaba7365.

Sterner RC and Sterner RM (2021) CAR-T cell therapy: Current limitations and potential strategies. Blood Cancer J 11:69.

Sung RS, Qin L and Bromberg JS (2001) TNFα and IFNγ Induced by innate anti-adenoviral immune responses inhibit adenovirus-mediated transgene expression. Mol Ther 3:757-767.

Talmadge JE and Cowan KH (2020) Gene therapy in oncology. In: Niederhuber JE, Armitage JO, Doroshow JH, Kastan MB and Tepper JE (eds) Abeloff's clinical oncology. 6th edition. Elsevier, pp 470-485.

Thapa B and Narain R (2016) Mechanism, current challenges and new approaches for non-viral gene delivery. In: Narain R (ed) Polymers and nanomaterials for gene therapy. Elsevier, pp 1-27.

Tremblay JP, Malouin F, Roy R, Huard J, Bouchard JP, Satoh A and Richards CL (1993) Results of a triple blind clinical study of myoblast transplantations without immunosuppressive treatment in young boys with Duchenne muscular dystrophy. Cell Transplant 2:99-112.

Tucci F, Scaramuzza S, Aiuti A and Mortellaro A (2021) Update on clinical *ex vivo* hematopoietic stem cell gene therapy for inherited monogenic diseases. Mol Ther 29:489-504.

Van den Driessche T, Thorrez L, Naldini L, Follenzi A, Moons L, Berneman Z, Collen D and Chuah MK (2002) Lentiviral vectors containing the human immunodeficiency virus type-1 central polypurine tract can efficiently transduce nondividing hepatocytes and antigen-presenting cells *in vivo*. Blood 100:813-822.

Wagner DL, Amini L, Wendering DJ, Burkhardt L-M, Akyüz L, Reinke P, Volk H-D and Schmueck-Henneresse M (2019)

High prevalence of Streptococcus pyogenes Cas9-reactive T cells within the adult human population. Nat Med 25:242-248.
Wagner DL, Peter L and Schmueck-Henneresse M (2021) Cas9-directed immune tolerance in humans-a model to evaluate regulatory T cells in gene therapy? Gene Ther 28:549-559.
Waldrop MA and Kolb SJ (2019) Current treatment options in neurology - SMA therapeutics. Curr Treat Options Neurol 21:25.
Wang D, Mou H, Li S, Li Y, Hough S, Tran K, Li J, Yin H, Anderson DG, Sontheimer EJ et al. (2015) Adenovirus-mediated somatic genome editing of Pten by CRISPR/Cas9 in mouse liver in spite of Cas9-specific immune responses. Hum Gene Ther 26:432-442.
Wang D, Tai PWL and Gao G (2019) Adeno-associated virus vector as a platform for gene therapy delivery. Nat Rev Drug Discov 18:358-378.
Wang Z, Kuhr CS, Allen JM, Blankinship M, Gregorevic P, Chamberlain JS, Tapscott SJ and Storb R (2007) Sustained AAV-mediated dystrophin expression in a canine model of Duchenne muscular dystrophy with a brief course of immunosuppression. Mol Ther 15:1160-1166.
Wanisch K and Yáñez-Muñoz RJ (2009) Integration-deficient lentiviral vectors: A slow coming of age. Mol Ther 17:1316-1332.
Wilson JM (2009) Lessons learned from the gene therapy trial for ornithine transcarbamylase deficiency. Mol Genet Metab 96:151-157.
Xu X, Chemparathy A, Zeng L, Kempton HR, Shang S, Nakamura M and Qi LS (2021) Engineered miniature CRISPR-Cas system for mammalian genome regulation and editing. Mol Cell 81:4333-4345.e4.
Zaiss A-K, Liu Q, Bowen GP, Wong NCW, Bartlett JS and Muruve DA (2002) Differential activation of innate immune responses by adenovirus and adeno-associated virus vectors. J Virol 76:4580-4590.
Zeng J, Li X, Sander M, Zhang H, Yan G and Lin Y (2021) Oncolytic viro-immunotherapy: An emerging option in the treatment of gliomas. Front Immunol 12:721830.
Zhang W-W, Li L, Li D, Liu J, Li X, Li W, Xu X, Zhang MJ, Chandler LA, Lin H *et al.* (2018) The first approved gene therapy product for cancer Ad-p53 (Gendicine): 12 years in the clinic. Hum Gene Ther 29:160-179.
Zhang Y, Chirmule N, Gao GP and Wilson J (2000) CD40 ligand-dependent activation of cytotoxic T lymphocytes by adeno-associated virus vectors *in vivo*: Role of immature dendritic cells. J Virol 74:8003-8010.
Zsengellér Z, Otake K, Hossain SA, Berclaz PY and Trapnell BC (2000) Internalization of adenovirus by alveolar macrophages initiates early proinflammatory signaling during acute respiratory tract infection. J Virol 74:9655-9667.

Internet Resources

Daiichi Press Release (2021), Daiichi Sankyo Launches DELYTACT® Oncolytic Virus G47Δ in Japan, https://www.daiichisankyo.com/files/news/pressrelease/pdf/202111/20211101_E.pdf (accessed 12 January 2022).
European Medicines Agency – EMA (2016a) Committee for medicinal products for human use (CHMP), https://www.ema.europa.eu/en/documents/assessment-report/strimvelis-epar-public-assessment-report_en.pdf (accessed 9 December 2021).
European Medicines Agency - EMA (2016b) Committee for medicinal products for human use (CHMP), https://www.ema.europa.eu/en/documents/assessment-report/zalmoxis-epar-public-assessment-report_en.pdf (accessed 23 November 2021).
European Medicines Agency - EMA (2017a) Glybera, https://www.ema.europa.eu/en/medicines/human/EPAR/glybera (accessed 12 December 2021).
European Medicines Agency - EMA (2018a) Annex I - Sumary of product characteristics, https://www.ema.europa.eu/en/documents/product-information/kymriah-epar-product-information_en.pdf (accessed 9 December 2021).
European Medicines Agency - EMA (2018b) Anexo I - Resumo das características do medicamento, https://www.ema.europa.eu/en/documents/product-information/yescarta-epar-product-information_pt.pdf (accessed 9 December 2021).
European Medicines Agency (EMA) (2017b) Annex I - Summary of product characteristics, https://www.ema.europa.eu/en/documents/product-information/glybera-epar-product-information_en.pdf (accessed 12 December 2021).
European Medicines Agency (EMA) (2018) Luxturna, https://www.ema.europa.eu/en/medicines/human/EPAR/luxturna (accessed 13 December 2021).
European Medicines Agency (EMA) (2019) Zalmoxis (nalotimagene carmaleucel), https://www.ema.europa.eu/en/documents/public-statement/public-statement-zalmoxis-withdrawal-marketing-authorisation-european-union_en.pdf (accessed 9 December 2021).
European Medicines Agency (EMA) (2021) Meeting highlights from the Pharmacovigilance Risk Assessment Committee (PRAC) 5-8 July 2021, https://www.ema.europa.eu/en/news/meeting-highlights-pharmacovigilance-risk-assessment-committee-prac-5-8-july-2021 (accessed 11 December 2021).
Kite Pharma (2017) Yescarta® (axicabtagene ciloleucel) demonstrates high rates of response in relapsed or refractory indolent non-hodgkin lymphoma, https://www.kitepharma.com/news/press-releases/2020/5/yescarta-axicabtagene-ciloleucel-demonstrates-high-rates-of-response-in-relapsed-or-refractory-indolent-nonhodgkin-lymphoma (accessed 9 December 2021).
MedlinePlus (2019) Genes and gene therapy, https://medlineplus.gov/genesandgenetherapy.html (accessed 2 November 2021).
National Library of Medicine (U.S.) (1999) Phase I pilot study of liver-directed gene therapy for partial ornithine transcarbamylase deficiency, https://clinicaltrials.gov/ct2/show/NCT00004386 (accessed 1 April 2022).
National Library of Medicine (U.S.) (2012) Gene therapy for Wiskott-Aldrich syndrome (TIGET-WAS), https://clinicaltrials.gov/ct2/show/NCT01515462 (accessed 14 November 2021).
National Library of Medicine (U.S.) (2015) Study evaluating the safety and efficacy of KTE-C19 in adult participants with refractory aggressive non-Hodgkin lymphoma (ZUMA-1), https://clinicaltrials.gov/ct2/show/NCT02348216 (accessed 9 December 2021).
National Library of Medicine (U.S.) (2018) Gene transfer clinical study in X-linked myotubular myopathy (ASPIRO), https://clinicaltrials.gov/ct2/show/NCT03199469 (accessed 15 January 2022).
Online Mendelian Inheritance in Man, OMIM®, MIM Number: {204100}: {06/12/2019}, https://www.omim.org/entry/204100 (accessed 15 January 2022).
Online Mendelian Inheritance in Man, OMIM®, MIM Number: {238600}: {05/19/2021}, https://www.omim.org/entry/238600 (accessed 22 January 2022).
Online Mendelian Inheritance in Man, OMIM®, MIM Number: {253550}: {07/26/2021}, https://www.omim.org/entry/253550 (accessed 20 January 2022).
Online Mendelian Inheritance in Man, OMIM®, MIM Number: {311250}: {11/16/2020}, https://www.omim.org/entry/311250 (accessed 20 January 2022).
Online Mendelian Inheritance in Man, OMIM®, MIM Number: {613794}: {05/24/2016}, https://www.omim.org/entry/613794 (accessed 15 January 2022).
Orphan Drug Designations (2008a) Treatment of soft tissue sarcoma, https://www.accessdata.fda.gov/scripts/opdlisting/oopd/detailedIndex.cfm?cfgridkey=262108 (accessed 13 December 2021).

Orphan Drug Designations (2008b) Treatment of osteosarcoma, https://www.accessdata.fda.gov/scripts/opdlisting/oopd/detailedIndex.cfm?cfgridkey=261208 (accessed 13 December 2021).

U.S. Food & Drug - FDA (2015a), https://wayback.archive-it.org/7993/20190425013436/https://www.fda.gov/downloads/BiologicsBloodVaccines/CellularGeneTherapyProducts/ApprovedProducts/UCM469670.pdf (accessed 13 December 2021).

U.S. Food & Drug - FDA (2015b), https://wayback.archive-it.org/7993/20190423064205/https://www.fda.gov/downloads/BiologicsBloodVaccines/CellularGeneTherapyProducts/ApprovedProducts/UCM571161.zip (accessed 25 November 2021).

U.S. Food & Drug - FDA (2018), https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/luxturna (accessed 13 December 2021).

U.S. Food & Drug - FDA (2021a), https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/kymriah-tisagenlecleucel (accessed 9 December 2021).

U.S. Food & Drug - FDA (2021b), https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/yescarta-axicabtagene-ciloleucel (accessed 9 December 2021).

U.S. Food & Drug - FDA (2021c), https://www.fda.gov/vaccines-blood-biologics/zolgensma (accessed 13 December 2021).

UMIN000015995 (2014) A phase 2 clinical trial of a replication-competent, recombinant herpes simplex virus type 1 in patients with glioblastoma, https://rctportal.niph.go.jp/en/detail?trial_id=UMIN000015995 (accessed 11 February 2022).

Associate Editor: Carlos F. M. Menck

Advanced Drug Delivery Reviews 191 (2022) 114616

Contents lists available at ScienceDirect

Advanced Drug Delivery Reviews

journal homepage: www.elsevier.com/locate/adr

Gene editing strategies to treat lysosomal disorders: The example of mucopolysaccharidoses

Flávia Nathiely Silveira Fachel [a,b], Lariane Frâncio [a,c], Édina Poletto [a], Roselena Silvestri Schuh [a,b], Helder Ferreira Teixeira [b], Roberto Giugliani [c,d,e], Guilherme Baldo [a,c,f], Ursula Matte [a,c,e,*]

[a] Laboratório de Células, Tecidos e Genes – Centro de Pesquisa Experimental, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil
[b] Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, UFRGS, Porto Alegre, RS, Brazil
[c] Programa de Pós-Graduação em Genética e Biologia Molecular, UFRGS, Porto Alegre, RS, Brazil
[d] Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil
[e] Departamento de Genética, UFRGS, Porto Alegre, RS, Brazil
[f] Departamento de Fisiologia, UFRGS, Porto Alegre, RS, Brazil

ARTICLE INFO

Article history:
Received 13 February 2022
Revised 20 September 2022
Accepted 2 November 2022
Available online 7 November 2022

Keywords:
Gene editing
Mucopolysaccharidoses
CRISPR/Cas
Zinc finger
TALEN
Gene delivery methods

ABSTRACT

Lysosomal storage disorders are a group of progressive multisystemic hereditary diseases with a combined incidence of 1:4,800. Here we review the clinical and molecular characteristics of these diseases, with a special focus on Mucopolysaccharidoses, caused primarily by the lysosomal storage of glycosaminoglycans. Different gene editing techniques can be used to ameliorate their symptoms, using both viral and nonviral delivery methods. Whereas these are still being tested in animal models, early results of phase I/II clinical trials of gene therapy show how this technology may impact the future treatment of these diseases. Hurdles related to specific hard-to-reach organs, such as the central nervous system, heart, joints, and the eye must be tackled. Finally, the regulatory framework necessary to advance into clinical practice is also discussed.

Contents

Abbreviations: MPS, mucopolysaccharidoses; GAG, glycosaminoglycans; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; ATMP, Advanced Therapy Medicinal Products.

* Corresponding author.
E-mail address: umatte@hcpa.edu.br (U. Matte).

https://doi.org/10.1016/j.addr.2022.114616
0169-409X/

1. Lysosomal storage disorders: Current treatments and challenges

Lysosomes are central organelles involved in the degradation and recycling of various macromolecules. The lysosomal system also plays a role in multiple fundamental cellular processes, such as nutrient sensing, cholesterol homeostasis, plasma membrane repair, bone and tissue remodeling, viral/bacterial infections, cell death, and Ca2 + signaling [1]. Performing these functions involves ion channels, transporters, integral lysosomal membrane proteins, soluble hydrolases and their modifying enzymes. Lysosomal disorders occur when the recycling of macromolecules within this organelle is impaired.

The deficiency of lysosomal hydrolases causes about 70 % of lysosomal disorders, and the rest are due to abnormalities in modifying enzymes, membrane-associated transport proteins, or other non-enzymatic proteins [2]. As a result, cells enlarge with an increased number of lysosomes filled with undegraded or partially degraded substrates. Lysosomal storage leads to a cascade of events triggered by the primary gene defect that results in the disturbance of complex cellular pathways [3,4]. For practical purposes, lysosomal diseases are classified according to the accumulated compound in sphingolipidoses oligosaccharidoses, neural ceroid lipofuscinosis, mucolipidoses, and mucopolysaccharidosis [2].

Clinical manifestations are dependent on the specific substrate as storage occurs primarily at the site of synthesis. In general, these disorders are progressive, and the age of onset can vary from prenatal to adulthood. Most have an autosomal recessive inheritance pattern, except for Hunter disease (OMIM 309900), Fabry disease (OMIM 301500), and Danon disease (OMIM 300257), which are X-linked [5].

When considered individually, lysosomal diseases are rare, and some are very rare, with few reports in the literature. However, their combined incidence is much quite high. In a pilot newborn screening study, five of the most common lysosomal disorders (Gaucher, Fabry, Pompe (OMIM 232300), Niemann Pick A/B (OMIM 257200), and MPS I (OMIM 607014)), were screened in 65,000 babies. Of these, 69 were initially positive and 23 confirmed, which gives an incidence of 1:2,826 live births [6]. The prevalence also varies based on geographic regions and/or ethnic origin. For example, Gaucher disease (OMIM 231000) occurs in 1 in 40,000 to 1 in 60,000 births. In Ashkenazi Jews, its incidence is as high as 1 in 800 [7]. However, the true incidence depends entirely on correct diagnosis, adequate reports, and the availability of screening methods. A comprehensive, 12-year newborn screening program in Australia showed a combined incidence of 1:4,800 live births [8].

Diagnosis is based on clinical symptoms, which are often unspecific. Imaging tests, including X-ray, echocardiogram, tomography, and magnetic resonance, can also be used [9]. Classically, laboratory examination involves biochemical tests for stored substrates, followed by enzyme assay and DNA analysis [10]. More recently, genomic analysis is taking precedence over biochemical tests, and whole-exome sequencing can be used to diagnose patients with lysosomal disorders [11].

Correct diagnosis allows the patient to be referred to a specific treatment. Treatment should be started as soon as possible to slow the progression of the disease and improve the patient's quality of life. Currently, palliative treatments, surgeries, and specific therapies such as Enzyme Replacement Therapy (ERT), Substrate Reduction Therapy (SRT), and Hematopoietic Stem Cell Transplantation (HSCT) are the main options for patients [12]. Despite the various treatment options, they are not available for all lysosomal disorders [2]. For HSCT, patients need a compatible donor at an early age, as its efficacy reduces significantly as patients mature. The risks associated with the procedure and its limited benefits also prevent more widespread use. In SRT, the objective is to reduce the biosynthesis levels of accumulated substrates to avoid their storage [13], but the therapy presents several side effects and limited use. ERT is considered the best treatment option when available. A recombinant enzyme is directly administered to the patient to decrease or normalize the storage levels. Despite its satisfactory results, its efficiency can be affected due to effects such as anti-ERT antibodies in treated patients [14]. It requires lifelong IV administration and is poorly distributed to some organs. The brain, heart, bones, and joints are often deprived of enzymes due to blood barriers or limited perfusion. Therefore, exploring new therapeutic techniques will be very important to improve the results of treatments for these diseases and overcome the limitations of specific therapies.

This review will focus on Mucopolysaccharidoses (MPS), a group of lysosomal diseases with progressive multisystemic involvement. The MPS are caused by the deficiency of enzymes directly involved in the catabolism of glycosaminoglycans (GAG). As a result, there is an intralysosomal accumulation of undegraded or partially degraded GAG [15].

There are eleven types of MPS, classified according to their enzyme deficiency (Table 1). Although each type has specific features, they have some common characteristics such as multisystemic, chronic, and progressive deterioration, with osteoarticular, audiovisual, and cardiovascular alterations. Other shared clinical signs and symptoms include, to variable degrees, coarse faces, neurological impairment (in MPS I, II, III, and VII), skeletal dysplasia, hepatosplenomegaly, joint stiffness, and heart valvular disease [16]. Diagnosis starts from clinical suspicion, and the first test performed is the analysis of GAGs in urine. Then, blood samples are tested for enzyme activity depending on the type of GAGs detected in screening tests. All suspected cases of MPS II, MPS IIIA, MPS IVA, and MPS VI must also be tested for a second sulphatase to exclude Multiple Sulphatase Deficiency [17].

