How to Cite:
Alhabib, N. A., Almaniea, M. A., Alehaidib, S. M., Almansour, M. A., Aldosary, M. S., Almegbel, M. T., Alsubaie, S. A., & Alzahrani, A. M. (2022). Advanced drug delivery systems for enhancing the efficacy of RNA-based therapeutics. *International Journal of Health Sciences*, *6*(S10), 1659–1683. https://doi.org/10.53730/ijhs.v6nS10.15029

Advanced drug delivery systems for enhancing the efficacy of RNA-based therapeutics

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***Abstract*---**Background: RNA-based therapeutics, including antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), and messenger RNAs (mRNAs), offer significant promise in treating genetic and acquired diseases by targeting specific RNA sequences, encoding therapeutic proteins, or facilitating genome editing. However, the effective delivery of these RNA therapeutics remains a major challenge due to their large size, negative charge, and susceptibility to degradation. Aim: This review aims to explore advanced drug delivery systems developed to enhance the efficacy of RNA-based therapeutics, focusing on both viral and non-viral methods, and to evaluate the progress and limitations of these systems in clinical applications. Methods: The review synthesizes recent advancements in RNA delivery technologies, including viral vectors, lipid nanoparticles (LNPs), polymer-based nanoparticles, and hybrid systems. It also examines

International Journal of Health Sciences E-ISSN 2550-696X © 2022.
Manuscript submitted: 01 Jan 2022, Manuscript revised: 09 Jan 2022, Accepted for publication: 15 Jan 2022

various targeting strategies such as passive and active targeting to improve the specificity and efficiency of RNA delivery. Results: Significant progress has been made with both viral and non-viral delivery systems. Viral vectors, though effective, face challenges related to immunogenicity and production costs. Non-viral systems, particularly lipid nanoparticles and polymer-based carriers, have shown promising results, with several FDA-approved products demonstrating clinical efficacy. Advances in targeting strategies, including ligand-based and antibody-based methods, have improved the precision of RNA delivery. Conclusion: The development of effective RNA delivery systems is crucial for advancing RNA-based therapeutics. Innovations in delivery vehicles and targeting strategies have led to significant clinical advancements, though challenges remain in optimizing delivery efficiency and minimizing off-target effects. Future research should focus on refining these delivery systems and addressing remaining hurdles to fully realize the potential of RNA-based therapies.

***Keywords*---**RNA therapeutics, delivery systems, lipid nanoparticles, polymer nanoparticles, viral vectors, targeted delivery, mRNA vaccines.

Introduction

RNA therapies have the potential to alter gene expression or produce therapeutic proteins, making them applicable to diseases with well-characterized genetic targets, such as infectious diseases, cancers, immune disorders, and Mendelian conditions (including neurological disorders). Additionally, advancements in genome sequencing, single-cell gene expression analysis, and programmable nucleases are driving the identification of new targets for gene therapies. However, the challenge of manipulating these targets, particularly non-coding DNA and the 85% of the genome that might be undruggable by small molecules [1], is compounded by the need for effective delivery of therapeutic RNA to diseased cells. This Review addresses therapeutic RNA, including antisense oligonucleotides (ASOs) like gapmers, which have DNA nucleotides flanked by RNA [2], small interfering RNAs (siRNAs), and larger RNAs such as messenger RNA (mRNA) (Fig. 1). These RNA therapies work by targeting RNA or proteins, encoding missing or defective proteins, or facilitating DNA or RNA editing. Despite their therapeutic mechanisms, the large size of some RNA therapies, such as mRNAs, their anionic charge, and their susceptibility to RNases present in the bloodstream and tissues complicate efficient cellular entry and function. To overcome these barriers, researchers have developed both viral and non-viral delivery systems designed to protect RNA from degradation, enhance delivery to target cells, and minimize off-target exposure. While viral gene therapies [3] have shown successful clinical outcomes [4,5,6,7,8,9], their effectiveness can be limited by pre-existing immunity [10], viral-induced immunogenicity [11], unintended genomic integration [12], payload size constraints [13], re-dosing challenges, upscaling issues [14], and high production costs. Although some of these limitations are being addressed [15], they have spurred interest in alternative

delivery methods. Advances in synthetic materials for RNA encapsulation, such as polymers, lipids, and lipid nanoparticles (LNPs), have invigorated research into non-viral delivery systems, leading to FDA approvals for subcutaneously administered N-acetylgalactosamine (GalNAc)–siRNA conjugates targeting hepatocytes [16,17,18], intravenously administered LNP-based siRNA drugs targeting hepatocytes [19], and emergency use authorization (EUA) and FDA approval [20] for intramuscularly administered LNP-based mRNA COVID-19 vaccines [21,22]. These approvals suggest that improvements in delivery to non-hepatic tissues, including the central nervous system, eye, and ear, may lead to new therapeutic options. Furthermore, nanoparticle-based delivery systems may offer potential for non-viral DNA delivery, which has been reviewed elsewhere [23].

Therapeutic RNA Payloads

- **Classification and Mechanisms of RNA Drugs:**
    - RNA-based therapeutics are classified by their biochemical mechanisms of action, which dictate the requirements for effective drug delivery (Fig. 1). Oligonucleotide drugs, such as antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs), use endogenous cellular enzymes—RNAse H1 and the RNA-induced silencing complex (RISC), respectively—to facilitate delivery. This approach avoids the need for introducing large enzymes into the system [24].
    - Significant advances have been made in delivering small molecules and macromolecules [24], yet many therapeutic oligonucleotides still require maintenance at high concentrations to achieve gene manipulation [25]. For instance:
        - **Givosiran** is administered subcutaneously at 2.5 mg/kg monthly.
        - **Lumasiran** is given subcutaneously at 3.0 mg/kg monthly for three months, followed by 3 mg/kg every three months.
        - **Inclisiran** involves a single subcutaneous dose of 284 mg on days 1 and 90, and every six months thereafter, with potential for annual dosing with further improvements [25].
- **Advantages and Limitations of Delivery Systems:**
    - DNA nucleases, including CRISPR-based systems, can induce long-term cellular effects even with transient expression [27]. MicroRNAs (miRNAs) recruit RISC to complementary mRNA sequences, facilitating targeted RNA interference. This has led to the development and clinical testing of miRNA mimics and anti-miRNAs. For example:
        - **MRX34**, a double-stranded miRNA-34a mimic delivered via liposomes, was tested in advanced solid tumors [29].
        - **Miravirsen**, an anti-miRNA-122 drug, was evaluated for hepatitis C treatment [30].
        - **RG-101**, an anti-miRNA-122 drug, initially reduced viral load in hepatitis C patients but was discontinued due to hyperbilirubinemia [31,32].

- mRNA drugs represent a versatile therapeutic option for a range of diseases, including vaccines (e.g., COVID-19), protein replacement therapies, and genome editing.

- **siRNA Therapeutics:**
  - siRNA-based gene silencing uses double-stranded RNAs approximately 13 kDa in size to suppress protein translation. This is achieved by recruiting RISC to mRNA through Watson–Crick base pairing, with Ago2 protein cleaving the target mRNA. Other Ago proteins (Ago1, Ago3, Ago4) may facilitate nonspecific mRNA degradation by localizing mRNA to processing (P)-bodies [33,34].
  - siRNAs have been approved by the FDA and EMA for several indications:
    - **Patisiran**: Treats hereditary transthyretin-mediated amyloidosis (hATTR) [19].
    - **Givosiran**: For acute hepatic porphyria [16].
    - **Lumasiran**: For primary hyperoxaluria type 1 [18].
    - **Inclisiran**: For hypercholesterolemia [17].
  - The FDA has also accepted a new drug application for **Vutrisiran**, an investigational RNA interference (RNAi) therapeutic for hATTR amyloidosis with polyneuropathy, following successful Phase III trials [36].
  - The rapid clinical implementation of siRNA is attributed to:
    - The small size of siRNA allowing for solid-phase synthesis with site-specific chemical modifications.
    - Use of RISC, which is endogenous to eukaryotic cells, avoiding the need for large enzyme delivery.
    - siRNA's requirement for only cytoplasmic delivery, which is simpler than nuclear delivery.
- **Antisense Oligonucleotides (ASOs):**
  - ASOs are oligonucleotides with a molecular weight of 6–9 kDa and share manufacturing advantages with siRNAs. They have been FDA-approved for several conditions, including familial hypercholesterolemia [41], hATTR amyloidosis with polyneuropathy [42], certain subtypes of Duchenne muscular dystrophy [43,44], and infantile-onset spinal muscular atrophy [45].
  - ASOs act through three mechanisms:
    - **RNase H1 Activation**: ASOs bind mRNA via Watson–Crick base pairing, recruiting RNase H1 to cleave the target RNA, a process known as gapmer function [46].
    - **Splicing Modulation**: ASOs can interfere with splicing machinery, promoting alternative splicing and increasing target protein expression [47].
    - **Translational Arrest**: ASOs can bind to the translation initiation codon of target mRNA, leading to downregulation of protein expression [48].
  - Chemical modifications of ASOs, such as gapmers with RNA-like and DNA regions, and other modifications like locked nucleic acids, impact their binding affinity and mechanism of action. These modifications can enhance pharmacokinetics, stability, and

immune response [50,51]. ASOs often have a phosphorothioate backbone to aid nuclear transport [52,53].

- **ADAR-Oligonucleotides**: A novel class of ASOs with engineered hairpin domains that recruit the RNA-editing enzyme adenosine deaminase acting on RNA (ADAR) for A-to-I editing. These oligonucleotides, with a molecular weight of 10–35 kDa, bind target mRNA and induce editing, representing an emerging approach for genetic disease treatment [56].

mRNA-Based Therapeutics

- **Overview and Applications of mRNA Therapies:**
  - **Protein Encoding and Therapeutic Functions:** mRNA therapeutics can encode proteins with therapeutic functions. Due to their size, mRNAs are synthesized in vitro and cannot yet be chemically modified with site-specific precision using solid-state synthesis (Fig. 1b) [57]. These mRNAs can serve various roles, including:
    - **Protein Replacement:** Replacing deficient or malfunctioning proteins [57].
    - **Protein Reduction:** Using Cas9-based methods to reduce levels of target proteins [58].
    - **Mutation Repair:** Employing base editing techniques to correct protein mutations at the DNA level [59,60].
- **Clinical Examples and Successes:**
  - **CRISPR-Based mRNA Therapies:** In 2021, a clinical study demonstrated that lipid nanoparticles (LNPs) encapsulating Streptococcus pyogenes Cas9 mRNA and a CRISPR guide RNA achieved an 87% reduction in blood transthyretin (TTR) levels in patients with hereditary transthyretin-mediated amyloidosis (hATTR) [58]. TTR mutations cause hATTR, a condition affecting vitamin A and thyroxine transport.
  - **mRNA Vaccines:** The successful FDA-approved mRNA vaccine against SARS-CoV-2 exemplifies the potential of mRNA-based therapies for viral infections [61,62]. Other clinical efforts include:
    - **Cystic Fibrosis:** Ongoing trials by Translate Bio for mRNA-mediated protein replacement, though improvements in lung function have been limited [63].
    - **Ornithine Transcarbamylase Deficiency:** Trials by Translate Bio were discontinued due to adverse pharmacokinetic and safety profiles [64].
    - **Arcturus Therapeutics:** Initiation of a Phase II trial for an mRNA therapeutic targeting ornithine transcarbamylase deficiency [65].
- **Immunological and Vaccine Applications:**
  - **Autoimmune and Vaccine Development:** mRNA therapies have led to immunological tolerance and potential treatments for autoimmune diseases in animal models, such as experimental autoimmune encephalomyelitis [66]. Conversely, mRNA vaccines aim to induce long-lasting immunity against specific antigens, with

research spanning viruses like Zika, HIV, and influenza, as well as cancers such as melanoma [67–71].

- **Cancer Vaccines:** BNT111, developed by BioNTech, targets a combination of melanoma-associated antigens and has shown partial responses and metastasis shrinkage in Phase I trials [73]. mRNA can also deliver immune checkpoint molecules like OX40L to treat solid tumors, with Moderna's mRNA-2416 showing promise in increasing OX40L expression and pro-inflammatory responses [74].

- **mRNA for Gene Editing and Nucleases:**
  - **Transient Expression and Gene Editing:** mRNA can transiently express nucleases, such as zinc finger nucleases, transcription activator-like nucleases, and CRISPR-Cas system components [75]. This transient expression is advantageous for creating long-lasting gene editing effects while minimizing risks associated with persistent nuclease activity [76,77].
    - **Clinical Trials and Delivery Challenges:** Trials using adeno-associated viral vectors for SaCas9 have been initiated [78]. However, mRNA-based nucleases might be preferred due to the risks of off-target effects and vector integration associated with persistent DNA nucleases [27,79].
  - **Cas Enzyme Improvements:** Cas enzymes can be modified in three primary ways to enhance their therapeutic potential:
    - **Design and Evolution:** Rational design or evolution of Cas enzymes to target diverse DNA sequences [80,81,82].
    - **Nickases and dCas9:** Modification of Cas enzymes to produce nickases or dead Cas9 (dCas9) for targeted applications [82].
    - **Functional Additions:** Fusion of Cas enzymes with domains for transcriptional activation, epigenome editing, base editing, and other modifications [83–89]. Cas12a enzymes, which require shorter guide RNAs and produce staggered cuts, are also noted [90].

- **RNA Nucleases and Delivery:**
  - **RNA Editing and Therapeutics:** RNA nucleases can bind and cleave RNA or be engineered with adenosine deaminase acting on RNA (ADAR) domains for RNA base editing [91–94]. These nucleases are suited for transient gene expression changes and are advantageous for short-term diseases and RNA pathogens [93,95,96].
  - **Delivery Strategies:** Effective delivery of CRISPR therapeutics requires concurrent delivery of Cas proteins and guide RNAs, addressed through various strategies including nanoparticle co-delivery, AAV-mediated sgRNA expression, and pre-complexed ribonucleoproteins [77,96–106]. Compact Cas enzymes like Cas12j (Casϕ), Cas12f, Cas13bt, and Cas13ct are being explored to simplify delivery [109–112].

Synthetic Vehicles for RNA Delivery

Challenges and Requirements for RNA Delivery:

- **Avoiding Clearance and Targeting:** RNA therapeutics must evade clearance by off-target organs, target the correct tissue, and interact with the desired cell type within a complex microenvironment [113].
- **Cellular Uptake and Endosomal Escape:** Successful delivery requires endocytosis and efficient endosomal escape, while minimizing immune responses [113].
- **Modifications and Delivery Vehicles:** Small oligonucleotide RNA therapeutics (e.g., antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), and ADAR-oligonucleotides) can be delivered using conjugates and stable chemical modifications. In contrast, mRNA and DNA-based therapeutics necessitate specialized delivery vehicles [113].