Together, the incidence of all types of MPS is around 1 in 20,000–25,000 live births [18]. However, MPS epidemiological incidence data are available for a few countries and regions [19]. Celik et al. [20] reviewed the overall prevalence of each MPS subtype in 29 countries. But as these are clinically heterogeneous diseases that require specific investigations, these prevalences may be underestimated. Therefore, using population databases to estimate rare disease frequencies was done for MPS with incidences higher than previously reported [21].

These diseases show a continuum of clinical involvement, with severe cases presenting early at birth as fetal hydrops in MPS I and MPS VII [22] to attenuated phenotypes with little organ involvement and average lifespan as seen in MPS IX [23]. Wide clinical variability is also observed within each MPS type and is related to the residual amount of enzyme activity. However, even patients with attenuated forms can have significant disabilities, requiring medical and surgical interventions. Moreover, a substantial group of patients shows intellectual disability, which is hard to treat with available therapeutic options [24].

All genes related to the MPS have been identified (Table 2), and MPS II is the only X-linked. Concerning gene defects, the MPS are heterogeneous, with deletions, rearrangements, and several single nucleotide variants described in each type of MPS. Nonsense variants and gene rearrangements are associated with a more severe phenotype, while missense and splice site variants can be found both in attenuated and severe phenotypes. However, genotype-phenotype correlation is not always absolute, suggesting that other variants in the same gene or other loci and/or environmental factors can modulate the patient's clinical phenotype [25].

Table 1
Main clinical characteristics of mucopolysaccharidoses (MPS).

Type	Syndrome	Enzyme deficiency	Storage product	Main clinical features
MPS I	Hurler, Hurler-Scheie or Scheie	Alpha-L-iduronidase	DS, HS	Ear/nose/throat infections, short stature, skeletal dysplasia, hernias, hepatomegaly, dysmorphology, organomegaly, joint stiffness, cardiac disease, corneal clouding, retinal degeneration, hearing deficits, severe learning and cognitive deficits, and progressive deterioration of the central nervous system functions.
MPS II	Hunter	Iduronate-2-sulfatase	DS, HS	Ear/nose/throat infections, short stature, skeletal dysplasia, hernias, hepatomegaly, dysmorphology, organomegaly, joint stiffness, cardiac disease, hearing deficits, severe learning and cognitive deficits and progressive deterioration of the central nervous system functions.
MPS IIIA	Sanfilippo A	Heparan *N*-sulfatase	HS	Behavioral difficulties, coarse facial features*, dysfunctions of the central nervous system***, mental retardation***, sleep disturbance***, hyperactivity, loss of communication skills and lack of mobility.
MPS IIIB	Sanfilippo B	Alpha-*N*-acetylglucosaminidase	HS	Behavioral difficulties, coarse facial features*, dysfunctions of the central nervous system***, mental retardation***, sleep disturbance***, hyperactivity, loss of communication skills and lack of mobility.
MPS IIIC	Sanfilippo C	Acetyl-CoA alpha-glucosaminide *N*-acetyltransferase	HS	Behavioral difficulties, coarse facial features*, dysfunctions of the central nervous system***, mental retardation***, sleep disturbance***, hyperactivity, loss of communication skills and lack of mobility.
MPS IIID	Sanfilippo D	*N*-acetylglucosamine-6-sulfatase	HS	Behavioral difficulties, coarse facial features*, dysfunctions of the central nervous system***, mental retardation***, sleep disturbance***, hyperactivity, loss of communication skills and lack of mobility.
MPS IVA	Morquio A	Galactosamine-6-sulfate sulfatase	CS, KS	Skeletal dysplasia***, odontoid dysplasia, acute or chronic cervical myelopathy, joint laxity and visual complications.
MPS IVB	Morquio B	Beta-Galactosidase	KS	Skeletal dysplasia***, odontoid dysplasia, acute or chronic cervical myelopathy, joint laxity and visual complications.
MPS VI	Maroteaux-Lamy	*N*-acetylgalactosamine-4-sulfatase	DS	Ear/nose/throat infections, coarse facial features, decreased growth velocity, decreased mobility and joint laxity.
MPS VII	Sly	Beta-glucuronidase	DS, HS	Coarse facial features, scaphocephaly, hydrops fetalis, mental retardation, skeletal dysplasia, and others.
MPS IX	Natowicz	Hyaluronidase 1	HA	Joint dysfunctions, short stature, and erosion of the hip joint.

Type: Mucopolysaccharidosis (MPS); Mucolipidosis (ML). Storage product: chondroitin sulfate (CS); dermatan sulfate (DS); glycosaminoglycans (GAGs); heparan sulfate (HS); hyaluronic acid or hyaluronan (HA); keratan sulfate (KS). Clinical features: * Attenuated; *** Severe.

Table 2
Main molecular characteristics of mucopolysaccharidoses (MPS).

Type	Gene	Chromosome location	Frequent variants
MPS I	IDUA	4p16.3	p.Trp402Ter, p.Gln70Ter, p.Pro533Arg, p.Arg89Gln, c.1190-1G > A
MPS II	IDS	Xq28	p.Arg468Gln, p.Arg468Trp, p.Ser333Leu, c.1122C > T
MPS IIIA	SGSH	17q25.3	p.Arg74Cys, p.Gln380Arg, p.Ser66Trp, p.Arg245His
MPS IIIB	NAGLU	17q21.3	p.Arg297Ter, p.Val334Phe
MPS IIIC	HGSNAT	8p11.1	p.Arg384Ter
MPS IIID	GNS	12q14.3	–
MPS IVA	GALNS	16q24.3	p.Arg386Cys, p.Gly301Cys, p.Iso113Phe
MPS IVB	GLB1	3p22.3	p.Tyr183His, p.Trp273Leu, p.Thr500Ala
MPS VI	ARSB	5q13-14	p.Leu321Pro, p.Arg152Trp, p.Tyr210Cys, p.Tyr251Ter, p.His178Leu, c.1143-8T > G
MPS VII	GUSB	7q11.21	p.Leu176Phe, p.Arg357Ter
MPS IX	HYAL1	3p21.3-p.21.2	p.Glu268Lys, p.Val35AlafsX25

MPS I (OMIM 607014, 607015, 607016) is caused by a deficiency of α-L-iduronidase (IDUA), involved in the degradation of glycosaminoglycans heparan sulfate (HS) and dermatan sulfate (DS). Historically it is classified into three subtypes: Hurler (severe), Hurler-Scheie (intermediate), and Scheie (attenuated). The severe form is about five times more frequent than the attenuated, but this may be due to the underdiagnosis of milder phenotypes. The main clinical features are recurrent ear/nose/throat infections, short stature, skeletal dysplasia, hernias, hepatomegaly, dysmorphology, organomegaly, joint stiffness, cardiac disease, corneal clouding, retinal degeneration, hearing deficits, severe learning and cognitive deficits and progressive deterioration of the central nervous system functions. Severely affected patients die in the first decade of life, whereas those with milder phenotypes reach adulthood [26]. MPS I's standard treatment is enzyme replacement therapy (ERT) with Laronidase (Aldurazyme®). This treatment can delay the progression of the disease, but it is not curative. Hematopoietic stem cell transplantation (HSCT) can also be performed in patients under two years. The IDUA gene contains 14 exons, and until now, more than 300 IDUA variants have been described. The three most common variants in the Americas, Europe, and Australia. are p.Trp402Ter, p.Gln70Ter, and p.Pro533Arg [27].

In MPS II or Hunter Syndrome (OMIM 309900), the deficiency of iduronate-2-sulfatase (IDS) results in the accumulation of heparan sulfate (HS) and dermatan sulfate (DS) in lysosomes. Its incidence is 1:100,000 and 1:170,000 male births [28]. The main clinical features of MPS II are similar to those of MPS I: recurrent ear/nose/throat infections, short stature, skeletal dysplasia, hernias, hepatomegaly, dysmorphology, organomegaly, joint stiffness, cardiac

disease, hearing deficits, severe learning and cognitive deficits and progressive deterioration of the central nervous system functions [29]. The treatment is ERT with Idursulfase (Elaprase®). The IDS gene is located on the long arm of the X chromosome (Xq28) [28], and the disease primarily affects hemizygous men. Until 2020, 658 variants had been described, including whole gene and exon deletions and complex gene rearrangements. The most frequent variants are p.Arg468Gln, p.Arg468Trp, and p.Ser333Leu [29].

MPS III (Sanfilippo Syndrome) is classified into four subtypes (IIIA, IIIB, IIIC, and IIID), depending on the deficient enzyme heparan *N*-sulfatase (SGHS), alpha-*N*-acetylglucosaminidase (NAGLU), heparan-alpha-glucosaminide *N*-Aacetyltransferase (HGSNAT), *N*-acetyltransferase and *N*-acetylglucosamine-6-sulfatase (GNS), respectively. The only GAG accumulated is heparan sulfate (HS). The main clinical aspects are behavioral difficulties, coarse facial features, severe dysfunctions of the central nervous system, severe mental retardation, severe sleep disturbance, hyperactivity, loss of communication skills, and lack of mobility [30]. To date, there is no specific treatment option for MPS III other than palliative and symptomatic measures. The incidence of MPS IIIA (OMIM 252900) is 1:100,000. To date, the SGSH gene has 145 variants, with p.Arg74Cys, p.Gln380Arg, p.Ser66Trp, and p.Arg245His being the most frequent. The incidence of MPS IIIB (OMIM 252920) is 1:200,000. The NAGLU gene has 166 gene variants, all occurring at low frequencies, p.Arg297Ter, p.Val334Phe being the most frequent. The HGSNAT gene is responsible for MPS IIIC (OMIM 252930), which has an incidence of 1:1,500,000. There are 68 variants identified until now; two of them, p.Arg344Cys and p.Ser518Phe, are frequent in the Dutch population. The least frequent subtype is IIID (OMIM 252940), with an estimated incidence of 1:1,000,000 and only 25 disease-causing variants in the GNS gene reported to date [31].

MPS IV (Morquio Syndrome) is classified into two subtypes: MPS IVA (OMIM 253000) and MPS IVB (OMIM 253010). MPS IVA is caused by deficiency of galactosamine-6-sulfatase (GALNS), which results in the accumulation of chondroitin sulfate (CS) and keratan sulfate (KS). MPS IVB is caused by a deficiency of beta-galactosidase (GLB1), and the main accumulation product is keratan sulfate (KS). The main clinical aspects are severe skeletal dysplasia, odontoid dysplasia, acute or chronic cervical myelopathy, joint laxity, and visual complications. MPS IVB patients usually present a milder phenotype [32]. ERT is available only for MPS VA, with Elosulfase alfa (VIMIZIM ®). In MPS IVA, until now, 362 variants are known in the GALNS gene, and p.Arg386Cys, p.Gly301Cys, and p.Iso113Phe are the most frequent [32]. MPS IVB, on the other hand, is caused by variants in the GLB1 gene, which is also involved in GM1 Gangliosidosis. Until 2018, 215 variants have been described in this gene, most associated with GM1. However, p.Trp273Leu is the most common variant in MPS IVB patients [33]. The incidences are from 1:71,000 to 1:179,000 for MPS IVA and < 1:2,000,000 for MPS IVB.

MPS VI (Maroteaux-Lamy Syndrome) (OMIM 253200) is a rare disease with an incidence of 1:240,000. It is caused by a deficiency of *N*-acetylgalactosamine-4-sulfatase (ARSB), resulting in the accumulation of dermatan sulfate (DS). Its main clinical features are recurrent ear/nose/throat infections, coarse facial features, decreased growth velocity, decreased mobility, and joint laxity [34]. The available treatment for MPS IV is ERT with galsulfase (Naglazyme®). Until now, the ARSB gene involved in MPS VI has 197 variants identified, but only two alleles have been reported multiple times in global samples: c.962 T > C [p. (Leu321Pro)] and c.454C > T [p. (Arg152Trp)]. Others frequent variants are: p. Leu321Pro, p.Arg152Trp, p.Tyr210Cys, p.Tyr251Ter, p.His178Leu, c.1143-8T > G, p.Arg315Gln [35].

In MPS VII (Sly Syndrome) (OMIM 253220), the incidence is < 1:2,000,000 [36]. The most frequent clinical features are coarse facial features, scaphocephaly, hydrops fetalis, mental retardation, and skeletal dysplasia. A deficiency of beta-glucuronidase causes it, and patients accumulate dermatan sulfate (DS) and heparan sulfate (HS). There is no specific treatment. The GUSB gene contains 12 exons, and until now, 64 variants are known. Variant p. Leu176Phe is the most prevalent in patients [37].

Finally, MPS IX (Natowicz Syndrome) (OMIM 601492), an extremely rare type of MPS, is caused by a deficiency of Hyaluronidase, resulting in the accumulation of hyaluronic acid or hyaluronan (HA). Its main clinical manifestations are joint dysfunctions, short stature, and erosion of the hip joint. Variants in the HYAL1 gene, which contains three exons, cause MPS IX. So far, until now, only three different mutations responsible for the disease have been described [23].

2. Gene editing

Genome editing is the precise DNA modification by adding, deleting, or substituting sequences in the genome. Depending on the desired outcome, it can be achieved using different techniques and approaches. The goal of former genome editing platforms was to cleave the double-strand DNA at a specific location to activate cellular repair mechanisms and consequently induce sequence changes at the cleaved site. Techniques such as Zinc Finger Nucleases (ZFN), Transcription Activator-Like Effector Nucleases (TALEN), or the basic CRISPR/Cas9 system rely on such a strategy. More recently, the genome editing toolbox has expanded and now comprises techniques that occur without double-stranded breaks, as we will discuss further below (Fig. 1).

Protein-based platforms: ZFN and TALEN

The first genome editing platforms are based on DNA recognition protein domains fused with endonucleases. These domains are customizable according to the DNA sequence of interest – for ZNF, for example, each finger binds to three nucleotides in the DNA [38], while for TALEN, each nucleotide is recognized by 33–35 amino acids sequences [39]. In addition, these domains are fused to the nuclease domain of *Fok*I, which requires dimerization at the target to cleave the DNA [39]. This dimerization increases the specificity and complexity of these systems, as it involves designing and synthesizing two different DNA-binding protein domains – one for each DNA strand – limiting its general use.

CRISPR/Cas9

The acronymous CRISPR/Cas stands for Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated proteins, which originally refers to a sequence found in bacteria and archaea that functions as a prokaryote adaptative immune system against pathogens [40,41]. Based on its activity in bacteria, CRISPR/Cas was developed as a tool for genome editing of eukaryote cells [42,43], allowing precise modifications in the DNA sequence of any cell of interest. The system requires a guide RNA (gRNA), a Cas nuclease, and a Protospacer Adjacent Motif (PAM) in the target sequence. The gRNA contains an invariable scaffold and a 20 nt customizable sequence that is designed to be complementary to the target sequence [44]. The nuclease, frequently Cas9 from Streptococcus pyogenes (SpCas9), cleaves the double-strand DNA in the designated location. Finally, the PAM (an "NGG" sequence recognized by SpCas9, where "N" stands for any nucleotide) must be found in the target, immediately downstream of the gRNA binding site, for target recognition and Cas9 activity. Once Cas9 finds the appropriate target (given by gRNA binding and the presence of PAM), it produces a blunt-end break in the DNA [45], which soon activates DNA repair mechanisms as double-strand breaks are extremely toxic for cells [46].

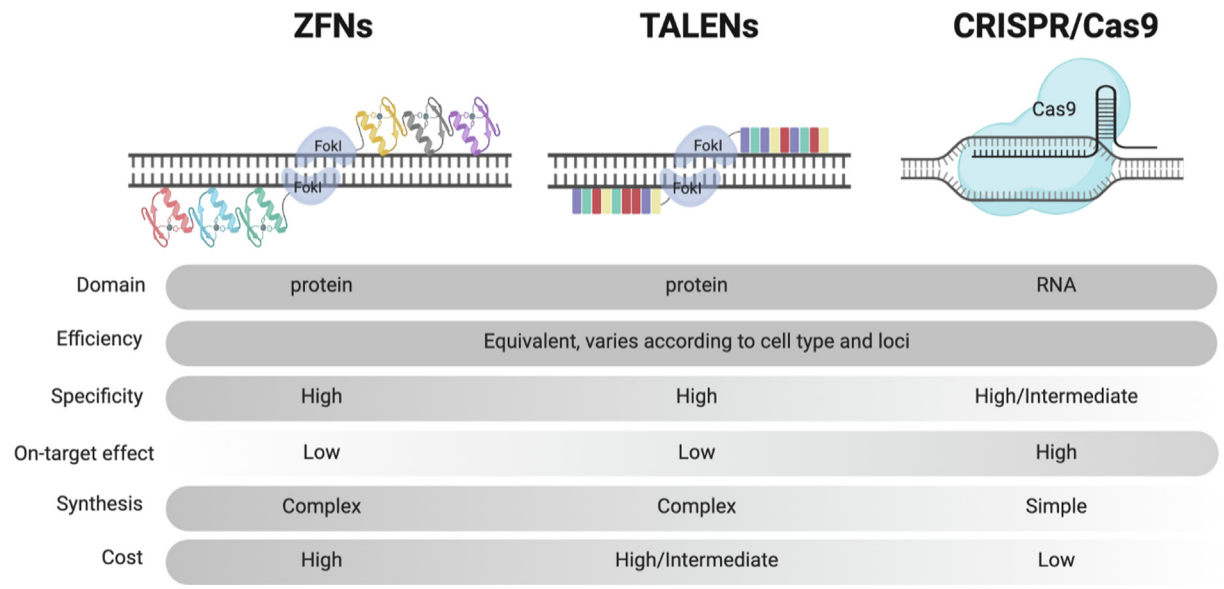

Fig. 1. Summary of ZNFs, Talens, and CRISPR/Cas9 techniques.

Editing strategies based on DNA breaks

The main cellular DNA repair mechanisms are non-homologous end-joining (NHEJ) and homology-directed repair (HDR), and both can be used in different strategies for genome editing. NHEJ is usually very effective in repairing the break and restoring the initial sequence [47]. Still, as soon as the target sequence is end-joined, CRISPR/Cas9 can act again and induce new breaks in the same molecule. Repetitive cycles of break and repair eventually end when a mistake occurs, changing the original sequence and stopping the process. These mistakes can take the form of insertions, deletions, or substitutions, varying from one to several base pairs and frequently knocking out the gene. For this reason, the NHEJ pathway is traditionally associated with mutation induction and is used to knockout genes [48]. Nevertheless, indel induction can also be used to recover gene function when, for example, the reading frame in a mutated gene is restored, ultimately producing a functional protein [49].