Lipids and Lipid-Based Nanoparticles (LNPs):

- **Key Components and Structures:** LNPs, crucial for drug delivery, are composed of lipids forming micelles, liposomes, or multilayered structures. FDA-approved LNPs for liver delivery of siRNA and mRNA vaccines include cationic or ionizable lipids, cholesterol, helper lipids, and PEG-lipids [19,61,62].
- **Lipid Structures and Delivery:** Variations in lipid structures influence LNP interactions with cells. Libraries of lipid delivery systems have been created using chemistries such as Michael addition, epoxide, and alcohol-based reactions [114,118,119].
- **Preclinical and Clinical Developments:**
    - **Hepatocyte Delivery:** Advances in lipid design reduced the dose required for effective hepatocyte gene silencing from 1.0 mg/kg to 0.002 mg/kg [121,122]. Key lipids include C12-200, cKK-E12, DLin-KC2-DMA, and DLin-MC3-DMA [120–123].
    - **mRNA Delivery:** LNPs have effectively delivered mRNA to the liver in various models. Recent LNPs, like LP0177 and Lipid H, have demonstrated efficacy in both preclinical and clinical studies [59,60]. LNPs used in vaccines and therapeutic trials include components such as DLin-MC3-DMA (Alnylam), SM-102 (Moderna), and ALC-0315 (Pfizer/BioNTech/Acuitas) [19,61,62].
    - **Modifications for Targeting:** Changes in cholesterol, PEG-lipid, or helper lipid structures can alter delivery efficiency and targeting specificity. For instance, modified cholesterol or PEG-lipids have enhanced delivery to specific tissues [124–139].

Polymers and Polymer-Based Nanoparticles:

- **Polymeric Systems:** Various polymers, including polyethylenimine (PEI), poly(l-lysine) (PLL), and poly(beta-amino ester) (PBAE), are utilized for RNA delivery due to their ability to form complexes with RNA [144,145].
    - **PLGA and Cationic Polymers:** PLGA, though commonly used for small molecules, requires modification with cationic groups for RNA delivery. PEI and PLL, which can be toxic in unmodified forms, are often modified for improved efficacy and tolerability [147–155].

- **PBAE Nanoparticles:** PBAEs, designed for better biodegradability and reduced cytotoxicity compared to PEI and PLL, have been used for delivering various RNA types [156–165].
- **Lipid-Polymer Hybrids and Dendrimers:** Lipid-polymer hybrids combine lipids with polymers to enhance stability and delivery. Dendrimers, such as PAMAM, offer another approach with well-defined structures for RNA delivery to various tissues, including the central nervous system [166–172].

Overall, the development of effective RNA delivery systems involves optimizing vehicle components to ensure targeted delivery, efficient cellular uptake, and minimal off-target effects.

Active vs. Passive Tissue Targeting in RNA Delivery

Passive Tissue Targeting:

- **Concept and Mechanism:** Passive targeting, or endogenous targeting, leverages the natural interactions between nanoparticles and serum proteins. This method does not require specific targeting ligands but relies on the adsorption of serum biomolecules onto the nanoparticle surface. This adsorption alters the nanoparticle's surface properties and affects how it interacts with tissues and immune cells [173].
- **Key Factors Influencing Passive Targeting:**
  - **Protein Corona:** When nanoparticles enter the bloodstream, they quickly adsorb proteins, forming a "corona" that modifies their behavior. For instance, apolipoprotein E (ApoE) can be critical for the delivery of certain LNPs to hepatocytes, whereas other LNPs may depend on different serum proteins like LDL or VLDL [178,179].
  - **Nanoparticle Size and Charge:** Size affects the surface area-to-volume ratio, influencing how nanoparticles interact with immune cells and target tissues. Smaller nanoparticles have a higher surface area relative to their volume, which can influence their interaction with biomolecules. Nanoparticle size and charge also affect delivery efficiency and tissue targeting [174,182,183].
  - **Example:** LNPs originally designed for liver delivery have been repurposed for targeting other organs. For example, altering the charge of LNPs has redirected their delivery from the liver to the spleen or lungs [142,125].

Active Tissue Targeting:

- **Concept and Mechanism:** Active targeting involves modifying the delivery system with ligands, antibodies, or aptamers that specifically bind to receptors on target cells. This approach enhances the precision of delivery by using these targeting moieties to direct the therapeutic agent to specific cell types or tissues [173].
- **Types of Active Targeting:**
  - **Ligand-Based Targeting:** Ligands like GalNAc bind to specific receptors (e.g., asialoglycoprotein receptor, ASGPR) on target cells. This method has been employed in FDA-approved drugs such as

givosiran and lumasiran, which use GalNAc–siRNA conjugates for targeted liver delivery [16,18].

- **Antibody-Based Targeting:** Antibodies or antibody fragments can be conjugated to RNA molecules or nanoparticles. For instance, anti-CD71 antibody fragments have been used to deliver siRNA to muscle tissues [197], and monoclonal antibodies have been used for long-term muscle silencing in preclinical models [198].
- **Aptamer-Based Targeting:** RNA aptamers, which fold into specific three-dimensional structures, can bind to receptors on target cells. An example is the use of an anti-PDGFRα RNA aptamer to deliver siRNA targeting STAT3, a key regulator in glioblastoma [196].
- **Nanoparticle Decoration:** mRNA, due to its large size, is often delivered using nanoparticles decorated with antibodies or aptamers. The ASSET platform uses monoclonal antibody-coated LNPs for targeted delivery to specific cell types or subsets [199–201].

Key Examples and Approaches:

- **Cholesterol and Lipid Conjugates:** Cholesterol-functionalized DNA–RNA heteroduplexes have shown promise in crossing the blood-brain barrier [194]. Hydrophobic conjugates have been used for liver delivery, while less hydrophobic conjugates improved delivery to extrahepatic tissues [192,193].
- **LNPs with Specific Antibodies:** LNPs conjugated with antibodies or antibody fragments can be targeted to specific receptors. For example, LNPs decorated with antibodies targeting plasmalemma vesicle-associated protein have been used for lung cell targeting [202].

The Pathway to Clinical RNA Delivery

1. Nanoparticle Discovery Pipeline:

- **Overview:** The discovery pipeline for RNA delivery systems involves several stages of preclinical testing before advancing to clinical trials. This pipeline begins with high-throughput screening of nanoparticles in cell culture, progresses to animal models, and culminates in non-human primate (NHP) studies if initial results are promising [Fig. 5a].
- **High-Throughput Screening:** Initially, thousands of nanoparticles are tested in vitro. Due to the limitations of in vitro models in predicting in vivo outcomes, this stage helps in optimizing nanoparticle traits, but not all nanoparticles will advance to the next stages.
- **In Vivo Testing:**
  - **Mouse Studies:** A smaller subset of nanoparticles is tested in mice to evaluate their in vivo performance. This step often involves testing thousands of nanoparticles, but logistical constraints limit the number of candidates.
  - **Rat and NHP Studies:** Nanoparticles that show promise in mice are then tested in rats and, subsequently, in non-human primates. NHPs are considered the closest model to humans, providing a better prediction of clinical outcomes.

- **Challenges and Improvements:**
    - **Species Variability:** Differences in metabolism, serum lipids, and organ size across species can affect nanoparticle delivery. For example, the liver size relative to body mass differs between mice, rats, and NHPs, which can impact nanoparticle targeting and efficacy [Fig. 5b].
    - **SANDS Approach:** Species-Agnostic Nanoparticle Delivery Screening (SANDS) is a method developed to address these challenges. It involves testing nanoparticles in various models, including mice with humanized livers, to improve predictions of clinical efficacy and safety.