Genome editing techniques also allow for targeted integration of sequences, which is more frequently achieved with homology-directed repair (HDR). This mechanism is used by the cell when a DNA template is available (naturally the sister chromatid during the S/G2 phase), with homology sequences to the damaged DNA molecule [50]. Therefore, in this strategy, a DNA template containing the intended edit must be delivered to the cell together with the genome editing machinery. After pairing the homologous sequences, the DNA polymerase incorporates into the genome, at the cut site, whatever is in between the homology arms of the DNA template. This process allows for precise modifications in the target, including insertions, deletions, or substitutions of one to thousands of nucleotides.

For therapeutic purposes, HDR-mediated genome editing can be used for single nucleotide variant (SNV) correction, coding sequence (cDNA) insertion, gene addition (which includes an exogenous promoter), and exon replacement [51], among other strategies. SNV correction is the simplest of the modifications above as it requires smaller DNA templates. Our group has applied this strategy using CRISPR/Cas9 in MPSI fibroblasts homozygous for p.Trp402*, the most common disease-causing variant observed in patients [27], as a proof-of-concept study demonstrating that IDUA enzyme activity can be restored if the point mutation is corrected by HDR-mediated genome editing [52,53]. Another strategy is cDNA insertion, which can be done in the original locus, allowing the usual regulatory elements to control the expression. Alternatively, it can be integrated into alternate regions to be expressed under other regulatory mechanisms, allowing for different expression patterns. For example, this strategy was used for MPS I and II, using ZFN to insert the cDNA in the highly expressed albumin locus in hepatocytes, resulting in enzyme overexpression without adding an exogenous promoter or other regulatory sequences [54,55].

Gene addition denotes the insertion of a whole gene containing the coding sequence and a promoter. It is usually done in safe harbors, genomic loci that are amenable to sequence changes with no effect on cellular function or the expression of other genes [56]. For MPS I, this strategy was applied to human cells for *ex vivo* gene therapy, using the CCR5 gene as a safe harbor [57,58]; and *in vivo* to MPSI mice, targeting IDUA to the Rosa26 murine locus [59]. Gene addition has advantages over cDNA insertion only, as the promoter can be designed according to intrinsic characteristics and specific needs. Taking the MPSI studies, strong promoters were used to compensate for predicted low editing efficiency or for low engraftment of edited cells after transplantation, allowing edited cells to secret enough enzyme to cross-correct the neighbor cells [57–59]. Besides increased expression, different promoters also allow for cell/lineage-specific expression of the gene, such as the CD68 promoter for restricted expression in the monocyte/-macrophage lineage, for example [60].

HDR is not the only mechanism by which a sequence can be added to the target after an induced double-strand break. Another option is homology-independent targeted integration, or HITI, which uses the NHEJ repair pathway to insert a sequence of interest at the break site [61–63]. This technique was described using CRISPR/Cas9, in which the Cas9, the gRNA, and a donor template containing the sequence of interest are delivered to the cells, similarly to HDR. Instead of homology arms, however, the sequence of interest is flanked by gRNA binding sites, the same as the target in the genome. This way, the genome and donor template are cleaved by the same gRNA, creating a fragment with blunt ends that can be end-joined at the cut site in the genome. In addition, this method is orientation-dependent, as the integration in the wrong orientation creates a novel gRNA binding site at each side of the transgene, allowing additional cutting until integration occurs in the correct orientation – thus losing the gRNA recognition sequence. The most

significant advantage of using HITI or other strategies independent of homology is the higher and constant activity of the NHEJ pathway in mammalian cells regardless of the cell cycle. At the same time, HDR usually is only active in dividing cells [50]. This allows the editing of quiescent cells, such as hematopoietic stem cells for *ex vivo* gene therapy [64] or neurons [62].

Latest CRISPR strategies

Finally, a couple of CRISPR-based platforms have been developed, relying on the ability of gRNA and Cas9 to find the target in the genome rather than inducing a DNA double-strand break, presumably minimizing off-target activity. The first was base editing, a system that uses either a catalytic inactive/dead Cas9 (dCas) or a nickase Cas9 (nCas9) linked to a base-modifying enzyme [65]. As the complex binds to the target, modifying enzymes such as cytosine deaminase or adenosine deaminase catalyze the conversion of C > T and A > G [66], respectively. Although the nucleotide changes are limited with this technique, it can be used to correct the MPSI nonsense mutation p.Trp402*, characterized by TGG > TAG mutation, for example, with a lower risk of off-target activity. The other tool, called prime editing, is the latest CRISPR-based system developed [67]. Like base editing, it also uses a nCas9, but this time fused with reverse transcriptase. The guiding molecule is a prime editing gRNA (pegRNA), which includes a recognition binding site in its 3′ (just as regular CRISPR), an RT template including the edit desired, and a homology sequence in its most 5′ end that binds to the DNA strand that nCas9 has nicked. Therefore, this pegRNA helps with the target recognition, provides a primer for RT, and serves as the template containing the intended modifications, all in one molecule.

On- and off-target effects

Double-strand DNA breaks induced by CRISPR/Cas9 or other nuclease-based tools have been associated with undesired events that can hinder the use of genome editing as it is in the clinical setting. The consequences of specific DNA breaks are called "on-target effects" and can be observed in up to 40 % of edited induced pluripotent stem cells [68]. Frequently reported effects are large deletions, with consequent loss-of-heterozygosity [69]; up-regulation of the p53 pathway [70], which may also be involved in major chromosomal truncations [71]; induction of structural nuclear defects, such as micronuclei and chromosome bridges [72] and even loss of the entire chromosome that has been cleaved [73]. These on-target effects can be potentially avoided by using nickases rather than nucleases, which induces the cut in one DNA strand instead of provoking the double-strand break [71].

Off targets effects are caused by cleavage of DNA sequences similar to the target site but located in other genome regions. Their consequences can be both activation or disruption and silencing of a specific gene, with little repercussion to the organism (such as no repercussion or apoptosis of a single cell), or they can also give rise to tumorigenic processes. Several methods have been developed to detect such effects, and these methods vary in their principles, characteristics, and, most importantly, their limit of detection of such events. In this sense, traditional Sanger sequencing of candidate regions can be used but only detect very frequent events. In contrast, genomic techniques based on next-gen sequencing, such as Digenome-Seq, can detect events as low as 0.1 % or even lower [74]. To reduce the chance of off-target events, investigators need to be very careful in designing their gRNAs, and the use of high-fidelity Cas9 is also recommended.

Regardless of the strategy chosen, CRISPR-based methods offer multiple possibilities for genome editing. The toolbox is continuously being improved to achieve higher efficiencies and safer options, and we may be seeing them soon in many clinical trials, including for mucopolysaccharidoses.

3. Delivery of gene editing tools

Gene editing platforms have been used to relieve disease symptoms partially or entirely by mutating or correcting defective genes. Nevertheless, the efficient transfer of these genetic tools into cells and the delivery to target organs remains a challenge [75]. This may be the most challenging barrier to overcome for the translation to clinical administration, directly affecting robust and precise genome editing achievement. Appropriate delivery methods are required to transport all gene editing components efficiently and selectively to specific target cells and tissues of interest while minimizing off-target effects. New approaches and vectors are being studied to achieve this goal, mainly focusing on a safe, reproducible, and efficient delivery method [76,77].

Various physical, chemical, and biological processes and reagents can transfer genes into cells for efficient, targeted genome editing *in vitro*. Still, new approaches must be developed for delivering gene editing tools into the target organs *in vivo* [78]. Following administration, the elements of gene editing systems should remain stable before reaching the target site. Afterward, they need to be effectively taken up by the targeted cell types and escape from the *endo*-lysosome system to avoid degradation [79]. After being released from the endosome or lysosome, the elements also need to enter the nucleus to initiate genome editing. In addition, in the case of plasmids, they must traffic through the cytosol to the nucleus for transcription [80]. There are many obstacles to effective gene editing, such as encapsulation of the editing tools in delivery vectors, stability of the complex under physiological conditions, recognition and clearance by the reticuloendothelial system (RES), host immune responses triggered by gene editing elements, insufficient accumulation of non-viral complexes in the targeted tissues, phagocytic clearance, cellular uptake, endosomal and lysosomal degradation, cytoplasmic mobility and nuclear import, among others [80]. Furthermore, on-target and off-target effects are related to the delivery vectors of the CRISPR-Cas9 system [81,82]. Therefore, delivery systems are crucial for eventual commercial platforms and complexes based on gene editing technology.

To summarize the various delivery technologies, cargo molecules and carrier types can be classified separately (Fig. 2). For example, as gene editing elements, CRISPR-Cas9 and guide RNA (gRNA) genome editing machinery are delivered in one of the three forms; DNA plasmid, mRNA, or ribonucleoprotein (RNP, Cas9 protein complexed with gRNA) [83]. Other gene editing tools such as Talen and Zinc finger may also be used. The carrier (or vector) type can be categorized into three major groups based on the cellular entry mechanism: biological methods, chemical methods, and physical methods, the less common [83,84].

Gene editing platforms may be delivered through direct transport and injection or viral and/or non-viral vectors, or even with both [85]. Direct transport may be achieved by co-microinjection of Cas9 and sgRNAs [86] and electroporation with Cas9 and sgRNAs [87], especially for *in vitro* gene editing. In contrast, hydrodynamic injection with Cas9 and sgRNAs may be used in *in vivo* experiments [88,89]. Unfortunately, although physical approaches are often successful in the laboratory, these methods are not very amenable for clinical translation [90].

Biological vectors such as exosomes or extracellular vesicles may also be used to carry gene editing systems [91,92], as they show unique properties: biocompatibility, safety, capacity for rational design, and ability to cross biological barriers. Kim and colleagues used cancer-derived exosomes for plasmid DNA delivery encoding the CRISPR/Cas system and showed efficient editing in an ovarian cancer mouse model [93]. Lin and colleagues developed

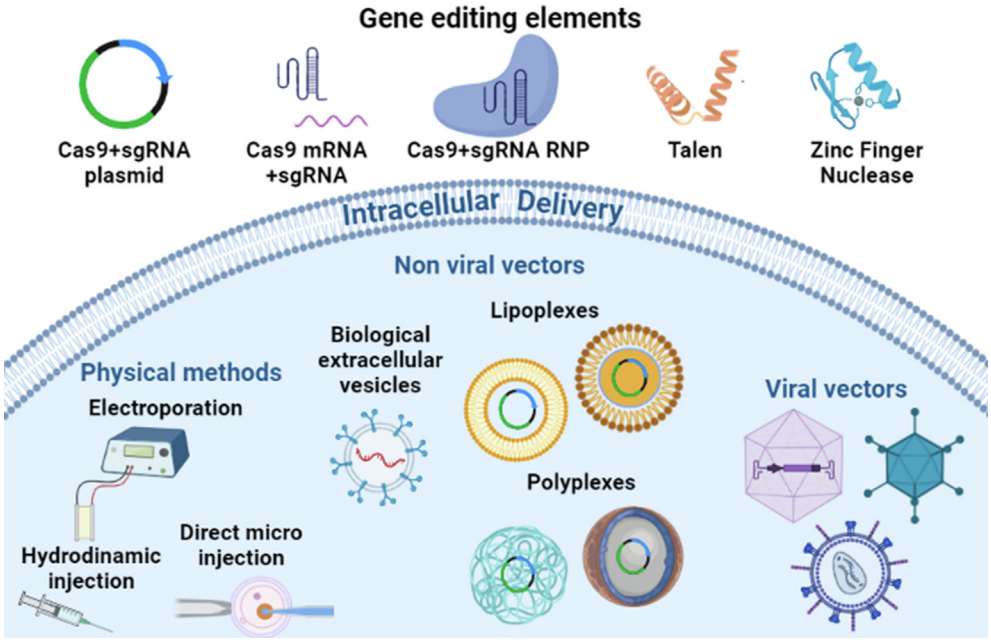

Fig. 2. Schematic figure showing the most common intracellular delivery platforms for gene editing elements (created with Biorender.com).

an exosome-liposome hybrid vector that enabled CRISPR interference in mesenchymal stem cells [94]. These biological carriers are rising as an alternative for protein delivery, such as RNP [95].

Viral vectors, such as adenovirus [96], lentivirus [97], and adeno-associated [98], have been widely used in gene delivery *in vitro* and *in vivo* due to the availability of well-established protocols and high transduction efficiencies. They facilitate *in vivo* delivery of gene-editing tools and have been engineered for safety by removing the genes required for viral replication. However, the risk of carcinogenesis, insertional mutagenesis, limited insertion size, immune responses, and difficulty in large-scale production critically limit their applications [80,99].

Despite displaying somewhat reduced efficacy compared to viral carriers, non-viral-based systems offer advantages in terms of biosafety and versatility. Non-viral vectors may carry the protein or genes encoding for Cas9 and guide RNA, also carrying genes encoding for the correct sequence aiming at homologous directed repair (HDR) of damaged genes. These carriers may be lipid-based, as commercial transfection reagent Lipofectamine® [53,100,101], cationic liposomes [52,102], cationic nanoemulsions [52,103], cholesterol-rich lipoplexes [104], multifunctional lipids [105], solid lipid nanoparticles [106], among others. Cationic nanostructured carriers contain cationic lipids that consist of a cationic head group, a hydrophobic tail, and a linker between these two domains [107,108]. Traditionally, monovalent cationic lipids such as 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) or 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA) have been used to form lipoplexes and are sometimes considered fusogenic [109,110]. These molecules bind to negatively charged polyanionic nucleic acids to carry them across the cellular membrane [107,111,112]. In addition, encapsulation of gene therapy elements in a lipid layer may protect from nucleases and other degradation enzymes [103]. Cationic nanostructures have been investigated for the delivery of siRNA [113], mRNA [114], as well as plasmid DNA [103]. Current challenges for gene delivery are low rates of transfection [81] and targeting specific tissues [115] and circumvention of the toxicity due to massive interaction with anionic cellular membranes. Therefore, there have been many attempts to modify the lipid-based vectors to provide safer and greater efficiency. Some concerns remain over potential cytotoxicity induced by cationic nanostructures, and new lipids are being investigated [80,116].

The addition of neutrally-charged helper lipids such as 1,2-dioleoyl-3-*syn*-*glycero*-phosphatidylethanolamine (DOPE), 1,2-dioleoyl-*sn*-*glycero*-*sn*-3-phosphatidylcholine (DOPC), or cholesterol, enables the formation of liposomes, aid in endosomal escape, promote membrane fusion, and enhance nanostructure stability [104,108,110]. The presence of DOPE promotes fusogenic, inverted hexagonal lipid structures, while DOPC promotes more stable laminar structures. Lipopolyplexes containing these helper lipids show substantially higher transfection efficiency, rapid endosomal trafficking, and nuclear accumulation of nucleic acids [117].

Another commonly used strategy is pegylation, in which the hydrophilic molecule polyethylene glycol (PEG) is used to reduce fouling of the nanoparticle surface, hielding from immune recognition and increasing blood circulation time. Besides having a cationic charge, pegylated nanostructures are also being investigated due to their high serum stability and endosomal escape abilities [118]. For example, Finn and colleagues reported that lipid nanoparticles formulated with PEG lipids showed excellent serum stability and, when used to deliver Cas9 mRNA and sgRNAs targeting the mouse transthyretin gene in liver cells, resulted in more than 97 % reduction in serum protein levels that persisted for at least 12 months after a single systemic injection [119].

Cas9/sgRNA ribonucleoprotein complexes (RNPs) may also be used to target and edit DNA. However, many therapeutic targets cannot currently be accessed due to the lack of carriers that can deliver RNPs systemically [99]. Cas9 protein has a positive net charge of + 22, and sgRNA has an anionic charge of − 103, resulting in the combined Cas9-sgRNA complex having an overall negative charge for binding to cationic nanostructures [102]. In this sense, Wei and colleagues reported a generalizable methodology that allows the engineering of modified dendrimer lipid nanoparticles, stable nucleic acid lipid particles, and lipid-like nanoparticles to efficiently deliver RNPs into cells and edit tissues, including muscle, brain, liver, and lungs. Intravenous injection facilitated tissue-specific, multiplexed editing of six genes in mouse lungs [115]. Ph-sensitive liposomes were also tested and disassembled

in the presence of the tumor microenvironment, releasing the Cas9 protein and allowing for gene editing [120]. Another avenue to explore is using polymeric materials to deliver gene editing proteins. Recently, a research group reported using zinc/imidazole-based metal–organic frameworks (MOF) for intracellular protein delivery [121]. These MOF nanoparticles have been demonstrated to protect protein cargo from protease digestion effectively and were used to enable intracellular delivery of CRISPR RNPs. However, the editing efficiency was relatively low (30 % gene knockout in CHO cells *in vitro*) [121]. Qiu et al. have developed a lipid nanoparticle (LNP) system to efficiently deliver the CRISPR-Cas9 and gRNA targeting the Angptl3 gene, a regulator of lipoprotein metabolism, in the mouse liver [122]. A single injection of LNP packed with CRISPR-Cas9 mRNA, and gRNA targeting Angptl3 disrupted the Angptl3 gene in mice and reduced low-density lipoprotein cholesterol by 57 % and triglyceride by about 29 % for over 100 days [122]. Overall, these strategies could be adapted to delivering gene editing protein complexes, which are mostly impermeable to cell membranes on their own.

Polymer-based biocompatible nanoparticles such as nanocapsules [123] may also be used [124]. One study showed a polymer (poly(N-(N-(2-aminoethyl)-2-aminoethyl) aspartamide)) coating of Cas9-sgRNA RNPs adsorbed onto gold nanoparticles to correct the dystrophin gene in a mouse model of Duchenne muscular dystrophy *in vivo* [125]. Recent work by the labs of Cheng and Leong have demonstrated the promise of a cationic α-helical polypeptide to deliver Cas9, and sgRNA plasmids for enhanced efficiency at gene editing *in vitro* and *in vivo* [126], leading to 67 % targeted protein knockdown in HeLa cells *in vivo* following repeated intratumoral injections and reducing tumor growth by >71 %, consequently significantly extending survival in the HeLa xenograft mouse model. These strategies, using cationic polymers to deliver either nucleic acids, nucleic acid neutralized RNP complexes, or anionic modified RNP complexes, have all relied upon local delivery due to the cationic nature of the particles being utilized, presenting potential systemic delivery challenges. While this relatively high efficacy of cationic polymer-based materials for local delivery is promising in mice, for certain applications, it may face difficulties in scaling up to patients due to the larger length scales required for sufficient transport and therapeutic coverage [118].

Even though CRISPR/Cas gene editing platform is relatively new [43], there are many suggested design principles for the development of materials and transfection strategies with lipid-based materials [127], which could be applied to rapidly optimize carriers for gene editing in a variety of tissue types to advance genomic medicine [128], regenerative biology, and drug discovery [80,129–131].

4. Gene editing for lysosomal storage diseases: The example of MPS

MPS patients present a set of common features that include progressive multisystemic involvement leading to signs that denote the involvement of all organs, having therapeutic targets widespread throughout their body [132]. In this case, effective treatments in different tissues and systems will be successful, and the better the treatment reaches the bloodstream and lymphatic system, the easier it will spread to all cells [133]. In this context, the best choice of administration route combined with the best strategy for gene and protein delivery is imperative.

Current treatments for MPS are based on HSCT and ERT and provide the amelioration of some symptoms, although they are not completely curative. The main difficulties are the widespread symptoms and the segregated tissues that are more difficult to treat, such as eye, bone, heart valves, and joint cartilages. In this sense, both systemic and in situ therapies are currently being studied to treat MPS patients, including gene therapy [134].

Some recent studies describe MPS genome editing *in vitro* as the first preclinical study performed to ensure the safety and efficacy of treatments before translating to *in vivo* studies. The first reports of genome editing *in vitro* for MPS used patients' fibroblasts. Cationic nanoemulsions and liposomes were used to co-deliver a CRISPR-Cas9 and gRNA expression plasmid and a single-stranded oligonucleotide donor template to correct the p.Trp402Ter variant. At seven days after transfection, IDUA enzyme activity reached 5 % of normal levels, enough to significantly reduce lysosome size in the whole cultured population of fibroblasts [52,53,103]. Fig. 3 shows a representative scheme of gene editing studies for MPS.

Another study showed a proof of concept for *ex vivo* gene editing therapy using induced pluripotent stem cell (iPSC) and CRISPR/Cas9 in a MPS I model mouse cell. Disease-affected iPSCs were generated from Idua knockout mouse embryonic fibroblasts, which carry a disrupting neomycin-resistant gene cassette (Neor) in exon VI of the Idua gene. Double guide RNAs were used to remove the Neor sequence, and various lengths of donor templates were used to reconstruct the exon VI sequence. After induced fibroblast differentiation, the gene-corrected iPSC-derived fibroblasts demonstrated Idua function equivalent to the wild-type iPSC-derived fibroblasts. In addition, the Idua-deficient cells were competent to be reprogrammed to iPSCs, and pluripotency was maintained through CRISPR/CAS9-mediated gene correction [135].

When considering widespread *in vivo* gene editing that would promote enzyme production and release for the majority of the tissues, some experimental treatments for MPS were performed with ZFN. A ZFN-targeting system was delivered systemically through recombinant AAV2/8 vectors to insert the IDS (hIDS) coding sequence into the albumin locus of hepatocytes of an MPS II mouse model [54]. Supraphysiological levels of IDS enzyme were observed in the circulation and peripheral organs and GAG reduction, showing prevention of neurocognitive deficit in young MPS II mice (6–9 weeks old). Another study describes liver-directed protein replacement therapy using ZFN-mediated site-specific integration of IDUA (encoding for alpha-L-iduronidase) and IDS (encoding for iduronate-2-sulfatase) transgenes into the albumin locus delivered by an adeno-associated viral (AAV) vector *in vivo* (via tail vein injection), which showed high expression of lysosomal enzymes at therapeutic levels [136].

An experimental *in vivo* approach delivered ZFN and a corrective copy of the IDUA gene, which was inserted at the albumin locus in hepatocytes of a MPS I murine model [137]. The authors demonstrated stable integration of the transgenes resulting in liver-specific expression and secretion of the enzymes to plasma. In contrast, IDUA expression remained stable in mice for 1–2 months. An additional design led to sustained enzyme expression, secretion from liver hepatocytes to circulation, and systemic uptake, promoting the correction of metabolic disease and prevention of neurobehavioral deficit in MPS I mice [55].

These promising results [54,55,137] suggested that this approach could be effective in humans [138], and the strategy is being tested in two clinical trials for MPS I. The studies described [55,137] delivered zinc finger nucleases to treat mice with MPS I, resulting in phase I/II clinical trial (ClinicalTrials.gov: NCT02702115). However, results showed that the efficacy needs improvement due to the low transgene expression level. In this sense, Ou and colleagues [139] designed a gene editing approach with CRISPR to insert a promoterless IDUA cDNA sequence into the albumin locus of hepatocytes through AAV8 vectors injected into neonatal and adult MPS I mice. As a result, IDUA enzyme activity in the brain significantly increased while storage levels were normalized. Furthermore, neurobehavioral tests showed that trea-

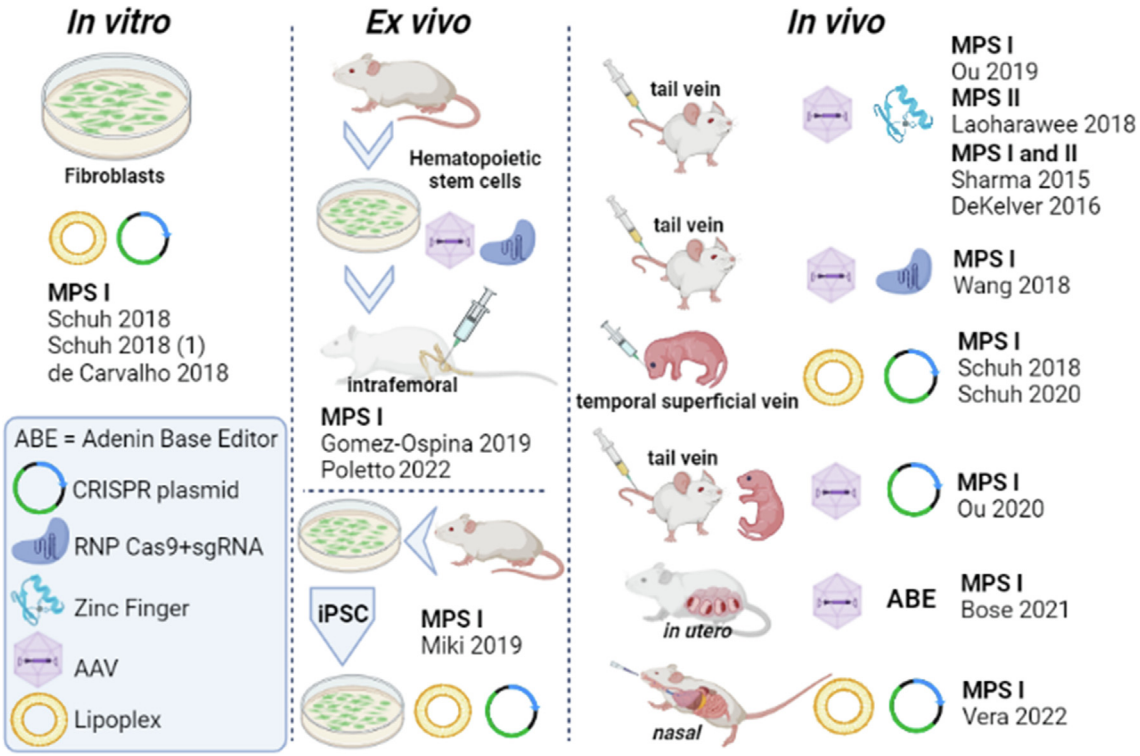

Fig. 3. Schematic figure representing *in vitro*, *ex vivo*, and *in vivo* gene editing studies performed for MPS (created with Biorender.com). MPS I: mucopolysaccharidosis type I; MPS II: mucopolysaccharidosis type II; ABE: Adenin Base Editor; RNP: ribonucleoprotein; AAV: adeno-associated virus vector; Lipoplex: lipid-based nanostructure as nanoemulsion or liposome complexed with a gene editing element.

ted mice had better memory and learning ability. Additionally, this therapeutic platform has the potential to treat other lysosomal diseases, and new applications may arise from these studies.

Wang et al. [140] reported the adeno-associated delivery of Cas9 and guide RNA, which induced allelic exchange and rescued the disease phenotype in a mouse model of MPS I. This procedure recombines non-mutated genetic information in two heterozygous alleles into one functional allele without using donor DNA templates. Several advantages distinguish this allelic exchange from other therapeutic *in vivo* genome-editing approaches as no exogenous DNA repair template is needed.

Gomez-Ospina et al. [57] presented an efficient *ex vivo* genome editing strategy using the sgRNA/Cas9 RNP, which inserted the IDUA gene into hematopoietic stem and progenitor cells from MPS I mice. Edited cells secreted supraphysiological enzyme levels and improved biochemical and phenotypic abnormalities in an immunocompromised murine model of MPS I generated through total body irradiation. The same group performed similar experiments, but the immunocompromised mice were achieved through busulfan conditioning, which allowed higher engraftment of human genome edited hematopoietic stem cells and improved central nervous system correction in the same MPS I mouse model (Fig. 4) [58]. This approach may apply to other lysosomal storage disorders and other types of MPS.

In another study, cationic liposomes carrying the CRISPR/Cas9 plasmid and a donor vector carrying the IDUA gene were administered through a single hydrodynamic injection in newborn MPS I mice. The authors reported a significant, sustained increase in serum IDUA levels, while vectors were markedly detected in the lungs, heart, and all organs but the brains. Furthermore, animals presented improvement in cardiovascular parameters [59]. The positive effects of gene editing on cardiovascular, respiratory, and skeletal functions in MPS I mice were reported after the same treatment. In contrast, behavioural abnormalities and neuroinflammation persisted, suggesting deterioration of the neurological functions [141]. The same group 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. The treatment increased IDUA activity in the lung, heart, and brain areas, reducing GAG levels in the same tissues. Furthermore, treated mice showed improvement in behavioural tests, suggesting prevention of the cognitive damage (Fig. 5) [142].

A gene editing treatment leading to enzyme production in major organs would be extremely interesting. However, some tissues are not easily accessible even though the enzyme is produced on a large scale and released into the bloodstream. These difficult-to-access tissues require in situ long-lasting treatments to ensure patient compliance. Likewise, the sooner the treatments are performed, the more efficiently the symptoms will be prevented, and there is still the possibility of avoiding the appearance of neurological impairment. In fact, in utero base editing has the potential to correct disease-causing mutations before the onset of pathology. Bose and colleagues [66] assessed in utero AAV9 delivery of an adenine base editor (ABE) targeting the Idua G → A (W392X) variant in MPS I mice, corresponding to the common IDUA G → A (W402X) variant in MPS I patients. They showed efficient long-term correction in hepatocytes and cardiomyocytes and low-level editing in the brain and improved survival and amelioration of metabolic, musculoskeletal, and cardiac disease. The study demonstrated the possibility of efficiently performing therapeutic base editing in multiple organs before birth via a clinically relevant delivery mechanism [66].

Several MPS patients present dysmorphic facial features, growth retardation, and skeletal and restricted joint movements. They also may present heart and respiratory complications, organomegaly, and neurological impairment, although this last characteristic is highly variable between and within MPS types [143,144].

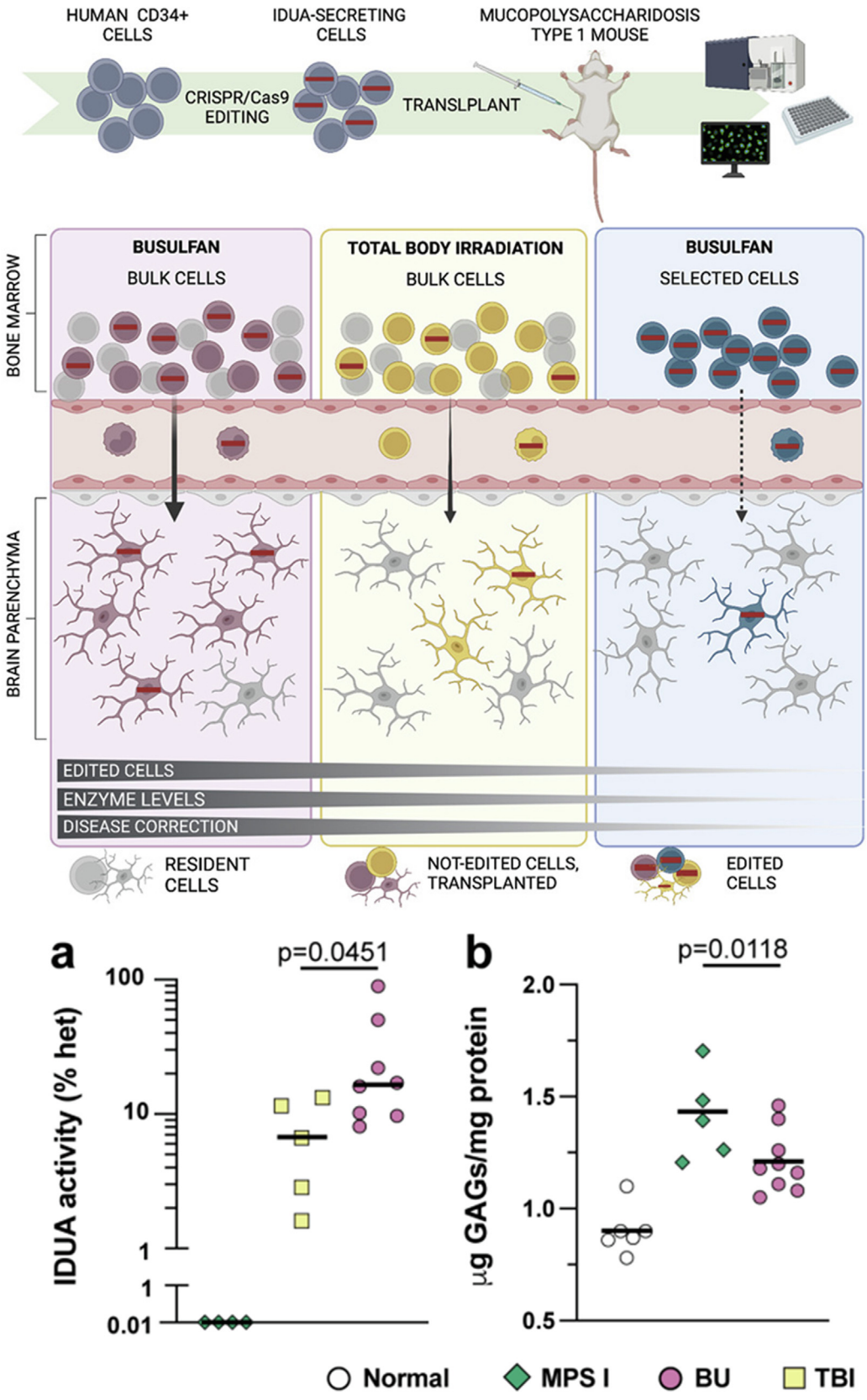

Fig. 4. Busulfan (BU) conditioning enhances the therapeutic efficacy of edited HSPCs in the CNS compared to total body irradiation (TBI). a. Enzyme activity of IDUA in the brain of BU and TBI treated mice. b. Storage of GAGs in the brain measured by the DMB assay. Reproduced with permission from Molecular Therapy – Methods & Clinical Development [58].

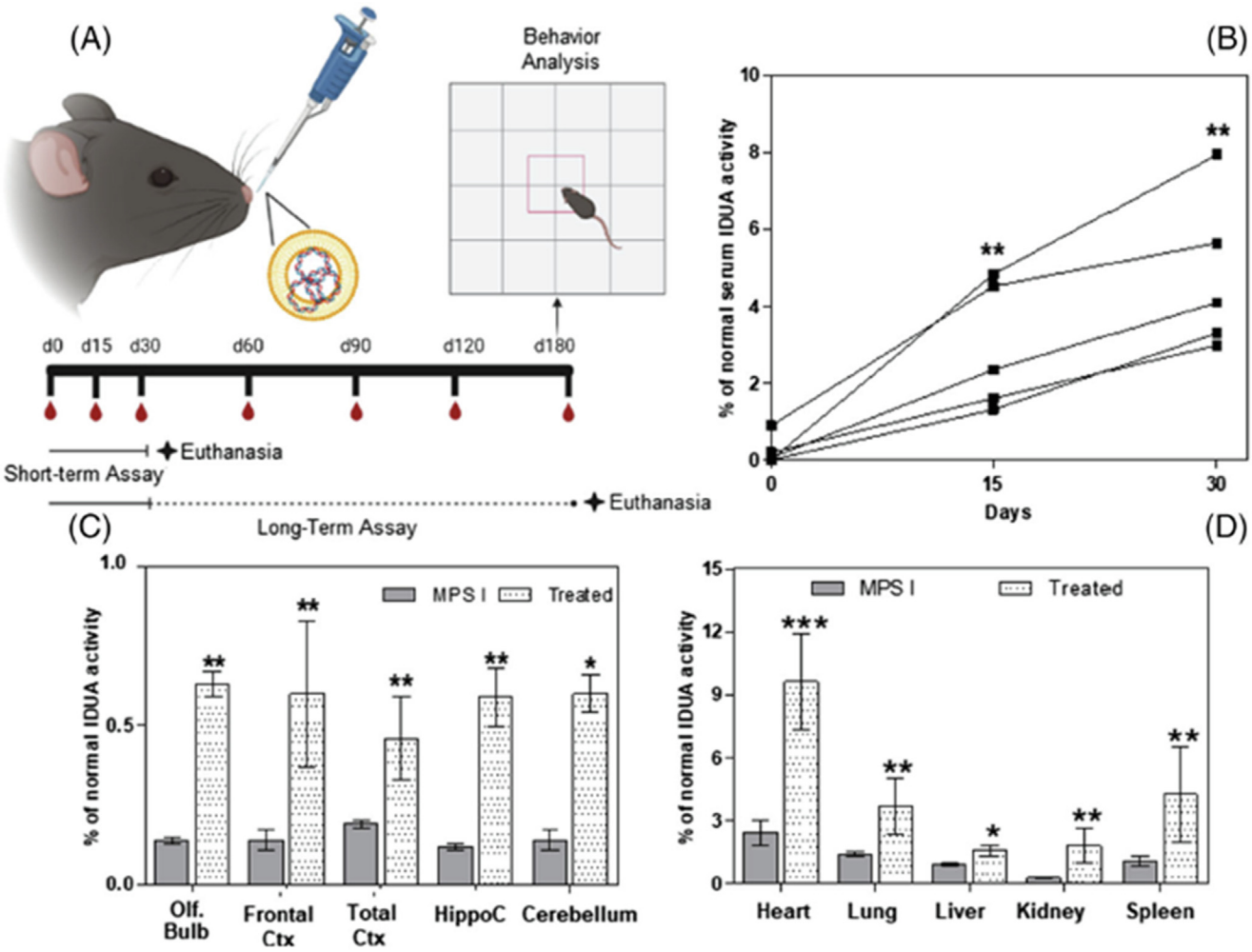

Fig. 5. Experimental design and results of nasal administration (NA) of liposomal complexes carrying two plasmids encoding for the CRISPR/Cas9 system and for the IDUA gene targeting the ROSA26 locus, aiming at brain delivery in MPS I mice. (A) Schematic timeline of short- and long-term studies; (B) serum 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). Reproduced with permission from [142].