2. Hallmarks of Clinically Relevant Delivery Systems:

- **Scalable Chemistry:** Successful clinical delivery systems are synthesized using scalable, often biodegradable chemistry. For instance, adding ester bonds to lipids can enhance safety and biodegradability [233].
- **Manufacturability:** The delivery system must be chemically simple enough to be manufactured at a large scale, complying with Current Good Manufacturing Practice (CGMP). For example, GalNAc conjugates are manufactured in large batches and conjugated to RNA or ASOs [234].
- **On-Target vs. Off-Target Delivery:** An acceptable ratio of on-target to off-target delivery is crucial. This involves measuring both biodistribution (where the delivery system travels) and functionality (where the payload affects cell function). For effective RNA delivery, the payload must reach its target cell and function correctly within the cell.
- **Dose and Safety:** The therapeutic dose should be much lower than the dose at which toxicity occurs. Non-human primate studies are preferred for assessing RNA toxicity due to their closer physiological resemblance to humans.
- **Consistency and Stability:** The delivery system should maintain consistent activity across batches and be stable during storage and shipping. Techniques such as lyophilization and cryoprotection are used to enhance the stability of mRNA-LNPs [237,238].
- **Re-dosing:** The ability to safely re-dose the RNA drug is important for maintaining therapeutic effects. Successful re-dosing has been demonstrated with siRNA and mRNA therapies, though the optimal dosing intervals need to be determined [19,21].

3. FDA and EMA Approved RNA Therapeutics:

- **GalNAc–siRNA Conjugates:** Drugs like givosiran, lumasiran, and inclisiran utilize GalNAc conjugates to target specific liver receptors. These conjugates have shown efficacy and safety in clinical trials [16,18,17].
- **Other Examples:** Fitusiran and vutrisiran are other GalNAc–siRNA conjugates with positive clinical outcomes. Fitusiran targets antithrombin mRNA to treat hemophilia, while vutrisiran treats hATTR amyloidosis [241,242].
- **Ongoing Trials:** Companies like Arrowhead, Silence Therapeutics, and Dicerna are exploring GalNAc–siRNA conjugates for various diseases, while Ionis Pharmaceuticals is using GalNAc to deliver ASOs [62,243].

The pathway to clinical RNA delivery involves a multi-stage preclinical pipeline, characterized by high-throughput screening, in vivo testing across multiple animal models, and eventual clinical trials. Success in this pipeline requires addressing challenges related to species variability, ensuring scalable and manufacturable delivery systems, and maintaining consistency, safety, and efficacy of the RNA therapeutics [244].

Conclusion

The advancement of RNA-based therapeutics has significantly impacted the treatment landscape for a variety of diseases, ranging from genetic disorders to viral infections. The efficacy of these therapies is highly dependent on overcoming challenges related to the delivery of therapeutic RNA molecules to their target cells. The review highlights the progress made in developing advanced drug delivery systems designed to address these challenges, focusing on both viral and non-viral strategies. Viral vectors have demonstrated substantial clinical success, but face limitations related to immunogenicity, production complexity, and payload capacity. In contrast, non-viral delivery systems, particularly lipid nanoparticles (LNPs) and polymer-based nanoparticles, offer promising alternatives. These systems have been instrumental in the success of several FDA-approved RNA therapeutics, such as mRNA vaccines and siRNA-based drugs. The ability to encapsulate RNA therapeutics within these delivery vehicles has been pivotal in protecting RNA from degradation and enhancing cellular uptake. Targeting strategies have also evolved, with advancements in passive and active targeting approaches improving the precision of RNA delivery. Passive targeting leverages natural interactions between nanoparticles and serum proteins, while active targeting employs specific ligands, antibodies, or aptamers to direct RNA therapeutics to precise cellular targets. These strategies have shown considerable potential in increasing the specificity of RNA therapeutics, thereby reducing off-target effects and improving therapeutic outcomes. Despite these advancements, significant challenges remain, including optimizing delivery efficiency, reducing off-target effects, and addressing the scalability and manufacturability of RNA delivery systems. Ongoing research and development are crucial to overcoming these hurdles and expanding the therapeutic applications of RNA-based technologies. Future efforts should continue to refine delivery systems, enhance targeting precision, and address the remaining limitations to fully leverage the potential of RNA-based therapeutics in clinical practice.

References

1. Hopkins, A. L. & Groom, C. R. The druggable genome. *Nat. Rev. Drug. Discov.* 1, 727–730 (2002).
2. Roberts, T. C., Langer, R. & Wood, M. J. A. Advances in oligonucleotide drug delivery. *Nat. Rev. Drug Discov.* 19, 673–694 (2020).
3. High, K. A. & Roncarolo, M. G. Gene therapy. *N. Engl. J. Med.* 381, 455–464 (2019).
4. Pasi, K. J. et al. Multiyear follow-up of AAV5-hFVIII-SQ gene therapy for hemophilia A. *N. Engl. J. Med.* 382, 29–40 (2020).

5. Mendell, J. R. et al. Single-dose gene-replacement therapy for spinal muscular atrophy. *N. Engl. J. Med.* 377, 1713–1722 (2017).
6. Frangoul, H. et al. CRISPR-Cas9 gene editing for sickle cell disease and β-thalassemia. *N. Engl. J. Med.* 384, 252–260 (2021).
7. Esrick, E. B. et al. Post-transcriptional genetic silencing of BCL11A to treat sickle cell disease. *N. Engl. J. Med.* 384, 205–215 (2021).
8. Kohn, D. B. et al. Autologous ex vivo lentiviral gene therapy for adenosine deaminase deficiency. *N. Engl. J. Med.* 384, 2002–2013 (2021).
9. Russell, S. et al. Efficacy and safety of voretigene neparvovec (AAV2-hRPE65v2) in patients with RPE65-mediated inherited retinal dystrophy: a randomised, controlled, open-label, phase 3 trial. *Lancet* 390, 849–860 (2017).
10. Aronson, S. J. et al. Prevalence and relevance of pre-existing anti-adeno-associated virus immunity in the context of gene therapy for Crigler–Najjar syndrome. *Hum. Gene Ther.* 30, 1297–1305 (2019).
11. Bryson, T. E., Anglin, C. M., Bridges, P. H. & Cottle, R. N. Nuclease-mediated gene therapies for inherited metabolic diseases of the liver. *Yale J. Biol. Med.* 90, 553–566 (2017).
12. Nguyen, G. N. et al. A long-term study of AAV gene therapy in dogs with hemophilia A identifies clonal expansions of transduced liver cells. *Nat. Biotechnol.* 39, 47–55 (2021).
13. Wu, Z., Yang, H. & Colosi, P. Effect of genome size on AAV vector packaging. *Mol. Ther.* 18, 80–86 (2010).
14. Chandler, M., Panigaj, M., Rolband, L. A. & Afonin, K. A. Challenges to optimizing RNA nanostructures for large scale production and controlled therapeutic properties. *Nanomedicine* 15, 1331–1340 (2020).
15. Leborgne, C. et al. IgG-cleaving endopeptidase enables in vivo gene therapy in the presence of anti-AAV neutralizing antibodies. *Nat. Med.* 26, 1096–1101 (2020).
16. Balwani, M. et al. Phase 3 trial of RNAi therapeutic givosiran for acute intermittent porphyria. *N. Engl. J. Med.* 382, 2289–2301 (2020).
17. Ray, K. K. et al. Two phase 3 trials of inclisiran in patients with elevated LDL cholesterol. *N. Engl. J. Med.* 382, 1507–1519 (2020).
18. Garrelfs, S. F. et al. Lumasiran, an RNAi therapeutic for primary hyperoxaluria type 1. *N. Engl. J. Med.* 384, 1216–1226 (2021).
19. Adams, D. et al. Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis. *N. Engl. J. Med.* 379, 11–21 (2018).
20. Parums, D. V. Editorial: first full regulatory approval of a COVID-19 vaccine, the BNT162b2 Pfizer-BioNTech vaccine, and the real-world implications for Public Health Policy. *Med. Sci. Monit.* 27, e934625 (2021).
21. Baden, L. R. et al. Efficacy and safety of the mRNA-1273 SARS-CoV-2 vaccine. *N. Engl. J. Med.* 384, 403–416 (2021).
22. Polack, F. P. et al. Safety and efficacy of the BNT162b2 mRNA Covid-19 vaccine. *N. Engl. J. Med.* 383, 2603–2615 (2020).
23. Buck, J., Grossen, P., Cullis, P. R., Huwyler, J. & Witzigmann, D. Lipid-based DNA therapeutics: hallmarks of non-viral gene delivery. *ACS Nano* 13, 3754–3782 (2019).
24. Vargason, A. M., Anselmo, A. C. & Mitragotri, S. The evolution of commercial drug delivery technologies. *Nat. Biomed. Eng.* 5, 951–967 (2021).