Even if the treatment successfully produces and spreads the deficient enzyme throughout the body, some specific organs such as the brain, bone, joint, heart valves, and eye may not receive the enzyme. In this sense, local treatments may be the alternative to some of these clinical features. Although these approaches are not yet available for gene editing of MPS, some existing options could be translated to these diseases.

Neurological manifestations could be treated by delivering gene editing products targeting the CNS through systemic, nasal, or local administration. Intravascular (IV) administration appears as an option in studies focusing on CNS diseases, with good vector distribution in the tissue when using AAV9 vectors, for example [145]. Nose-to-brain delivery may also be achieved and is a non-invasive alternative for reaching the brain [146–148]. There are advances in base editing technologies and current techniques for cell and gene delivery to the CNS in patients with severe neurological forms of MPS. However, none was assessed to date [149].

Regarding bone therapy, MPS patients certainly would benefit from new alternatives of treatment [150]. Gene therapy is ineffective in reaching the bone unless the systems are modified to have an affinity for this tissue. Some studies report viral gene therapy as an alternative for the reversal of established bone pathology of MPS mice [151–153]. Another procedure would combine gene editing with a bone-targeting strategy, as non-viral vectors attached to a hydroxyapatite-binding site or alendronate (a drug with a high affinity for bone). Although there are no specific gene editing strategies to date, these could be potential options for selective gene delivery to the bone [154].

For joints, intra-articular injections could be an approach, as some studies show potential [155]. The drawback would be site injections in all joints, such as each finger joint, which would be very difficult to perform and obtain patient compliance. However, the procedures could be performed just one time if gene editing was effective.

The eye structures are very difficult to reach, and 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 [145]. AAV gene therapy for MPS1-associated corneal blindness was assessed, and AAV8G9-opt-IDUA administered to human corneas via intrastromal injection demonstrated widespread transduction, which included cells that naturally produced IDUA and resulted in a supraphysiological increase in IDUA activity [156,157]. A recent study showed intrastromal gene therapy that prevents and reverses advanced corneal clouding in a canine model of MPS I [158]. These procedures could be performed with gene editing tools to obtain long-lasting enzyme production.

Similar strategies studied for MPS could be adopted for other lysosomal diseases. Also, novel effective viral and non-viral vectors for gene editing tools delivery are still one of the bottlenecks for the successful clinical translation of genome editing. However, despite these challenges, the fast advances in genome editing and vectors science will undoubtedly pave the way for translating these promising approaches to clinical disease treatment soon.

5. Clinical perspectives

The encouraging data from animal models allowed researchers and companies to go one step forward toward clinical trials. Promising results from *ex vivo* and *in vivo* studies involving the CRISPR/Cas system achieved therapeutic benefits in mice. However, questions regarding the safety and scalability of the process still need to be evaluated, particularly for *in vivo* approaches.

More traditional gene editing approaches, however, have clinical trials already ongoing. This is the case for a gene editing platform based on zinc finger nucleases, developed for MPS I and MPS II [159,160]. In this platform, the zinc finger system cuts the genome at the albumin locus, and a functional copy of the transgene is inserted using AAV2/6 vectors. Therefore, the lysosomal enzyme is produced by liver cells under the control of the albumin promoter and secreted to the circulation, reaching other organs. The trials are registered at https://clinicaltrials.gov/ under the IDs NCT02702115 (for MPS I, called CHAMPIONS) and NCT03041324 (for MPS II, called EMPOWERS).

The study "CHAMPIONS" for MPS II [159] was the first trial to attempt to edit the genome *in vivo* in humans. It consists of a Phase 1/2 clinical trial evaluating the safety, tolerability, and effect on IDS enzyme activity of the product SB-913. Nine participants were assigned to four different cohorts with rising doses of the gene editing product. The first results published in meetings suggested that the treatment was well-tolerated, and no drug-related adverse events were reported at any dose, up to 10 months after dosing. However, plasma IDS values were very low, and one subject in cohort 2 showed an increase in ALT levels, suggesting an immune response to the therapy [161]. Additional analyses of the trial data are still pending.

The second trial [160] is called "EMPOWERS", which is directed to individuals with MPS I (NCT02702115). The platform is similar to the "CHAMPIONS" trial, but the transgene, in this case, is IDUA. Three participants were assigned to three different cohorts with increasing therapeutic doses of SB-318, the therapeutic product. The vector was well tolerated in all doses tested. No serious adverse events related to the study drug were reported up to 3 months after dosing. However, the plasma IDUA values remained unchanged from pre-treatment levels. In addition, two patients showed a decrease in the urinary excretion of GAGs, while one patient remained with GAGs values above the normal range. The final data were not published to date.

Despite promising, this approach is not likely to correct the brain disease since the enzyme produced by the liver cells is not able to cross the blood–brain-barrier. Furthermore, based on the preliminary data, it seems that *in vivo* gene editing for MPS will need a more efficient system, considering that even at the highest doses tested, enzyme levels produced were barely detectable. One third point to be considered is that, when using AAV vectors to deliver the Cas9 gene, the presence of the Cas9 that is continuously produced by the AAV vector could cause off-target effects, which needs to be investigated. In this sense, either ex-vivo approaches, the use of non-viral vectors, the purified Cas9, or its mRNA are other options to be tested soon [57,162].

6. Regulatory framework in genome editing-based therapy

In recent decades, extensive research has been conducted globally in the field of gene therapy for monogenic and rare diseases, including MPS, and different strategies are being investigated, ranging from conventional direct gene replacement or addition to the latest promising gene editing-based therapy [24,75,134,163,164]. Nevertheless, the regulatory framework of genome editing technologies as a therapeutic product class is comparatively new. It raises questions about how these products should be regulated for successful clinical implementation due to their novelty, complexity, and technical specificity associated with important challenges in terms of safety, efficacy, quality, manufacturing process, and ethical conflicts [163,165,166].

In the European Union (EU), genome editing-based therapy products have fallen under the regulatory framework for Advanced Therapy Medicinal Products (ATMPs) since 2008, per Regulation (EC) N°. 1394/2007 and European Medicines Agency (EMA) classification procedure [163,165–167]. ATMPs consist of complex and innovative biological products, including gene therapy, somatic cell therapy, and tissue engineering [163,167,168]. The first gene therapy authorized by EMA in the EU was Glybera® (alipogene tiparvovec) in 2012, an adeno-associated viral (AAV) vector expressing the human lipoprotein lipase gene used to treat patients with familial lipoprotein lipase deficiency. The EMA withdrew Glybera® in 2017 at the time of the withdrawal of the marketing authorization. [106]. Presently, several gene therapy products are available on the market, such as Luxturna® (voretigene neparvovec) used for congenital retinal dystrophy, Zolgensma® (onasemnogen abeparvovec) used for spinal muscular atrophy, Strimvelis® the first *ex vivo* stem cell gene therapy for the treatment of severe combined immunodeficiency caused by adenosine deaminase deficiency, and Libmeldy® (autologous haematopoietic stem cell) employed for metachromatic leukodystrophy [169].

No ATMPs authorized for use worldwide employ genome editing, even with many companies developing therapies based on these technologies, and some in the clinical trials stage for various applications [170], including for MPS. As can be seen, the time lag between the theoretical conceptualization, clinical trials, and the approval of a new ATMP is quite long. Moreover, it can be associated with various complexities, not only scientific but also regulatory, that require specific knowledge of manufacture and biological characterization that transcends the traditional pharmaceutical field [165,168,171].

While EMA uses the term ATMPs in the EU, the Food and Drug Administration (FDA) regulatory authority in the United States (US) and other regulatory agencies from Australia, Brazil, Canada, Japan, Korea, and other countries also have specific regulations for these therapeutic products. However, despite their differences, the main regulatory milestones achieved by the approved ATMPs are similar and converge in their primary goals of ensuring the quality, safety, and efficacy of these products [171–173].

In this context, the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) emerged as an alternative to accomplish international harmonization in scientific, technical, and regulatory requirements for pharmaceutical products, including the ATMPs, considering that the global regulatory convergence among the leading regulatory agencies may be one of the critical factors in simplifying and advancing its approval [171–173]. Currently, there are several guidelines on ATMPs, including quality, non-clinical and clinical considerations, with may offer a basis for the development and regulatory evaluation of genome editing-based therapies, even as

these therapies have not yet been approved and can face similar ATMPs concerns.

The main regulatory quality issues for ATMPs, include unreliable manufacturing process, deficits in comparability after manufacturing changes, and failures in product characterization and potency assays [166,174]. Furthermore, especially for genome editing tools, the delivery systems will include electroporation, plasmids, viral vectors, exosomes, nanotechnology-based delivery systems, and/or microfluidic devices, which will vary in their effectiveness in different *in vitro* / *ex vivo* settings and in their ability to target cells *in vivo*. Thus, biodistribution and immunogenicity studies are extremely important for in vivo applications [170,175].

In non-clinical development, the critical issue is the lack of adequate animal models, as these may not be representative of the clinical situation [166,174]. In addition, particularly for genome editing, preclinical efficacy models' treatments have several questions, including accounting for the impact of the *in vivo* cellular environment on genome-editing efficiency [170,175].

Regarding clinical development, the main challenges are frequently the absence of good therapeutic outcomes, the selection of the comparator, or inadequate statistical analysis (often associated with the limited number of patients) [166,174]. In addition, specific to genome editing, the clinical development is dependent on comprehensively identifying on- and off-target effects and any correlated toxicities. In this context, some of the challenges that need to be overcome include improving methods for delivering engineered nucleases and donor DNA, increasing nuclease activity in specific cell types and loci, increasing nuclease specificity, and reducing toxicity. Therefore, it is important to have assays, including *in vivo*, *in vitro*, and *in silico* methods, to quantify genome editing rates on- and off-target [170,175,176]. In addition, there is also a risk of spontaneous germline alteration in embryos, the correct disease-causing mutations that would be passed on to future generations [177,178].

Besides the regulatory issues, according to Barkholt et al. [179] for ATMPs approval, it is essential to carefully plan the product definition, characterize the product in advance, choose the potency assay that reflects the product's activity, recognize the critical in-process parameters and implement a risk-based approach. Correspondingly, Schacker et al. [166] suggest that it is possible to transform what appear to be regulatory challenges into essential considerations that must be addressed in product development through initial strategic planning.

Finally, it is possible to visualize that the genome editing-based therapy field has made significant progress in recent years, including for MPS, given the various advanced steps for these products, ranging from preclinical development to human clinical trials. However, several regulatory challenges must be considered and addressed before gene editing therapy products reach the market. In conclusion, in view of its constant advancement and technological innovation, the regulations must be adaptable to allow an individual approach (case-by-case), based on the potential therapeutic benefit versus the potential health risks that each of these products, a comprehensive benefit-risk analysis of data available, facilitating the approval process and the products reaching patients.

7. Conclusions

There are many challenges to overcome before gene editing can be clinically used for treating MPS. Safety issues must be solved, the regulatory framework must be cleared, and the challenge of targeting different hard-to-reach organs, like the brain, the heart, joints, and the bones, must be surpassed. Still, this is a promising therapeutic strategy for MPS and other lysosomal disorders. First, this is a long-lasting genetic modification, suitable for lifelong diseases. Second, the therapeutic target is often a soluble lysosomal enzyme that can be uptake by neighbouring cells. Also, the observation of attenuated phenotypes caused by residual enzyme activity shows that small increases in the amount of functional protein are sufficient to improve clinical outcomes. As scientific research in this field progresses novel delivery vectors will be designed, and we shall see the first clinical trials using gene editing for lysosomal disorders.

Funding

This work was supported by grants from the National Research Council (CNPq – 403587/2021–2 RSS; 312714/2018–1 UM; 442425/2020–1 FF and UM; 442545/2020-7 GB), State Agency of Research (FAPERGS - 22/2551-0000385-0), and Fundo de Incentivo a Pesquisa (FIPE/HCPA).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] P. Saftig, J. Klumperman, Lysosome biogenesis and lysosomal membrane proteins: Trafficking meets function, Nat. Rev. Mol. Cell Biol. 10 (2009) 623–635, https://doi.org/10.1038/nrm2745.
[2] F. Poswar, F. Vairo, M. Burin, K. Michelin-Tirelli, A. Brusius-Facchin, F. Kubaski, C. Desouza, G. Baldo, R. Giugliani, Lysosomal diseases: Overview on current diagnosis and treatment, Genet. Mol. Biol. 42 (2019) 165–177, https://doi.org/10.1590/1678-4685-gmb-2018-0159.
[3] T. Corrêa, B.C. Feltes, R. Giugliani, U. Matte, Disruption of morphogenic and growth pathways in lysosomal storage diseases, WIREs Mech. Dis. 13 (2021) 1–16, https://doi.org/10.1002/wsbm.1521.
[4] F.M. Platt, B. Boland, A.C. van der Spoel, Lysosomal storage disorders: The cellular impact of lysosomal dysfunction, J. Cell Biol. 199 (2012) 723–734, https://doi.org/10.1083/jcb.201208152.
[5] V. Rajkumar, V. Dumpa, Lysosomal storage disease, 2022. //www.ncbi.nlm.nih.gov/books/NBK563270/.
[6] M.P. Wasserstein, M. Caggana, S.M. Bailey, R.J. Desnick, L. Edelmann, L. Estrella, I. Holzman, N.R. Kelly, R. Kornreich, S.G. Kupchik, M. Martin, S.M. Nafday, R. Wasserman, A. Yang, C. Yu, J.J. Orsini, The New York pilot newborn screening program for lysosomal storage diseases: Report of the First 65,000 Infants, Genet. Med. 21 (2019) 631–640, https://doi.org/10.1038/s41436-018-0129-y.
[7] J.Ô. Stirnemann, N. Belmatoug, F. Camou, C. Serratrice, R. Froissart, C. Caillaud, T. Levade, L. Astudillo, J. Serratrice, A. Brassier, C. Rose, T.B. De Villemeur, M.G. Berger, A review of gaucher disease pathophysiology, clinical presentation and treatments, Int. J. Mol. Sci. 18 (2017) 1–30, https://doi.org/10.3390/ijms18020441.
[8] S.J. Chin, M. Fuller, Prevalence of lysosomal storage disorders in Australia from 2009 to 2020, Lancet Reg. Health Wets. Pac. 19 (2022), https://doi.org/10.1016/j.lanwpc.2021.100344.
[9] G. Parenti, D.L. Medina, A. Ballabio, The rapidly evolving view of lysosomal storage diseases, EMBO Mol. Med. 13 (2021) 1–21, https://doi.org/10.15252/emmm.202012836.
[10] H.J. Vernon, Inborn errors of metabolism: Advances in diagnosis and therapy, JAMA Pediatr. 169 (2015) 778–782, https://doi.org/10.1001/jamapediatrics.2015.0754.
[11] F.P. Vairo, N.J. Boczek, M.A. Cousin, C. Kaiwar, P.R. Blackburn, E. Conboy, B.C. Lanpher, R.H. Gavrilova, P.N. Pichurin, K.N. Lazaridis, D. Babovic-Vuksanovic, E.W. Klee, The prevalence of diseases caused by lysosome-related genes in a cohort of undiagnosed patients, Mol. Genet. Metab. Reports. 13 (2017) 46–51, https://doi.org/10.1016/j.ymgmr.2017.08.001.
[12] F.M. Platt, Emptying the stores: Lysosomal diseases and therapeutic strategies, Nat. Rev. Drug Discov. 17 (2018) 133–150, https://doi.org/10.1038/nrd.2017.214.
[13] M.F. Coutinho, J.I. Santos, S. Alves, Less is more: Substrate reduction therapy for lysosomal storage disorders, Int. J. Mol. Sci. 17 (2016), https://doi.org/10.3390/ijms17071065.
[14] C. Fernández-Pereira, B.S. Millán-Tejado, M. Gallardo-Gómez, T. Pérez-Márquez, M. Alves-Villar, C. Melcón-Crespo, J. Fernández-Martín, S. Ortolano, Therapeutic approaches in lysosomal storage diseases, Biomolecules. 11 (2021) 1–19, https://doi.org/10.3390/biom11121775.
[15] M. Stapleton, N. Arunkumar, F. Kubaski, R.W. Mason, O. Tadao, S. Tomatsu, Clinical presentation and diagnosis of mucopolysaccharidoses, Mol. Genet. Metab. 125 (2018) 4–17, https://doi.org/10.1016/j.ymgme.2018.01.003.

[16] K.L. McBride, K.M. Flanigan, Update in the Mucopolysaccharidoses, Semin. Pediatr. Neurol. 37 (2021), https://doi.org/10.1016/j.spen.2021.100874.
[17] F. Kubaski, F. De Oliveira Poswar, K. Michelin-Tirelli, M.G. Burin, D. Rojas-Málaga, A.C. Brusius-Facchin, S. Leistner-Segal, R. Giugliani, Diagnosis of mucopolysaccharidoses, Diagnostics. 10 (2020), https://doi.org/10.3390/diagnostics10030172.
[18] J. Zhou, J. Lin, W.T. Leung, L. Wang, A basic understanding of mucopolysaccharidosis: Incidence, clinical features, diagnosis, and management, Intractable Rare Dis. Res. 9 (2020) P1–P9, https://doi.org/10.5582/irdr.2020.01011.
[19] R. Giugliani, Mucopolysacccharidoses: From understanding to treatment, a century of discoveries, Genet. Mol. Biol. 35 (2012) 924–931.
[20] B. Celik, S.C. Tomatsu, S. Tomatsu, S.A. Khan, Epidemiology of mucopolysaccharidoses, Diagnostics. 11 (2021), https://doi.org/10.1016/j.ymgme.2017.12.311.
[21] P. Borges, G. Pasqualim, R. Giugliani, F. Vairo, U. Matte, Estimated prevalence of mucopolysaccharidoses from population-based exomes and genomes, Orphanet J. Rare Dis. 15 (2020) 1–9, https://doi.org/10.1186/s13023-020-01608-0.
[22] J. Sheth, M. Mistri, K. Shah, M. Chaudhary, K. Godbole, F. Sheth, Lysosomal Storage Disorders in Nonimmune Hydrops Fetalis (NIHF): An Indian Experience BT - JIMD Reports, Volume 35, in: E. Morava, M. Baumgartner, M. Patterson, S. Rahman, J. Zschocke, V. Peters (Eds.), Springer Berlin Heidelberg, Berlin, Heidelberg, 2017: pp. 47–52. 10.1007/8904_2016_24.
[23] B. Triggs-Raine, Biology of hyaluronan: Insights from genetic disorders of hyaluronan metabolism, World J. Biol. Chem. 6 (2015) 110, https://doi.org/10.4331/wjbc.v6.i3.110.
[24] M. Stapleton, H. Hoshina, K. Sawamoto, F. Kubaski, R.W. Mason, W.G. Mackenzie, M. Theroux, H. Kobayashi, S. Yamaguchi, Y. Suzuki, T. Fukao, O. Tadao, H. Ida, S. Tomatsu, Critical review of current MPS guidelines and management, Mol. Genet. Metab. 126 (2019) 238–245, https://doi.org/10.1016/j.ymgme.2018.07.001.
[25] L. Gaffke, K. Pierzynowska, M. Podlacha, D. Hoinkis, E. Rintz, J. Brokowska, Z. Cyske, G. Wegrzyn, Underestimated aspect of mucopolysaccharidosis pathogenesis: Global changes in cellular processes revealed by transcriptomic studies, Int. J. Mol. Sci. 21 (2020), https://doi.org/10.3390/ijms21041204.
[26] F. Kubaski, F. de Oliveira Poswar, K. Michelin-Tirelli, U. da Silveira Matte, D.D. Horovitz, A.L. Barth, G. Baldo, F. Vairo, R. Giugliani, Mucopolysaccharidosis type I, Diagnostics. (2020). 10.3390/diagnostics10030161.
[27] E. Poletto, G. Pasqualim, R. Giugliani, U. Matte, G. Baldo, Worldwide distribution of common IDUA pathogenic variants, Clin. Genet. 94 (2018) 95–102, https://doi.org/10.1111/cge.13224.
[28] F. D'Avanzo, L. Rigon, A. Zanetti, R. Tomanin, Mucopolysaccharidosis type II: One hundred years of research, diagnosis, and treatment, Int. J. Mol. Sci. 21 (2020). 10.3390/ijms21041258.
[29] S. Verma, S. Pantoom, J. Petters, A.K. Pandey, A. Hermann, J. Lukas, A molecular genetics view on Mucopolysaccharidosis Type II, Mutat. Res. - Rev. Mutat. Res. 788 (2021), https://doi.org/10.1016/j.mrrev.2021.108392.
[30] M.J. Valstar, G.J.G. Ruijter, O.P. van Diggelen, B.J. Poorthuis, F.A. Wijburg, Sanfilippo syndrome: A mini-review, J. Inherit. Metab. Dis. 31 (2008) 240–252, https://doi.org/10.1007/s10545-008-0838-5.
[31] N. Benetó, L. Vilageliu, D. Grinberg, I. Canals, Sanfilippo syndrome: Molecular basis, disease models and therapeutic approaches, Int. J. Mol. Sci. 21 (2020) 1–20, https://doi.org/10.3390/ijms21217819.
[32] K. Sawamoto, J.V.Á. González, M. Piechnik, F.J. Otero, M.L. Couce, Y. Suzuki, S. Tomatsu, Mucopolysaccharidosis IVA: Diagnosis, treatment, and management, Int. J. Mol. Sci. 21 (2020), https://doi.org/10.3390/ijms21041517.
[33] A. Caciotti, L. Cellai, R. Tonin, D. Mei, E. Procopio, M. Di Rocco, A. Andaloro, D. Antuzzi, A. Rampazzo, M. Rigoldi, G. Forni, G. la Marca, R. Guerrini, A. Morrone, Morquio B disease: From pathophysiology towards diagnosis, Mol. Genet. Metab. 132 (2021) 180–188, https://doi.org/10.1016/j.ymgme.2021.01.008.
[34] R. Tomanin, L. Karageorgos, A. Zanetti, M. Al-Sayed, M. Bailey, N. Miller, H. Sakuraba, J.J. Hopwood, Mucopolysaccharidosis type VI (MPS VI) and molecular analysis: Review and classification of published variants in the ARSB gene, Hum. Mutat. 39 (2018) 1788–1802, https://doi.org/10.1002/humu.23613.
[35] F. D'Avanzo, A. Zanetti, C. De Filippis, R. Tomanin, Mucopolysaccharidosis type VI, an updated overview of the disease, Int. J. Mol. Sci. 22 (2021), https://doi.org/10.3390/ijms222413456.
[36] R. Giugliani, A.L. Barth, M.R.C. Dumas, J.F. da Silva Franco, L. de Rosso Giuliani, C.H.P. Grangeiro, D.D.G. Horovitz, C.A. Kim, E.K.E. de Araújo Leão, P.F.V. de Medeiros, D.S.C.G. Miguel, M.E.S.A. Moreira, H.M.G.P. dos Santos, L.C.S. da Silva, L.R. da Silva, I.N. de Souza, T. Nalin, D. Garcia, Mucopolysaccharidosis VII in Brazil: natural history and clinical findings, Orphanet J. Rare Dis. 16 (2021) 1–9, https://doi.org/10.1186/s13023-021-01870-w.
[37] F.I. Khan, M. Shahbaaz, K. Bisetty, A. Waheed, W.S. Sly, F. Ahmad, M.I. Hassan, Large scale analysis of the mutational landscape in β-glucuronidase: A major player of mucopolysaccharidosis type VII, Gene 576 (2016) 36–44, https://doi.org/10.1016/j.gene.2015.09.062.
[38] F.D. Urnov, E.J. Rebar, M.C. Holmes, H.S. Zhang, P.D. Gregory, Genome editing with engineered zinc finger nucleases, Nat. Rev. Genet. 11 (2010) 636–646, https://doi.org/10.1038/nrg2842.
[39] H. Kim, J.-S. Kim, A guide to genome engineering with programmable nucleases, Nat. Rev. Genet. 15 (2014) 321–334, https://doi.org/10.1038/nrg3686.
[40] R. Barrangou, C. Fremaux, H. Deveau, M. Richards, P. Boyaval, S. Moineau, D.A. Romero, P. Horvath, CRISPR provides acquired resistance against viruses in prokaryotes, Science 315 (2007) 1709–1712, https://doi.org/10.1126/science.1138140.
[41] F.J.M. Mojica, C. Díez-Villaseñor, J. García-Martínez, E. Soria, Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements, J. Mol. Evol. 60 (2005) 174–182, https://doi.org/10.1007/s00239-004-0046-3.
[42] M. Jinek, K. Chylinski, I. Fonfara, M. Hauer, J.A. Doudna, E. Charpentier, A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity, Science 337 (2012) 816–821, https://doi.org/10.1126/science.1225829.
[43] P. Mali, L. Yang, K.M. Esvelt, J. Aach, M. Guell, J.E. DiCarlo, J.E. Norville, G.M. Church, RNA-guided human genome engineering via Cas9, Science 339 (2013) 823–826, https://doi.org/10.1126/science.1232033.
[44] M. Jinek, A. East, A. Cheng, S. Lin, E. Ma, J. Doudna, RNA-programmed genome editing in human cells, Elife. 2 (2013) e00471.
[45] F. Jiang, J.A. Doudna, CRISPR-Cas9 Structures and Mechanisms, Annu. Rev. Biophys. 46 (2017) 505–529, https://doi.org/10.1146/annurev-biophys-062215-010822.
[46] A.C. Vítor, P. Huertas, G. Legube, S.F. de Almeida, Studying DNA Double-Strand Break Repair: An Ever-Growing Toolbox, Front. Mol. Biosci. 7 (2020).
[47] M. Bétermier, P. Bertrand, B.S. Lopez, Is Non-Homologous End-Joining Really an Inherently Error-Prone Process?, PLOS Genet 10 (2014) e1004086.
[48] E. Poletto, G. Baldo, Creating cell lines for mimicking diseases, Prog. Mol. Biol. Transl. Sci. 181 (2021) 59–87, https://doi.org/10.1016/bs.pmbts.2021.01.014.
[49] D.G. Ousterout, P. Perez-Pinera, P.I. Thakore, A.M. Kabadi, M.T. Brown, X. Qin, O. Fedrigo, V. Mouly, J.P. Tremblay, C.A. Gersbach, Reading frame correction by targeted genome editing restores dystrophin expression in cells from Duchenne muscular dystrophy patients, Mol. Ther. 21 (2013) 1718–1726, https://doi.org/10.1038/mt.2013.111.
[50] H. Yang, S. Ren, S. Yu, H. Pan, T. Li, S. Ge, J. Zhang, N. Xia, Methods Favoring Homology-Directed Repair Choice in Response to CRISPR/Cas9 Induced-Double Strand Breaks, Int. J. Mol. Sci. 21 (2020), https://doi.org/10.3390/ijms21186461.
[51] E. Poletto, G. Baldo, N. Gomez-Ospina, Genome editing for mucopolysaccharidoses, Int. J. Mol. Sci. 21 (2020) 1–20, https://doi.org/10.3390/ijms21020500.
[52] R.S. Schuh, T.G. de Carvalho, R. Giugliani, U. Matte, G. Baldo, H.F. Teixeira, Gene editing of MPS I human fibroblasts by co-delivery of a CRISPR/Cas9 plasmid and a donor oligonucleotide using nanoemulsions as nonviral carriers, Eur. J. Pharm. Biopharm. 122 (2018) 158–166, https://doi.org/10.1016/j.ejpb.2017.10.017.
[53] T.G. de Carvalho, R. Schuh, G. Pasqualim, F.M. Pellenz, E.C. Filippi-Chiela, R. Giugliani, G. Baldo, U. Matte, CRISPR-Cas9-mediated gene editing in human MPS I fibroblasts, Gene 678 (2018) 33–37, https://doi.org/10.1016/j.gene.2018.08.004.
[54] K. Laoharawee, R.C. DeKelver, K.M. Podetz-Pedersen, M. Rohde, S. Sproul, H.-O. Nguyen, T. Nguyen, S.J. St Martin, L. Ou, S. Tom, R. Radeke, K.E. Meyer, M.C. Holmes, C.B. Whitley, T. Wechsler, R.S. McIvor, Dose-Dependent Prevention of Metabolic and Neurologic Disease in Murine MPS II by ZFN-Mediated In Vivo Genome Editing., Mol. Ther. 26 (2018) 1127–1136. 10.1016/j.ymthe.2018.03.002.
[55] L. Ou, R.C. DeKelver, M. Rohde, S. Tom, R. Radeke, S.J. St Martin, Y. Santiago, S. Sproul, M.J. Przybilla, B.L. Koniar, K.M. Podetz-Pedersen, K. Laoharawee, R.D. Cooksley, K.E. Meyer, M.C. Holmes, R.S. McIvor, T. Wechsler, C.B. Whitley, ZFN-Mediated In Vivo Genome Editing Corrects Murine Hurler Syndrome., Mol. Ther. 27 (2019) 178–187. 10.1016/j.ymthe.2018.10.018.
[56] S. Pellenz, M. Phelps, W. Tang, B.T. Hovde, R.B. Sinit, W. Fu, H. Li, E. Chen, R.J.J. Monnat, New Human Chromosomal Sites with "Safe Harbor" Potential for Targeted Transgene Insertion, Hum. Gene Ther. 30 (2019) 814–828, https://doi.org/10.1089/hum.2018.169.
[57] N. Gomez-Ospina, S.G. Scharenberg, N. Mostrel, R.O. Bak, S. Mantri, R.M. Quadros, C.B. Gurumurthy, C. Lee, G. Bao, C.J. Suarez, S. Khan, K. Sawamoto, S. Tomatsu, N. Raj, L.D. Attardi, L. Aurelian, M.H. Porteus, Human genome-edited hematopoietic stem cells phenotypically correct Mucopolysaccharidosis type I, Nat. Commun. 10 (2019) 4045, https://doi.org/10.1038/s41467-019-11962-8.
[58] E. Poletto, P. Colella, L.N. Pimentel Vera, S. Khan, S. Tomatsu, G. Baldo, N. Gomez-Ospina, Improved engraftment and therapeutic efficacy by human genome edited hematopoietic stem cells with Busulfan-based myeloablation, Mol. Ther. - Methods Clin. Dev. 25 (2022) 392–409, https://doi.org/10.1016/j.omtm.2022.04.009.
[59] R.S. Schuh, E. Poletto, G. Pasqualim, A.M.V. Tavares, F.S. Meyer, E.A. Gonzalez, R. Giugliani, U. Matte, H.F. Teixeira, G. Baldo, In vivo genome editing of mucopolysaccharidosis I mice using the CRISPR/Cas9 system, J. Control. Release. 288 (2018) 23–33, https://doi.org/10.1016/j.jconrel.2018.08.031.
[60] S.G. Scharenberg, E. Poletto, K.L. Lucot, P. Colella, A. Sheikali, T.J. Montine, M. H. Porteus, N. Gomez-Ospina, Engineering monocyte/macrophage-specific glucocerebrosidase expression in human hematopoietic stem cells using genome editing, Nat. Commun. 11 (2020) 3327, https://doi.org/10.1038/s41467-020-17148-x.