25. Watts, J. K. & Corey, D. R. Silencing disease genes in the laboratory and the clinic. *J. Pathol.* 226, 365–379 (2012).
26. Kosmas, C. E. et al. Inclisiran for the treatment of cardiovascular disease: a short review on the emerging data and therapeutic potential. *Ther. Clin. Risk Manag.* 16, 1031–1037 (2020).
27. Chen, F., Alphonse, M. & Liu, Q. Strategies for nonviral nanoparticle-based delivery of CRISPR/Cas9 therapeutics. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* 12, e1609 (2020).
28. Hanna, J., Hossain, G. S. & Kocerha, J. The potential for microRNA therapeutics and clinical research. *Front. Genet.* 10, 478 (2019).
29. Hong, D. S. et al. Phase 1 study of MRX34, a liposomal miR-34a mimic, in patients with advanced solid tumours. *Br. J. Cancer* 122, 1630–1637 (2020).
30. van der Ree, M. H. et al. Miravirsen dosing in chronic hepatitis C patients results in decreased microRNA-122 levels without affecting other microRNAs in plasma. *Aliment. Pharmacol. Ther.* 43, 102–113 (2016).
31. van der Ree, M. H. et al. Safety, tolerability, and antiviral effect of RG-101 in patients with chronic hepatitis C: a phase 1B, double-blind, randomised controlled trial. *Lancet* 389, 709–717 (2017).
32. Regulus announces pipeline updates and advancements. *Regulus* http://ir.regulusrx.com/news-releases/news-release-details/regulus-announces-pipeline-updates-and-advancements (2017).
33. Wilson, R. C. & Doudna, J. A. Molecular mechanisms of RNA interference. *Annu. Rev.*
34. Liu, J., Valencia-Sanchez, M. A., Hannon, G. J. & Parker, R. MicroRNA-dependent localization of
35. Alnylam announces U.S. Food and Drug Administration acceptance of new drug application for investigational vutrisiran for the treatment of the polyneuropathy of hereditary ATTR amyloidosis. *Alnylam* https://investors.alnylam.com/press-release?id=25811 (2021).
36. HELIOS-A: 9-month results from the phase 3 study of vutrisiran in patients with hereditary transthyretin-mediated amyloidosis with polyneuropathy. *Alnylam* https://www.alnylam.com/wp-content/uploads/2021/04/Adams_HELIOS-A-9-Month-Results.pdf (2021).
37. Fire, A. et al. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811 (1998).
38. Adachi, H., Hengesbach, M., Yu, Y. T. & Morais, P. From antisense RNA to RNA modification: therapeutic potential of RNA-based technologies. *Biomedicines* 9, 550 (2021).
39. Humphreys, S. C. et al. Emerging siRNA design principles and consequences for biotransformation and disposition in drug development. *J. Med. Chem.* 63, 6407–6422 (2020).
40. Evers, M. M., Toonen, L. J. & van Roon-Mom, W. M. Antisense oligonucleotides in therapy for neurodegenerative disorders. *Adv. Drug Deliv. Rev.* 87, 90–103 (2015).
41. Santos, R. D. et al. Mipomersen, an antisense oligonucleotide to apolipoprotein B-100, reduces lipoprotein(a) in various populations with hypercholesterolemia: results of 4 phase III trials. *Arterioscler. Thromb. Vasc. Biol.* 35, 689–699 (2015).

42. Benson, M. D. et al. Inotersen treatment for patients with hereditary transthyretin amyloidosis. *N. Engl. J. Med.* 379, 22–31 (2018).
43. Lim, K. R., Maruyama, R. & Yokota, T. Eteplirsen in the treatment of Duchenne muscular dystrophy. *Drug Des. Devel Ther.* 11, 533–545 (2017).
44. Frank, D. E. et al. Increased dystrophin production with golodirsen in patients with Duchenne muscular dystrophy. *Neurology* 94, e2270–e2282 (2020).
45. Finkel, R. S. et al. Nusinersen versus sham control in infantile-onset spinal muscular atrophy. *N. Engl. J. Med.* 377, 1723–1732 (2017).
46. Crooke, S. T. Molecular mechanisms of antisense oligonucleotides. *Nucleic Acid Ther.* 27, 70–77 (2017).
47. Lim, K. H. et al. Antisense oligonucleotide modulation of non-productive alternative splicing upregulates gene expression. *Nat. Commun.* 11, 3501 (2020).
48. Kilanowska, A. & Studzińska, S. In vivo and in vitro studies of antisense oligonucleotides — a review. *RSC Adv.* 10, 34501–34516 (2020).
49. Bennett, C. F., Baker, B. F., Pham, N., Swayze, E. & Geary, R. S. Pharmacology of antisense drugs. *Annu. Rev. Pharmacol. Toxicol.* 57, 81–105 (2017).
50. Burdick, A. D. et al. Sequence motifs associated with hepatotoxicity of locked nucleic acid — modified antisense oligonucleotides. *Nucleic Acids Res.* 42, 4882–4891 (2014).
51. Yamamoto, T. et al. Highly potent GalNAc-conjugated tiny LNA anti-miRNA-122 antisense oligonucleotides. *Pharmaceutics* 13, 817 (2021).
52. Shen, W. et al. Chemical modification of PS-ASO therapeutics reduces cellular protein-binding and improves the therapeutic index. *Nat. Biotechnol.* 37, 640–650 (2019).
53. Miller, C. M. et al. Stabilin-1 and stabilin-2 are specific receptors for the cellular internalization of phosphorothioate-modified antisense oligonucleotides (ASOs) in the liver. *Nucleic Acids Res.* 44, 2782–2794 (2016).
54. Merkle, T. et al. Precise RNA editing by recruiting endogenous ADARs with antisense oligonucleotides. *Nat. Biotechnol.* 37, 133–138 (2019).
55. Qu, L. et al. Programmable RNA editing by recruiting endogenous ADAR using engineered RNAs. *Nat. Biotechnol.* 37, 1059–1069 (2019).
56. Aquino-Jarquin, G. Novel engineered programmable systems for ADAR-mediated RNA editing. *Mol. Ther. Nucleic Acids* 19, 1065–1072 (2020).
57. Da Silva Sanchez, A., Paunovska, K., Cristian, A. & Dahlman, J. E. Treating cystic fibrosis with mRNA and CRISPR. *Hum. Gene Ther.* 31, 940–955 (2020).
58. Gillmore, J. D. et al. CRISPR-Cas9 in vivo gene editing for transthyretin amyloidosis. *N. Engl. J. Med.* 385, 493–502 (2021).
59. Musunuru, K. et al. In vivo CRISPR base editing of PCSK9 durably lowers cholesterol in primates. *Nature* 593, 429–434 (2021).
60. Rothgangl, T. et al. In vivo adenine base editing of PCSK9 in macaques reduces LDL cholesterol levels. *Nat. Biotechnol.* 39, 949–957 (2021).
61. Thompson, M. G. et al. Interim estimates of vaccine effectiveness of BNT162b2 and mRNA-1273 COVID-19 vaccines in preventing SARS-CoV-2 infection among health care personnel, first responders, and other essential and frontline workers — eight U.S. locations, December 2020-March 2021. *MMWR* 70, 495–500 (2021).