[61] S. Sawatsubashi, Y. Joko, S. Fukumoto, T. Matsumoto, S.S. Sugano, Development of versatile non-homologous end joining-based knock-in module for genome editing, Sci. Rep. 8 (2018) 593, https://doi.org/10.1038/s41598-017-18911-9.
[62] K. Suzuki, Y. Tsunekawa, R. Hernandez-Benitez, J. Wu, J. Zhu, E.J. Kim, F. Hatanaka, M. Yamamoto, T. Araoka, Z. Li, M. Kurita, T. Hishida, M. Li, E. Aizawa, S. Guo, S. Chen, A. Goebl, R.D. Soligalla, J. Qu, T. Jiang, X. Fu, M. Jafari, C.R. Esteban, W.T. Berggren, J. Lajara, E. Nuñez-Delicado, P. Guillen, J.M. Campistol, F. Matsuzaki, G.-H. Liu, P. Magistretti, K. Zhang, E.M. Callaway, K. Zhang, J.C.I. Belmonte, In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration, Nature 540 (2016) 144–149, https://doi.org/10.1038/nature20565.
[63] K. Suzuki, J.C. Izpisua Belmonte, In vivo genome editing via the HITI method as a tool for gene therapy, J. Hum. Genet. 63 (2018) 157–164, https://doi.org/10.1038/s10038-017-0352-4.
[64] S. Byambaa, H. Uosaki, T. Ohmori, H. Hara, H. Endo, O. Nureki, Y. Hanazono, Non-viral ex vivo genome-editing in mouse bona fide hematopoietic stem cells with CRISPR/Cas9, Mol. Ther. - Methods Clin. Dev. 20 (2021) 451–462, https://doi.org/10.1016/j.omtm.2021.01.001.
[65] A.C. Komor, Y.B. Kim, M.S. Packer, J.A. Zuris, D.R. Liu, Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage, Nature 533 (2016) 420–424, https://doi.org/10.1038/nature17946.
[66] S.K. Bose, B.M. White, M. V Kashyap, A. Dave, F.R. De Bie, H. Li, K. Singh, P. Menon, T. Wang, S. Teerdhala, V. Swaminathan, H.A. Hartman, S. Jayachandran, P. Chandrasekaran, K. Musunuru, R. Jain, D.B. Frank, P. Zoltick, W.H. Peranteau, In utero adenine base editing corrects multi-organ pathology in a lethal lysosomal storage disease., Nat. Commun. 12 (2021) 4291. 10.1038/s41467-021-24443-8.
[67] A.V. Anzalone, P.B. Randolph, J.R. Davis, A.A. Sousa, L.W. Koblan, J.M. Levy, P.J. Chen, C. Wilson, G.A. Newby, A. Raguram, D.R. Liu, Search-and-replace genome editing without double-strand breaks or donor DNA, Nature 576 (2019) 149–157, https://doi.org/10.1038/s41586-019-1711-4.
[68] I. Weisheit, J.A. Kroeger, R. Malik, J. Klimmt, D. Crusius, A. Dannert, M. Dichgans, D. Paquet, Detection of Deleterious On-Target Effects after HDR-Mediated CRISPR Editing, Cell Rep. 31 (2020), https://doi.org/10.1016/j.celrep.2020.107689.
[69] F. Adikusuma, S. Piltz, M.A. Corbett, M. Turvey, S.R. McColl, K.J. Helbig, M.R. Beard, J. Hughes, R.T. Pomerantz, P.Q. Thomas, Large deletions induced by Cas9 cleavage, Nature 560 (2018) E8–E9, https://doi.org/10.1038/s41586-018-0380-z.
[70] O.M. Enache, V. Rendo, M. Abdusamad, D. Lam, D. Davison, S. Pal, N. Currimjee, J. Hess, S. Pantel, A. Nag, A.R. Thorner, J.G. Doench, F. Vazquez, R. Beroukhim, T.R. Golub, U. Ben-David, Cas9 activates the p53 pathway and selects for p53-inactivating mutations, Nat. Genet. 52 (2020) 662–668, https://doi.org/10.1038/s41588-020-0623-4.
[71] G. Cullot, J. Boutin, J. Toutain, F. Prat, P. Pennamen, C. Rooryck, M. Teichmann, E. Rousseau, I. Lamrissi-Garcia, V. Guyonnet-Duperat, A. Bibeyran, M. Lalanne, V. Prouzet-Mauléon, B. Turcq, C. Ged, J.-M. Blouin, E. Richard, S. Dabernat, F. Moreau-Gaudry, A. Bedel, CRISPR-Cas9 genome editing induces megabase-scale chromosomal truncations, Nat. Commun. 10 (2019) 1136, https://doi.org/10.1038/s41467-019-09006-2.
[72] M.L. Leibowitz, S. Papathanasiou, P.A. Doerfler, L.J. Blaine, L. Sun, Y. Yao, C.-Z. Zhang, M.J. Weiss, D. Pellman, Chromothripsis as an on-target consequence of CRISPR–Cas9 genome editing, Nat. Genet. 53 (2021) 895–905, https://doi.org/10.1038/s41588-021-00838-7.
[73] M.V. Zuccaro, J. Xu, C. Mitchell, D. Marin, R. Zimmerman, B. Rana, E. Weinstein, R.T. King, K.L. Palmerola, M.E. Smith, S.H. Tsang, R. Goland, M. Jasin, R. Lobo, N. Treff, D. Egli, Allele-Specific Chromosome Removal after Cas9 Cleavage in Human Embryos, Cell 183 (2020) 1650–1664.e15, https://doi.org/10.1016/j.cell.2020.10.025.
[74] M. Naeem, S. Majeed, M.Z. Hoque, I. Ahmad, Latest Developed Strategies to Minimize the Off-Target Effects in CRISPR-Cas-Mediated Genome Editing, Cells. 9 (2020), https://doi.org/10.3390/cells9071608.
[75] J. van Haasteren, J. Li, O.J. Scheideler, N. Murthy, D.V. Schaffer, The delivery challenge: fulfilling the promise of therapeutic genome editing, Nat. Biotechnol. 38 (2020) 845–855, https://doi.org/10.1038/s41587-020-0565-5.
[76] Z.-Y. He, K. Men, Z. Qin, Y. Yang, T. Xu, Y.-Q. Wei, Non-viral and viral delivery systems for CRISPR-Cas9 technology in the biomedical field, Sci. China Life Sci. 60 (2017) 458–467, https://doi.org/10.1007/s11427-017-9033-0.
[77] C.A. Lino, J.C. Harper, J.P. Carney, J.A. Timlin, Delivering CRISPR: a review of the challenges and approaches, Drug Deliv. 25 (2018) 1234–1257, https://doi.org/10.1080/10717544.2018.1474964.
[78] L. Wang, W. Zheng, S. Liu, B. Li, X. Jiang, Delivery of CRISPR/Cas9 by Novel Strategies for Gene Therapy, ChemBioChem 20 (2019) 634–643, https://doi.org/10.1002/cbic.201800629.
[79] A.L.J. Marschall, Targeting the Inside of Cells with Biologicals: Chemicals as a Delivery Strategy, BioDrugs. 35 (2021) 643–671, https://doi.org/10.1007/s40259-021-00500-y.
[80] L. Li, S. Hu, X. Chen, Non-viral delivery systems for CRISPR/Cas9-based genome editing: Challenges and opportunities, Biomaterials 171 (2018) 207–218, https://doi.org/10.1016/j.biomaterials.2018.04.031.
[81] H. Yin, C.-Q. Song, J.R. Dorkin, L.J. Zhu, Y. Li, Q. Wu, A. Park, J. Yang, S. Suresh, A. Bizhanova, A. Gupta, M.F. Bolukbasi, S. Walsh, R.L. Bogorad, G. Gao, Z. Weng, Y. Dong, V. Koteliansky, S.A. Wolfe, R. Langer, W. Xue, D.G. Anderson, Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo, Nat. Biotechnol. 34 (2016) 328–333, https://doi.org/10.1038/nbt.3471.
[82] J. Liu, S. Shui, Delivery methods for site-specific nucleases: Achieving the full potential of therapeutic gene editing, J. Control. Release. 244 (2016) 83–97, https://doi.org/10.1016/j.jconrel.2016.11.014.
[83] E.A. Taha, J. Lee, A. Hotta, Delivery of CRISPR-Cas tools for in vivo genome editing therapy: Trends and challenges, J. Control. Release. 342 (2022) 345–361, https://doi.org/10.1016/j.jconrel.2022.01.013.
[84] H. Cheng, F. Zhang, Y. Ding, CRISPR/Cas9 Delivery System Engineering for Genome Editing in Therapeutic Applications, Pharm. 13 (2021), https://doi.org/10.3390/pharmaceutics13101649.
[85] J.B. Miller, S. Zhang, P. Kos, H. Xiong, K. Zhou, S.S. Perelman, H. Zhu, D.J. Siegwart, Non-Viral CRISPR/Cas Gene Editing In Vitro and In Vivo Enabled by Synthetic Nanoparticle Co-Delivery of Cas9 mRNA and sgRNA, Angew. Chem. Int. Ed. Engl. 56 (2017) 1059–1063, https://doi.org/10.1002/anie.201610209.
[86] Y. Niu, B. Shen, Y. Cui, Y. Chen, J. Wang, L. Wang, Y. Kang, X. Zhao, W. Si, W. Li, A.P. Xiang, J. Zhou, X. Guo, Y. Bi, C. Si, B. Hu, G. Dong, H. Wang, Z. Zhou, T. Li, T. Tan, X. Pu, F. Wang, S. Ji, Q. Zhou, X. Huang, W. Ji, J. Sha, Generation of Gene-Modified Cynomolgus Monkey via Cas9/RNA-Mediated Gene Targeting in One-Cell Embryos, Cell 156 (2014) 836–843, https://doi.org/10.1016/j.cell.2014.01.027.
[87] Y. Feng, S. Sassi, J.K. Shen, X. Yang, Y. Gao, E. Osaka, J. Zhang, S. Yang, C. Yang, H.J. Mankin, F.J. Hornicek, Z. Duan, Targeting Cdk11 in osteosarcoma cells using the CRISPR-cas9 system, J. Orthop. Res. 33 (2015) 199–207, https://doi.org/10.1002/jor.22745.
[88] S.-R. Lin, H.-C. Yang, Y.-T. Kuo, C.-J. Liu, T.-Y. Yang, K.-C. Sung, Y.-Y. Lin, H.-Y. Wang, C.-C. Wang, Y.-C. Shen, F.-Y. Wu, J.-H. Kao, D.-S. Chen, P.-J. Chen, The CRISPR/Cas9 System Facilitates Clearance of the Intrahepatic HBV Templates In Vivo, Mol. Ther. - Nucleic Acids. 3 (2014), https://doi.org/10.1038/mtna.2014.38.
[89] H. Yin, W. Xue, S. Chen, R.L. Bogorad, E. Benedetti, M. Grompe, V. Koteliansky, P.A. Sharp, T. Jacks, D.G. Anderson, Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype, Nat. Biotechnol. 32 (2014) 551–553, https://doi.org/10.1038/nbt.2884.
[90] D. Rosenblum, A. Gutkin, N. Dammes, D. Peer, Progress and challenges towards CRISPR/Cas clinical translation, Adv. Drug Deliv. Rev. 154–155 (2020) 176–186, https://doi.org/10.1016/j.addr.2020.07.004.
[91] D. Kostyushev, A. Kostyusheva, S. Brezgin, V. Smirnov, E. Volchkova, A. Lukashev, V. Chulanov, Gene Editing by Extracellular Vesicles., Int. J. Mol. Sci. 21 (2020). 10.3390/ijms21197362.
[92] L. Duan, K. Ouyang, J. Wang, L. Xu, X. Xu, C. Wen, Y. Xie, Y. Liang, J. Xia, Exosomes as Targeted Delivery Platform of CRISPR/Cas9 for Therapeutic Genome Editing, ChemBioChem 22 (2021) 3360–3368, https://doi.org/10.1002/cbic.202100359.
[93] S.M. Kim, Y. Yang, S.J. Oh, Y. Hong, M. Seo, M. Jang, Cancer-derived exosomes as a delivery platform of CRISPR/Cas9 confer cancer cell tropism-dependent targeting, J. Control. Release Off. J. Control. Release Soc. 266 (2017) 8–16, https://doi.org/10.1016/j.jconrel.2017.09.013.
[94] Y. Lin, J. Wu, W. Gu, Y. Huang, Z. Tong, L. Huang, J. Tan, Exosome-Liposome Hybrid Nanoparticles Deliver CRISPR/Cas9 System in MSCs, Adv. Sci. (Weinheim, Baden-Wurttemberg, Ger. 5 (2018) 1700611. 10.1002/advs.201700611.
[95] S. Le Saux, A. Aubert-Pouëssel, K.E. Mohamed, P. Martineau, L. Guglielmi, J.-M. Devoisselle, P. Legrand, J. Chopineau, M. Morille, Interest of extracellular vesicles in regards to lipid nanoparticle based systems for intracellular protein delivery, Adv. Drug Deliv. Rev. 176 (2021), https://doi.org/10.1016/j.addr.2021.113837.
[96] Q. Ding, A. Strong, K.M. Patel, S.-L. Ng, B.S. Gosis, S.N. Regan, C.A. Cowan, D.J. Rader, K. Musunuru, Permanent alteration of PCSK9 with in vivo CRISPR-Cas9 genome editing, Circ. Res. 115 (2014) 488–492, https://doi.org/10.1161/CIRCRESAHA.115.304351.
[97] W. Wang, C. Ye, J. Liu, D. Zhang, J.T. Kimata, P. Zhou, CCR5 Gene Disruption via Lentiviral Vectors Expressing Cas9 and Single Guided RNA Renders Cells Resistant to HIV-1 Infection, PLoS ONE 9 (2014) e115987.
[98] D. Wang, F. Zhang, G. Gao, CRISPR-Based Therapeutic Genome Editing: Strategies and In Vivo Delivery by AAV Vectors, Cell 181 (2020) 136–150, https://doi.org/10.1016/j.cell.2020.03.023.
[99] I. Ates, T. Rathbone, C. Stuart, P.H. Bridges, R.N. Cottle, Delivery Approaches for Therapeutic Genome Editing and Challenges, Genes (Basel) 11 (2020), https://doi.org/10.3390/genes11101113.
[100] S. Blinka, M.H. Reimer Jr., K. Pulakanti, S. Rao, Super-Enhancers at the Nanog Locus Differentially Regulate Neighboring Pluripotency-Associated Genes, Cell Rep. 17 (2016) 19–28, https://doi.org/10.1016/j.celrep.2016.09.002.
[101] K.M. Davis, V. Pattanayak, D.B. Thompson, J.A. Zuris, D.R. Liu, Small molecule–triggered Cas9 protein with improved genome-editing specificity, Nat. Chem. Biol. 11 (2015) 316–318, https://doi.org/10.1038/nchembio.1793.
[102] J.A. Zuris, D.B. Thompson, Y. Shu, J.P. Guilinger, J.L. Bessen, J.H. Hu, M.L. Maeder, J.K. Joung, Z.-Y. Chen, D.R. Liu, Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo, Nat. Biotechnol. 33 (2015) 73–80, https://doi.org/10.1038/nbt.3081.
[103] R.S. Schuh, É. Poletto, F.N.S. Fachel, U. Matte, G. Baldo, H.F. Teixeira, Physicochemical properties of cationic nanoemulsions and liposomes obtained by microfluidization complexed with a single plasmid or along with an oligonucleotide: implications for CRISPR/Cas technology, J. Colloid Interface Sci. 530 (2018) 243–255, https://doi.org/10.1016/j.jcis.2018.06.058.

[104] E.S. Hosseini, M. Nikkhah, S. Hosseinkhani, Cholesterol-rich lipid-mediated nanoparticles boost of transfection efficiency, utilized for gene editing by CRISPR-Cas9, Int. J. Nanomed. 14 (2019) 4353–4366, https://doi.org/10.2147/IJN.S199104.
[105] D. Sun, Z. Sun, H. Jiang, A.M. Vaidya, R. Xin, N.R. Ayat, A.L. Schilb, P.L. Qiao, Z. Han, A. Naderi, Z.-R. Lu, Synthesis and Evaluation of pH-Sensitive Multifunctional Lipids for Efficient Delivery of CRISPR/Cas9 in Gene Editing, Bioconjug. Chem. 30 (2019) 667–678, https://doi.org/10.1021/acs.bioconjchem.8b00856.
[106] H. Akbaba, G. Erel-Akbaba, S. Senturk, Special Focus Issue Part II: Recruitment of solid lipid nanoparticles for the delivery of CRISPR/Cas9: primary evaluation of anticancer gene editing, Nanomedicine (Lond). 16 (2021) 963–978, https://doi.org/10.2217/nnm-2020-0412.
[107] P.V. Khachane, A.S. Jain, V.V. Dhawan, G.V. Joshi, A.A. Date, R. Mulherkar, M.S. Nagarsenker, Cationic nanoemulsions as potential carriers for intracellular delivery, Saudi Pharm. J. (2014), https://doi.org/10.1016/j.jsps.2014.07.007.
[108] A. Gupta, J.L. Andresen, R.S. Manan, R. Langer, Nucleic acid delivery for therapeutic applications, Adv. Drug Deliv. Rev. 178 (2021), https://doi.org/10.1016/j.addr.2021.113834.
[109] L. Wasungu, D. Hoekstra, Cationic lipids, lipoplexes and intracellular delivery of genes, J. Control. Release. 116 (2006) 255–264, https://doi.org/10.1016/j.jconrel.2006.06.024.
[110] H.F. Teixeira, F. Bruxel, M. Fraga, R.S. Schuh, G.K. Zorzi, U. Matte, E. Fattal, Cationic nanoemulsions as nucleic acids delivery systems, Int. J. Pharm. 534 (2017) 356–367, https://doi.org/10.1016/j.ijpharm.2017.10.030.
[111] L. Cong, F.A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, P.D. Hsu, X. Wu, W. Jiang, L.A. Marraffini, F. Zhang, Multiplex genome engineering using CRISPR/Cas systems, Science 339 (2013) 819–823, https://doi.org/10.1126/science.1231143.
[112] H.F. Teixeira, F. Bruxel, M. Fraga, R.S. Schuh, G.K. Zorzi, U. Matte, E. Fattal, Cationic nanoemulsions for nucleic acids delivery, Int. J. Pharm. 534 (2017) 356–367, https://doi.org/10.1016/j.ijpharm.2017.10.030.
[113] J.H. Azambuja, R.S. Schuh, L.R. Michels, N.E. Gelsleichter, L.R. Beckenkamp, I.C. Iser, G.S. Lenz, F.H. de Oliveira, G. Venturin, S. Greggio, J.C. daCosta, M.R. Wink, J. Sevigny, M.A. Stefani, A.M.O. Battastini, H.F. Teixeira, E. Braganhol, Nasal Administration of Cationic Nanoemulsions as CD73-siRNA Delivery System for Glioblastoma Treatment: a New Therapeutical Approach, Mol. Neurobiol. (2019), https://doi.org/10.1007/s12035-019-01730-6.
[114] H.-X. Zhang, Y. Zhang, H. Yin, Genome Editing with mRNA Encoding ZFN, TALEN, and Cas9, Mol. Ther. 27 (2019) 735–746, https://doi.org/10.1016/j.ymthe.2019.01.014.
[115] T. Wei, Q. Cheng, Y.-L. Min, E.N. Olson, D.J. Siegwart, Systemic nanoparticle delivery of CRISPR-Cas9 ribonucleoproteins for effective tissue specific genome editing, Nat. Commun. 11 (2020) 3232, https://doi.org/10.1038/s41467-020-17029-3.
[116] Z. Glass, M. Lee, Y. Li, Q. Xu, Engineering the Delivery System for CRISPR-Based Genome Editing, Trends Biotechnol. 36 (2018) 173–185, https://doi.org/10.1016/j.tibtech.2017.11.006.
[117] Z. Du, M.M. Munye, A.D. Tagalakis, M.D.I. Manunta, S.L. Hart, The role of the helper lipid on the DNA transfection efficiency of lipopolyplex formulations, Sci. Rep. 4 (2014) 7107, https://doi.org/10.1038/srep07107.
[118] Y. Rui, D.R. Wilson, J.J. Green, Non-Viral Delivery To Enable Genome Editing, Trends Biotechnol. 37 (2019) 281–293, https://doi.org/10.1016/j.tibtech.2018.08.010.
[119] J.D. Finn, A.R. Smith, M.C. Patel, L. Shaw, M.R. Youniss, J. van Heteren, T. Dirstine, C. Ciullo, R. Lescarbeau, J. Seitzer, R.R. Shah, A. Shah, D. Ling, J. Growe, M. Pink, E. Rohde, K.M. Wood, W.E. Salomon, W.F. Harrington, C. Dombrowski, W.R. Strapps, Y. Chang, D.V. Morrissey, A Single Administration of CRISPR/Cas9 Lipid Nanoparticles Achieves Robust and Persistent In Vivo Genome Editing, Cell Rep. 22 (2018) 2227–2235, https://doi.org/10.1016/j.celrep.2018.02.014.
[120] S. Zhen, Y. Liu, J. Lu, X. Tuo, X. Yang, H. Chen, W. Chen, X. Li, Human Papillomavirus Oncogene Manipulation Using Clustered Regularly Interspersed Short Palindromic Repeats/Cas9 Delivered by pH-Sensitive Cationic Liposomes, Hum. Gene Ther. 31 (2020) 309–324, https://doi.org/10.1089/hum.2019.312.
[121] S.K. Alsaiari, S. Patil, M. Alyami, K.O. Alamoudi, F.A. Aleisa, J.S. Merzaban, M. Li, N.M. Khashab, Endosomal Escape and Delivery of CRISPR/Cas9 Genome Editing Machinery Enabled by Nanoscale Zeolitic Imidazolate Framework, J. Am. Chem. Soc. 140 (2018) 143–146, https://doi.org/10.1021/jacs.7b11754.
[122] M. Qiu, Z. Glass, J. Chen, M. Haas, X. Jin, X. Zhao, X. Rui, Z. Ye, Y. Li, F. Zhang, Q. Xu, Lipid nanoparticle-mediated codelivery of Cas9 mRNA and single-guide RNA achieves liver-specific in vivo genome editing of Angptl3, Proc. Natl. Acad. Sci. 118 (2021) e2020401118. 10.1073/pnas.2020401118.
[123] G. Chen, A.A. Abdeen, Y. Wang, P.K. Shahi, S. Robertson, R. Xie, M. Suzuki, B.R. Pattnaik, K. Saha, S. Gong, A biodegradable nanocapsule delivers a Cas9 ribonucleoprotein complex for in vivo genome editing, Nat. Nanotechnol. 14 (2019) 974–980, https://doi.org/10.1038/s41565-019-0539-2.
[124] F. Chen, M. Alphonse, Q. Liu, Strategies for nonviral nanoparticle-based delivery of CRISPR/Cas9 therapeutics., Wiley Interdiscip, Rev. Nanomed. Nanobiotechnol. 12 (2020) e1609.
[125] K. Lee, M. Conboy, H.M. Park, F. Jiang, H.J. Kim, M.A. Dewitt, V.A. Mackley, K. Chang, A. Rao, C. Skinner, T. Shobha, M. Mehdipour, H. Liu, W.-C. Huang, F. Lan, N.L. Bray, S. Li, J.E. Corn, K. Kataoka, J.A. Doudna, I. Conboy, N. Murthy, Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA in vivo induces homology-directed DNA repair, Nat. Biomed. Eng. 1 (2017) 889–901, https://doi.org/10.1038/s41551-017-0137-2.
[126] H.-X. Wang, Z. Song, Y.-H. Lao, X. Xu, J. Gong, D. Cheng, S. Chakraborty, J.S. Park, M. Li, D. Huang, L. Yin, J. Cheng, K.W. Leong, Nonviral gene editing via CRISPR/Cas9 delivery by membrane-disruptive and endosomolytic helical polypeptide, Proc. Natl. Acad. Sci. U. S. A. 115 (2018) 4903–4908, https://doi.org/10.1073/pnas.1712963115.
[127] M. Qiu, Y. Li, H. Bloomer, Q. Xu, Developing Biodegradable Lipid Nanoparticles for Intracellular mRNA Delivery and Genome Editing, Acc. Chem. Res. 54 (2021) 4001–4011, https://doi.org/10.1021/acs.accounts.1c00500.
[128] Q. Cheng, T. Wei, L. Farbiak, L.T. Johnson, S.A. Dilliard, D.J. Siegwart, Selective organ targeting (SORT) nanoparticles for tissue-specific mRNA delivery and CRISPR-Cas gene editing, Nat. Nanotechnol. 15 (2020) 313–320, https://doi.org/10.1038/s41565-020-0669-6.
[129] B. Steyer, J. Carlson-Stevermer, N. Angenent-Mari, A. Khalil, T. Harkness, K. Saha, High content analysis platform for optimization of lipid mediated CRISPR-Cas9 delivery strategies in human cells, Acta Biomater. 34 (2016) 143–158, https://doi.org/10.1016/j.actbio.2015.12.036.
[130] J.S. Chin, W.H. Chooi, H. Wang, W. Ong, K.W. Leong, S.Y. Chew, Scaffold-mediated non-viral delivery platform for CRISPR/Cas9-based genome editing, Acta Biomater. 90 (2019) 60–70, https://doi.org/10.1016/j.actbio.2019.04.020.
[131] C.-F. Xu, G.-J. Chen, Y.-L. Luo, Y. Zhang, G. Zhao, Z.-D. Lu, A. Czarna, Z. Gu, J. Wang, Rational designs of in vivo CRISPR-Cas delivery systems, Adv. Drug Deliv. Rev. 168 (2021) 3–29, https://doi.org/10.1016/j.addr.2019.11.005.
[132] J. Neufeld, E. Muenzer, The Mucopolysaccharidosis., in: V. CR, Scriver AL, Beaudet WS, Sly D, Valle B, Childs KW, Kinzler B (Ed.), Metab. Mol. Basis Inherit. Dis., 8 ed., New York, 2001: pp. 3421–52.
[133] R. Penati, F. Fumagalli, V. Calbi, M.E. Bernardo, A. Aiuti, Gene therapy for lysosomal storage disorders: recent advances for metachromatic leukodystrophy and mucopolysaccaridosis I, J. Inherit. Metab. Dis. 40 (2017) 543–554, https://doi.org/10.1007/s10545-017-0052-4.
[134] S.C. Hurt, P.I. Dickson, D.T. Curiel, Mucopolysaccharidoses type I gene therapy, J. Inherit. Metab. Dis. (2021) 1088–1098, https://doi.org/10.1002/jimd.12414.
[135] T. Miki, L. Vazquez, L. Yanuaria, O. Lopez, I.M. Garcia, K. Ohashi, N.S. Rodriguez, Induced Pluripotent Stem Cell Derivation and Ex Vivo Gene Correction Using a Mucopolysaccharidosis Type 1 Disease Mouse Model, Stem Cells Int. (2019 (2019)) 6978303, https://doi.org/10.1155/2019/6978303.
[136] R. Sharma, X.M. Anguela, Y. Doyon, T. Wechsler, R.C. DeKelver, S. Sproul, D.E. Paschon, J.C. Miller, R.J. Davidson, D. Shivak, S. Zhou, J. Rieders, P.D. Gregory, M.C. Holmes, E.J. Rebar, K.A. High, In vivo genome editing of the albumin locus as a platform for protein replacement therapy, Blood 126 (2015) 1777–1784, https://doi.org/10.1182/blood-2014-12-615492.
[137] R. DeKelver, M. Rohde, S. Tom, Y. Santiago, S. Sproul, P.D. Gregory, M.C. Holmes, T. Wechsler, ZFN-mediated genome editing of albumin safe harbor in vivo results in supraphysiological levels of human IDS, IDUA and GBA in mice, Mol. Genet. Metab. 114 (2016) S36, https://doi.org/10.1016/j.ymgme.2014.12.065.
[138] M.P.T. Ernst, M. Broeders, P. Herrero-Hernandez, E. Oussoren, A.T. van der Ploeg, W.W.M.P. Pijnappel, Ready for Repair? Gene Editing Enters the Clinic for the Treatment of Human Disease, Mol. Ther. - Methods Clin. Dev. 18 (2020) 532–557, https://doi.org/10.1016/j.omtm.2020.06.022.
[139] L. Ou, M.J. Przybilla, O. Ahlat, S. Kim, P. Overn, J. Jarnes, M.G. O'Sullivan, C.B. Whitley, A Highly Efficacious PS Gene Editing System Corrects Metabolic and Neurological Complications of Mucopolysaccharidosis Type I, Mol. Ther. 28 (2020) 1442–1454, https://doi.org/10.1016/j.ymthe.2020.03.018.
[140] D. Wang, J. Li, C.-Q. Song, K. Tran, H. Mou, P.-H. Wu, P.W.L. Tai, C.A. Mendonca, L. Ren, B.Y. Wang, Q. Su, D.J. Gessler, P.D. Zamore, W. Xue, G. Gao, Cas9-mediated allelic exchange repairs compound heterozygous recessive mutations in mice, Nat. Biotechnol. 36 (2018) 839–842, https://doi.org/10.1038/nbt.4219.
[141] R.S. Schuh, E.A. Gonzalez, A.M.V. Tavares, B.G. Seolin, L. de S. Elias, L.N.P. Vera, F. Kubaski, E. Poletto, R. Giugliani, H.F. Teixeira, U. Matte, G. Baldo, Neonatal nonviral gene editing with the CRISPR/Cas9 system improves some cardiovascular, respiratory, and bone disease features of the mucopolysaccharidosis I phenotype in mice, Gene Ther. 27 (2020) 74–84. 10.1038/s41434-019-0113-4.
[142] L.N.P. Vera, R.S. Schuh, F.N.S. Fachel, E. Poletto, E. Piovesan, F. Kubaski, E. Couto, B. Brum, G. Rodrigues, H. Souza, R. Giugliani, U. Matte, G. Baldo, H.F. Teixeira, Brain and visceral gene editing of mucopolysaccharidosis I mice by nasal delivery of the CRISPR/Cas9 system, J. Gene Med. 24 (2022) e3410.
[143] J. Muenzer, Overview of the mucopolysaccharidoses., Rheumatology (Oxford). 50 Suppl 5 (2011) v4-12. 10.1093/rheumatology/ker394.
[144] S.A. Khan, S.C. Tomatsu, Mucolipidoses Overview: Past, Present, and Future, Int. J. Mol. Sci. 21 (2020), https://doi.org/10.3390/ijms21186812.
[145] H.S. Santos, L. Rodrigues, L.N.P. Vera, E. Poletto, E. Filippi-Chiela, L.F.R. Dos Santos Bruschi, R. Schuh, G. Baldo, I.S.G. Therapy, Curr. Gene Ther. (2021), https://doi.org/10.2174/1566523221666210504103323.
[146] R.S. Schuh, J. Bidone, E. Poletto, C.V. Pinheiro, G. Pasqualim, T.G. de Carvalho, M. Farinon, D. da Silva Diel, R.M. Xavier, G. Baldo, U. Matte, H.F. Teixeira, Nasal Administration of Cationic Nanoemulsions as Nucleic Acids Delivery Systems Aiming at Mucopolysaccharidosis Type I Gene Therapy, Pharm. Res. 35 (2018) 221, https://doi.org/10.1007/s11095-018-2503-5.