62. Dobrowolski, C., Paunovska, K., Hatit, M. Z. C., Lokugamage, M. P. & Dahlman, J. E. Therapeutic RNA delivery for COVID and other diseases. *Adv. Health. Mater.* 10, e2002022 (2021).
63. Translate Bio announces results from second interim data analysis from ongoing phase 1/2 clinical trial of MRT5005 in patients with cystic fibrosis (CF). *Translate Bio* https://investors.translate.bio/news-releases/news-release-details/translate-bio-announces-results-second-interim-data-analysis (2021).
64. Translate Bio announces pipeline program update. *Translate Bio* https://investors.translate.bio/news-releases/news-release-details/translate-bio-announces-pipeline-program-update (2021).
65. Arcturus Therapeutics announces first quarter 2021 company overview and financial results and provides new clinical data. *Arcturus Therapeutics* https://ir.arcturusrx.com/news-releases/news-release-details/arcturus-therapeutics-announces-first-quarter-2021-company (2021).
66. Krienke, C. et al. A noninflammatory mRNA vaccine for treatment of experimental autoimmune encephalomyelitis. *Science* 371, 145–153 (2021).
67. Pardi, N., Hogan, M. J., Porter, F. W. & Weissman, D. mRNA vaccines — a new era in vaccinology. *Nat. Rev. Drug Discov.* 17, 261–279 (2018).
68. Luisi, K. et al. Development of a potent Zika virus vaccine using self-amplifying messenger RNA. *Sci. Adv.* 6, eaba5068 (2020).
69. Leal, L. et al. Phase I clinical trial of an intranodally administered mRNA-based therapeutic vaccine against HIV-1 infection. *AIDS* 32, 2533–2545 (2018).
70. Feldman, R. A. et al. mRNA vaccines against H10N8 and H7N9 influenza viruses of pandemic potential are immunogenic and well tolerated in healthy adults in phase 1 randomized clinical trials. *Vaccine* 37, 3326–3334 (2019).
71. Sahin, U. et al. Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. *Nature* 547, 222–226 (2017).
72. Conry, R. M. et al. Characterization of a messenger RNA polynucleotide vaccine vector. *Cancer Res.* 55, 1397–1400 (1995).
73. Sahin, U. et al. An RNA vaccine drives immunity in checkpoint-inhibitor-treated melanoma. *Nature* 585, 107–112 (2020).
74. Jimeno, A. et al. Abstract CT032: A phase 1/2, open-label, multicenter, dose escalation and efficacy study of mRNA-2416, a lipid nanoparticle encapsulated mRNA encoding human OX40L, for intratumoral injection alone or in combination with durvalumab for patients with advanced malignancies. *Cancer Res.* 80, CT032 (2020).
75. Zhang, H. X., Zhang, Y. & Yin, H. Genome editing with mRNA encoding ZFN, TALEN, and Cas9. *Mol. Ther.* 27, 735–746 (2019).
76. Pardi, N. et al. Expression kinetics of nucleoside-modified mRNA delivered in lipid nanoparticles to mice by various routes. *J. Controlled Rel.* 217, 345–351 (2015).
77. Finn, J. D. et al. A single administration of CRISPR/Cas9 lipid nanoparticles achieves robust and persistent in vivo genome editing. *Cell Rep.* 22, 2227–2235 (2018).
78. Allergan and Editas Medicine announce dosing of first patient in landmark phase 1/2 clinical trial of CRISPR medicine AGN-151587 (EDIT-101) for the treatment of LCA10. *Editas Medicine* https://ir.editasmedicine.com/news-

releases/news-release-details/allergan-and-editas-medicine-announce-dosing-first-patient (2020).
79. Hanlon, K. S. et al. High levels of AAV vector integration into CRISPR-induced DNA breaks. *Nat. Commun.* 10, 4439 (2019).
80. Jiang, F. & Doudna, J. A. CRISPR-Cas9 structures and mechanisms. *Annu. Rev. Biophys.* 46,
81. Slaymaker, I. M. et al. Rationally engineered Cas9 nucleases with improved specificity. *Science* 351, 84–88 (2016).
82. Kleinstiver, B. P. et al. Engineered CRISPR-Cas9 nucleases with altered PAM
83. Gilbert, L. A. et al. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* 154, 442–451 (2013).
84. Thakore, P. I., Black, J. B., Hilton, I. B. & Gersbach, C. A. Editing the epigenome: technologies for programmable transcription and epigenetic modulation. *Nat. Methods* 13, 127–137 (2016).
85. Nuñez, J. K. et al. Genome-wide programmable transcriptional memory by CRISPR-based epigenome editing. *Cell* 184, 2503–2519.e2517 (2021).
86. Porto, E. M., Komor, A. C., Slaymaker, I. M. & Yeo, G. W. Base editing: advances and therapeutic opportunities. *Nat. Rev. Drug Discov.* 19, 839–859 (2020).
87. Mok, B. Y. et al. A bacterial cytidine deaminase toxin enables CRISPR-free mitochondrial base editing. *Nature* 583, 631–637 (2020).
88. Anzalone, A. V. et al. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* 576, 149–157 (2019).
89. Saito, M. et al. Dual modes of CRISPR-associated transposon homing. *Cell* 9, 2441–2453.e18 (2021).
90. Zetsche, B. et al. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* 163, 759–771 (2015).
91. Abudayyeh, O. O. et al. C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. *Science* 353, aaf5573 (2016).
92. Özcan, A. et al. Programmable RNA targeting with the single-protein CRISPR effector Cas7-11. *Nature* 597, 720–725 (2021).
93. Cox, D. B. T. et al. RNA editing with CRISPR-Cas13. *Science* 358, 1019–1027 (2017).
94. Abudayyeh, O. O. et al. A cytosine deaminase for programmable single-base RNA editing. *Science* 365, 382–386 (2019).
95. Abbott, T. R. et al. Development of CRISPR as an antiviral strategy to combat SARS-CoV-2 and influenza. *Cell* 181, 865–876.e812 (2020).
96. Blanchard, E. L. et al. Treatment of influenza and SARS-CoV-2 infections via mRNA-encoded Cas13a in rodents. *Nat. Biotechnol.* 39, 717–726 (2021).
97. Miller, J. B. et al. 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. Edn Engl.* 56, 1059–1063 (2017).
98. Jiang, C. et al. A non-viral CRISPR/Cas9 delivery system for therapeutically targeting HBV DNA and pcsk9 in vivo. *Cell Res.* 27, 440–443 (2017).
99. Sago, C. D. et al. High-throughput in vivo screen of functional mRNA delivery identifies nanoparticles for endothelial cell gene editing. *Proc. Natl Acad. Sci. USA* 115, E9944–E9952 (2018).
100. Yin, H. et al. Structure-guided chemical modification of guide RNA enables potent non-viral in vivo genome editing. *Nat. Biotechnol.* 35, 1179–1187 (2017).