[147] Z. Wang, G. Xiong, W.C. Tsang, A.G. Schätzlein, I.F. Uchegbu, Nose-to-brain delivery, J. Pharmacol. Exp. Ther. (2019), https://doi.org/10.1124/jpet.119.258152.
[148] L.N.P. Vera, G. Baldo, The potential of gene therapy for mucopolysaccharidosis type I, Expert Opin. Orphan Drugs. (2020), https://doi.org/10.1080/21678707.2020.1715208.
[149] C.L. Christensen, R.E. Ashmead, F.Y.M. Choy, Cell and Gene Therapies for Mucopolysaccharidoses: Base Editing and Therapeutic Delivery to the CNS., Dis. (Basel, Switzerland). 7 (2019). 10.3390/diseases7030047.
[150] S. Tomatsu, C.J. Alméciga-Díaz, A.M. Montaño, H. Yabe, A. Tanaka, V.C. Dung, R. Giugliani, F. Kubaski, R.W. Mason, E. Yasuda, K. Sawamoto, W. Mackenzie, Y. Suzuki, K.E. Orii, L.A. Barrera, W.S. Sly, T. Orii, Therapies for the bone in mucopolysaccharidoses, Mol. Genet. Metab. 114 (2015) 94–109, https://doi.org/10.1016/j.ymgme.2014.12.001.
[151] A.L.K. Derrick-Roberts, K. Panir, C.E. Pyragius, K.H. Zarrinkalam, G.J. Atkins, S. Byers, Reversal of established bone pathology in MPS VII mice following lentiviral-mediated gene therapy, Mol. Genet. Metab. 119 (2016) 249–257, https://doi.org/10.1016/j.ymgme.2016.09.003.
[152] K. Sawamoto, S. Karumuthil-Melethil, S. Khan, M. Stapleton, J.T. Bruder, O. Danos, S. Tomatsu, Liver-Targeted AAV8 Gene Therapy Ameliorates Skeletal and Cardiovascular Pathology in a Mucopolysaccharidosis IVA Murine Model, Mol. Ther. Methods Clin. Dev. 18 (2020) 50–61, https://doi.org/10.1016/j.omtm.2020.05.015.
[153] M. Wada, Y. Shimada, S. Iizuka, N. Ishii, H. Hiraki, T. Tachibana, K. Maeda, M. Saito, S. Arakawa, T. Ishimoto, T. Nakano, H. Ida, T. Ohashi, H. Kobayashi, Ex Vivo Gene Therapy Treats Bone Complications of Mucopolysaccharidosis Type II Mouse Models through Bone Remodeling Reactivation, Mol. Ther. - Methods Clin. Dev. 19 (2020) 261–274, https://doi.org/10.1016/j.omtm.2020.09.012.
[154] R. Schuh, G. Baldo, H. Teixeira, Nanotechnology applied to treatment of mucopolysaccharidoses, Expert Opin. Drug Deliv. 13 (2016) 1709–1718, https://doi.org/10.1080/17425247.2016.1202235.
[155] J. Bidone, R.S. Schuh, M. Farinon, É. Poletto, G. Pasqualim, P.G. de Oliveira, M. Fraga, R.M. Xavier, G. Baldo, H.F. Teixeira, U. Matte, Intra-articular nonviral gene therapy in mucopolysaccharidosis I mice, Int. J. Pharm. (2018), https://doi.org/10.1016/j.ijpharm.2018.06.049.
[156] M. Vance, T. Llanga, W. Bennett, K. Woodard, G. Murlidharan, N. Chungfat, A. Asokan, B. Gilger, J. Kurtzberg, R.J. Samulski, M.L. Hirsch, AAV Gene Therapy for MPS1-associated Corneal Blindness, Sci. Rep. 6 (2016) 22131, https://doi.org/10.1038/srep22131.
[157] L. Song, J.J. Bower, T. Llanga, J.H. Salmon, M.L. Hirsch, B.C. Gilger, Ocular Tolerability and Immune Response to Corneal Intrastromal AAV-IDUA Gene Therapy in New Zealand White Rabbits, Mol. Ther. Methods Clin. Dev. 18 (2020) 24–32, https://doi.org/10.1016/j.omtm.2020.05.014.
[158] K. Miyadera, L. Conatser, T.A. Llanga, K. Carlin, P. O'Donnell, J. Bagel, L. Song, J. Kurtzberg, R.J. Samulski, B. Gilger, M.L. Hirsch, Intrastromal Gene Therapy Prevents and Reverses Advanced Corneal Clouding in a Canine Model of Mucopolysaccharidosis I, Mol. Ther. 28 (2020) 1455–1463, https://doi.org/10.1016/j.ymthe.2020.04.004.
[159] J. Muenzer, C.E. Prada, B. Burton, H.A. Lau, C. Ficicioglu, C.W.P. Foo, S.A. Vaidya, C.B. Whitley, P. Harmatz, CHAMPIONS: A phase 1/2 clinical trial with dose escalation of SB-913 ZFN-mediated in vivo human genome editing for treatment of MPS II (Hunter syndrome), Mol. Genet. Metab. 126 (2019) S104, https://doi.org/10.1016/j.ymgme.2018.12.263.
[160] P. Harmatz, C. Lampe, R. Parini, R. Sharma, E. Leão-Teles, J. Johnson, D. Sivam, Z. Sisic, EMPOWERS: A phase 1/2 clinical trial of SB-318 ZFN-mediated in vivo human genome editing for treatment of MPS I (Hurler syndrome) Paul, Mol. Genet. Metab. 126 (2019) S68, https://doi.org/10.1016/j.ymgme.2018.12.163.
[161] C. Sheridan, Sangamo's landmark genome editing trial gets mixed reception, Nat. Biotechnol. 36 (2018) 907–908, https://doi.org/10.1038/nbt1018-907.
[162] J.D. Gillmore, E. Gane, J. Taubel, J. Kao, M. Fontana, M.L. Maitland, J. Seitzer, D. O'Connell, K.R. Walsh, K. Wood, J. Phillips, Y. Xu, A. Amaral, A.P. Boyd, J.E. Cehelsky, M.D. McKee, A. Schiermeier, O. Harari, A. Murphy, C.A. Kyratsous, B. Zambrowicz, R. Soltys, D.E. Gutstein, J. Leonard, L. Sepp-Lorenzino, D. Lebwohl, CRISPR-Cas9 In Vivo Gene Editing for Transthyretin Amyloidosis, N. Engl. J. Med. 385 (2021) 493–502, https://doi.org/10.1056/nejmoa2107454.
[163] M. Carvalho, B. Sepodes, A.P. Martins, Regulatory and scientific advancements in gene therapy: State-of-the-art of clinical applications and of the supporting european regulatory framework, Front. Med. 4 (2017), https://doi.org/10.3389/fmed.2017.00182.
[164] J.R. Mendell, S.A. Al-Zaidy, L.R. Rodino-Klapac, K. Goodspeed, S.J. Gray, C.N. Kay, S.L. Boye, S.E. Boye, L.A. George, S. Salabarria, M. Corti, B.J. Byrne, J.P. Tremblay, Current Clinical Applications of In Vivo Gene Therapy with AAVs, Mol. Ther. 29 (2021) 464–488, https://doi.org/10.1016/j.ymthe.2020.12.007.
[165] H. Bachtarzi, Ex vivo and in vivo genome editing: A regulatory scientific framework from early development to clinical implementation, Regen. Med. 12 (2017) 1015–1030, https://doi.org/10.2217/rme-2017-0095.
[166] M. Schacker, D. Seimetz, From fiction to science: clinical potentials and regulatory considerations of gene editing, Clin. Transl. Med. 8 (2019), https://doi.org/10.1186/s40169-019-0244-7.
[167] European Parliament, Regulation (EC) No. 1394/2007 of the European Parliament and of the Council of 13 November 2007 on Advanced Therapy Medicinal Products and Amending Directive 2001/83/EC and Regulation /EC) No 726/2004., (2007). https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2007:324:0121:0137:en:PDF (accessed August 31, 2021).
[168] C. Iglesias-López, A. Agustí, M. Obach, A. Vallano, Regulatory framework for advanced therapy medicinal products in Europe and United States, Front. Pharmacol. 10 (2019) 1–14, https://doi.org/10.3389/fphar.2019.00921.
[169] P. Zapolnik, A. Pyrkosz, Gene therapy for mucopolysaccharidosis type ii—a review of the current possibilities, Int. J. Mol. Sci. 22 (2021), https://doi.org/10.3390/ijms22115490.
[170] P.A. Hines, E. Agricola, J. Llinares Garcia, L. O'Dwyer, R. Herold, Therapeutic genome editing: regulatory horizons, Nat. Rev. Drug Discov. (2021) 1–7, https://doi.org/10.1038/d41573-021-00130-7.
[171] C. Iglesias-Lopez, M. Obach, A. Vallano, A. Agustí, Comparison of regulatory pathways for the approval of advanced therapies in the European Union and the United States, Cytotherapy. 23 (2021) 261–274, https://doi.org/10.1016/j.jcyt.2020.11.008.
[172] M. López-Paniagua, A. de la Mata, S. Galindo, F. Blázquez, M. Calonge, T. Nieto-Miguel, Advanced therapy medicinal products for the eye: Definitions and regulatory framework, Pharmaceutics. 13 (2021) 1–18, https://doi.org/10.3390/pharmaceutics13030347.
[173] C. Pimenta, V. Bettiol, T. Alencar-Silva, O.L. Franco, R. Pogue, J.L. Carvalho, M.S. S. Felipe, Advanced Therapies and Regulatory Framework in Different Areas of the Globe: Past, Present, and Future, Clin. Ther. 43 (2021) e103–e138, https://doi.org/10.1016/j.clinthera.2021.02.006.
[174] G. Shim, D. Kim, G.T. Park, H. Jin, S.K. Suh, Y.K. Oh, Therapeutic gene editing: Delivery and regulatory perspectives, Acta Pharmacol. Sin. 38 (2017) 738–753, https://doi.org/10.1038/aps.2017.2.
[175] T. Cathomen, S. Schüle, M. Schüßler-Lenz, M. Abou-El-Enein, The Human Genome Editing Race: Loosening Regulatory Standards for Commercial Advantage?, Trends Biotechnol 37 (2019) 120–123, https://doi.org/10.1016/j.tibtech.2018.06.005.
[176] A. Hendel, E.J. Fine, G. Bao, M.H. Porteus, Quantifying on- and off-target genome editing, Trends Biotechnol. 33 (2015) 132–140, https://doi.org/10.1016/j.tibtech.2014.12.001.
[177] D.B. Kohn, M.H. Porteus, A.M. Scharenberg, Ethical and regulatory aspects of genome editing, Blood 127 (2016) 2553–2560, https://doi.org/10.1182/blood-2016-01-678136.
[178] J.A. Doudna, The promise and challenge of therapeutic genome editing, Nature 578 (2020) 229–236, https://doi.org/10.1038/s41586-020-1978-5.
[179] L. Barkholt, C. Voltz-Girolt, J. Raine, T. Salmonson, M. Schüssler-Lenz, European regulatory experience with advanced therapy medicinal products, Nat. Rev. Drug Discov. 18 (2018) 8–9, https://doi.org/10.1038/nrd.2018.200.