101. Cheng, Q. et al. Selective organ targeting (SORT) nanoparticles for tissue-specific mRNA delivery and CRISPR-Cas gene editing. *Nat. Nanotechnol.* 15, 313–320 (2020).
102. Rosenblum, D. et al. CRISPR-Cas9 genome editing using targeted lipid nanoparticles for cancer therapy. *Sci. Adv.* 6, eabc9450 (2020).
103. Zhang, X. et al. Functionalized lipid-like nanoparticles for in vivo mRNA delivery and base editing. *Sci. Adv.* 6, eabc2315 (2020).
104. Qiu, M. et al. Lipid nanoparticle-mediated codelivery of Cas9 mRNA and single-guide RNA achieves liver-specific in vivo genome editing of Angptl3. *Proc. Natl Acad. Sci. USA* 118, e2020401118 (2021).
105. Yin, H. et al. Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo. *Nat. Biotechnol.* 34, 328–333 (2016).
106. Lee, B. et al. Nanoparticle delivery of CRISPR into the brain rescues a mouse model of fragile X syndrome from exaggerated repetitive behaviours. *Nat. Biomed. Eng.* 2, 497–507 (2018).
107. Lee, K. et al. Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA in vivo induces homology-directed DNA repair. *Nat. Biomed. Eng.* 1, 889–901 (2017).
108. Wei, T., Cheng, Q., Min, Y.-L., Olson, E. N. & Siegwart, D. J. Systemic nanoparticle delivery of CRISPR-Cas9 ribonucleoproteins for effective tissue specific genome editing. *Nat. Commun.* 11, 3232 (2020).
109. Pausch, P. et al. CRISPR-CasΦ from huge phages is a hypercompact genome editor. *Science* 369, 333–337 (2020).
110. Kim, D. Y. et al. Efficient CRISPR editing with a hypercompact Cas12f1 and engineered guide RNAs delivered by adeno-associated virus. *Nat. Biotechnol.* https://doi.org/10.1038/s41587-021-01009-z (2021).
111. Xu, X. et al. Engineered miniature CRISPR-Cas system for mammalian genome regulation and editing. *Mol. Cell* 81, 4333–4345.e4 (2021).
112. Kannan, S. et al. Compact RNA editors with small Cas13 proteins. *Nat. Biotechnol.* https://doi.org/10.1038/s41587-021-01030-2 (2021).
113. Cheng, C. J., Tietjen, G. T., Saucier-Sawyer, J. K. & Saltzman, W. M. A holistic approach to targeting disease with polymeric nanoparticles. *Nat. Rev. Drug Discov.* 14, 239–247 (2015).
114. Israelachvili, J. N., Mitchell, D. J. & Ninham, B. W. Theory of self-assembly of lipid bilayers and vesicles. *Biochim. Biophys. Acta* 470, 185–201 (1977).
115. Kulkarni, J. A. et al. On the formation and morphology of lipid nanoparticles containing ionizable cationic lipids and siRNA. *ACS Nano* 12, 4787–4795 (2018).
116. Herrera, M., Kim, J., Eygeris, Y., Jozic, A. & Sahay, G. Illuminating endosomal escape of polymorphic lipid nanoparticles that boost mRNA delivery. *Biomater. Sci.* 9, 4289–4300 (2021).
117. Semple, S. C. et al. Rational design of cationic lipids for siRNA delivery. *Nat. Biotechnol.* 28, 172–176 (2010).
118. Altınoglu, S., Wang, M. & Xu, Q. Combinatorial library strategies for synthesis of cationic lipid-like nanoparticles and their potential medical applications. *Nanomedicine* 10, 643–657 (2015).
119. Zhang, Y., Sun, C., Wang, C., Jankovic, K. E. & Dong, Y. Lipids and lipid derivatives for RNA delivery. *Chem. Rev.* 121, 12181–12277 (2021).

120. Love, K. T. et al. Lipid-like materials for low-dose, in vivo gene silencing. *Proc. Natl Acad. Sci. USA* 107, 1864–1869 (2010).
121. Zimmermann, T. S. et al. RNAi-mediated gene silencing in non-human primates. *Nature* 441, 111–114 (2006).
122. Dong, Y. et al. Lipopeptide nanoparticles for potent and selective siRNA delivery in rodents and nonhuman primates. *Proc. Natl Acad. Sci. USA* 111, 3955–3960 (2014).
123. Jayaraman, M. et al. Maximizing the potency of siRNA lipid nanoparticles for hepatic gene silencing in vivo. *Angew. Chem. Int. Edn Engl.* 51, 8529–8533 (2012).
124. Paunovska, K. et al. Nanoparticles containing oxidized cholesterol deliver mRNA to the liver microenvironment at clinically relevant doses. *Adv. Mater.* 31, 1807748 (2019).
125. Kauffman, K. J. et al. Rapid, single-cell analysis and discovery of vectored mRNA transfection in vivo with a loxP-flanked tdtomato reporter mouse. molecular therapy. *Nucleic Acids* 10, 55–63 (2018).
126. Kauffman, K. J. et al. Optimization of lipid nanoparticle formulations for mRNA delivery in vivo with fractional factorial and definitive screening designs. *Nano Lett.* 15, 7300–7306 (2015).
127. Sedic, M. et al. Safety evaluation of lipid nanoparticle-formulated modified mRNA in the Sprague–Dawley rat and cynomolgus monkey. *Vet. Pathol.* 55, 341–354 (2018).
128. ModernaTx. Compounds and compositions for intracellular delivery of therapeutic agents. US patent US20170210697A1 (2021).
129. Sabatine, M. S. et al. Evolocumab and clinical outcomes in patients with cardiovascular disease. *N. Engl. J. Med.* 376, 1713–1722 (2017).
130. Beam Therapeutics announces updated preclinical data highlighting optimized LNP delivery approaches for in vivo base editing to the liver and other tissues. *Beam Therapeutics* https://investors.beamtx.com/news-releases/news-release-details/beam-therapeutics-announces-updated-preclinical-data (2021).
131. Kulkarni, J. A., Cullis, P. R. & van der Meel, R. Lipid nanoparticles enabling gene therapies: from concepts to clinical utility. *Nucleic Acid. Ther.* 28, 146–157 (2018).
132. Cheng, X. & Lee, R. J. The role of helper lipids in lipid nanoparticles (LNPs) designed for oligonucleotide delivery. *Adv. Drug Deliv. Rev.* 99, 129–137 (2016).
133. Dahlman, J. E. et al. In vivo endothelial siRNA delivery using polymeric nanoparticles with low molecular weight. *Nat. Nano* 9, 648–655 (2014).
134. Khan, O. F. et al. Endothelial siRNA delivery in nonhuman primates using ionizable low-molecular weight polymeric nanoparticles. *Sci. Adv.* 4, eaar8409 (2018).
135. Sago, C. D. et al. Nanoparticles that deliver RNA to bone marrow identified by in vivo directed evolution. *J. Am. Chem. Soc.* 140, 17095–17105 (2018).
136. Paunovska, K. et al. Analyzing 2000 in vivo drug delivery data points reveals cholesterol structure impacts nanoparticle delivery. *ACS Nano* 12, 8341–8349 (2018).
137. Lokugamage, M. P. et al. Optimization of lipid nanoparticles for the delivery of nebulized therapeutic mRNA to the lungs. *Nat. Biomed. Eng.* 5, 1059–1068 (2021).

138. Mui, B. L. et al. Influence of polyethylene glycol lipid desorption rates on pharmacokinetics and pharmacodynamics of siRNA lipid nanoparticles. *Mol. Ther. Nucleic acids* 2, e139 (2013).
139. Ryals, R. C. et al. The effects of PEGylation on LNP based mRNA delivery to the eye. *PLoS ONE* 15, e0241006 (2020).
140. Suk, J. S., Xu, Q., Kim, N., Hanes, J. & Ensign, L. M. PEGylation as a strategy for improving nanoparticle-based drug and gene delivery. *Adv. Drug Deliv. Rev.* 99, 28–51 (2016).
141. Eygeris, Y., Patel, S., Jozic, A. & Sahay, G. Deconvoluting lipid nanoparticle structure for messenger RNA delivery. *Nano Lett.* 20, 4543–4549 (2020).
142. Kranz, L. M. et al. Systemic RNA delivery to dendritic cells exploits antiviral defence for cancer immunotherapy. *Nature* 534, 396–401 (2016).
143. Intellia Therapeutics presents preclinical proof of concept for CRISPR-based in vivo editing of bone marrow at Keystone eSymposium. *Intellia Therapeutics* https://ir.intelliatx.com/news-releases/news-release-details/intellia-therapeutics-presents-preclinical-proof-concept-crispr (2021).
144. Rai, R., Alwani, S. & Badea, I. Polymeric nanoparticles in gene therapy: new avenues of design and optimization for delivery applications. *Polymers* 11, 745 (2019).
145. Kamaly, N., Yameen, B., Wu, J. & Farokhzad, O. C. Degradable controlled-release polymers and polymeric nanoparticles: mechanisms of controlling drug release. *Chem. Rev.* 116, 2602–2663 (2016).
146. Crucho, C. I. C. & Barros, M. T. Polymeric nanoparticles: A study on the preparation variables and characterization methods. *Mater. Sci. Eng. C* 80, 771–784 (2017).
147. Zhong, H., Chan, G., Hu, Y., Hu, H. & Ouyang, D. A comprehensive map of FDA-approved pharmaceutical products. *Pharmaceutics* 10, 263 (2018).
148. Xiao, B. et al. Combination therapy for ulcerative colitis: orally targeted nanoparticles prevent mucosal damage and relieve inflammation. *Theranostics* 6, 2250–2266 (2016).
149. Harada-Shiba, M. et al. Polyion complex micelles as vectors in gene therapy — pharmacokinetics and in vivo gene transfer. *Gene Ther.* 9, 407–414 (2002).
150. Ewe, A. et al. Optimized polyethylenimine (PEI)-based nanoparticles for siRNA delivery, analyzed in vitro and in an ex vivo tumor tissue slice culture model. *Drug Deliv. Transl. Res.* 7, 206–216 (2017).
151. Gao, X. et al. The association of autophagy with polyethylenimine-induced cytotoxicity in nephritic and hepatic cell lines. *Biomaterials* 32, 8613–8625 (2011).
152. Breunig, M., Lungwitz, U., Liebl, R. & Goepferich, A. Breaking up the correlation between efficacy and toxicity for nonviral gene delivery. *Proc. Natl Acad. Sci. USA* 104, 14454–14459 (2007).
153. Ke, X. et al. Surface-functionalized PEGylated nanoparticles deliver messenger RNA to pulmonary immune cells. *ACS Appl. Mater. Interf.* 12, 35835–35844 (2020).
154. Tan, L. et al. Optimization of an mRNA vaccine assisted with cyclodextrin–polyethyleneimine conjugates. *Drug. Deliv. Transl. Res.* 10, 678–689 (2020).
155. Xiang, J. J. et al. IONP-PLL: a novel non-viral vector for efficient gene delivery. *J. Gene Med.* 5, 803–817 (2003).

156. Yin, H. et al. Non-viral vectors for gene-based therapy. *Nat. Rev. Genet.* 15, 541–555 (2014).
157. Choi, J. et al. Nonviral polymeric nanoparticles for gene therapy in pediatric CNS malignancies. *Nanomedicine* 23, 102115 (2020).
158. Akinc, A., Lynn, D. M., Anderson, D. G. & Langer, R. Parallel synthesis and biophysical characterization of a degradable polymer library for gene delivery. *J. Am. Chem. Soc.* 125, 5316–
159. Green, J. J., Langer, R. & Anderson, D. G. A combinatorial polymer library approach yields insight into nonviral gene delivery. *Acc. Chem. Res.* 41, 749–759 (2008).
160. Vandenbroucke, R. E. et al. Prolonged gene silencing in hepatoma cells and primary hepatocytes after small interfering RNA delivery with biodegradable poly(beta-amino esters). *J. Gene Med.* 10, 783–794 (2008).
161. Anderson, D. G., Lynn, D. M. & Langer, R. Semi-automated synthesis and screening of a large library of degradable cationic polymers for gene delivery. *Angew. Chem. Int. Edn Engl.* 42, 3153–3158 (2003).
162. Anderson, D. G., Akinc, A., Hossain, N. & Langer, R. Structure/property studies of polymeric gene delivery using a library of poly(beta-amino esters). *Mol. Ther.* 11, 426–434 (2005).
163. Mastorakos, P. et al. Highly compacted biodegradable DNA nanoparticles capable of overcoming the mucus barrier for inhaled lung gene therapy. *Proc. Natl Acad. Sci. USA* 112, 8720–8725 (2015).
164. Su, X., Fricke, J., Kavanagh, D. G. & Irvine, D. J. In vitro and in vivo mRNA delivery using lipid-enveloped pH-responsive polymer nanoparticles. *Mol. Pharm.* 8, 774–787 (2011).
165. Kozielski, K. L. et al. Cancer-selective nanoparticles for combinatorial siRNA delivery to primary human GBM in vitro and in vivo. *Biomaterials* 209, 79–87 (2019).
166. Eltoukhy, A. A., Chen, D., Alabi, C. A., Langer, R. & Anderson, D. G. Degradable terpolymers with alkyl side chains demonstrate enhanced gene delivery potency and nanoparticle stability. *Adv. Mater.* 25, 1487–1493 (2013).
167. Kaczmarek, J. C. et al. Polymer–lipid nanoparticles for systemic delivery of mRNA to the lungs. *Angew. Chem. Int. Edn Engl.* 55, 13808–13812 (2016).
168. Xu, L., Zhang, H. & Wu, Y. Dendrimer advances for the central nervous system delivery of therapeutics. *ACS Chem. Neurosci.* 5, 2–13 (2014).
169. Chahal, J. S. et al. Dendrimer-RNA nanoparticles generate protective immunity against lethal Ebola, H1N1 influenza, and *Toxoplasma gondii* challenges with a single dose. *Proc. Natl Acad. Sci. USA* 113, E4133–E4142 (2016).
170. Khan, O. F. et al. Ionizable amphiphilic dendrimer-based nanomaterials with alkyl-chain-substituted amines for tunable siRNA delivery to the liver endothelium in vivo. *Angew. Chem. Int. Edn Engl.* 53, 14397–14401 (2014).
171. Bielinska, A. U., Kukowska-Latallo, J. F. & Baker, J. R. Jr The interaction of plasmid DNA with polyamidoamine dendrimers: mechanism of complex formation and analysis of alterations induced in nuclease sensitivity and transcriptional activity of the complexed DNA. *Biochim. Biophys. Acta* 1353, 180–190 (1997).

172. Sonawane, N. D., Szoka, F. C. Jr & Verkman, A. S. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. *J. Biol. Chem.* 278, 44826–44831 (2003).
173. Yoo, J., Park, C., Yi, G., Lee, D. & Koo, H. Active targeting strategies using biological ligands for nanoparticle drug delivery systems. *Cancers* 11, 640 (2019).
174. Nel, A. E. et al. Understanding biophysicochemical interactions at the nano-bio interface. *Nat. Mater.* 8, 543–557 (2009).
175. Dawson, K. A. & Yan, Y. Current understanding of biological identity at the nanoscale and future prospects. *Nat. Nanotechnol.* 16, 229–242 (2021).
176. Schöttler, S. et al. Protein adsorption is required for stealth effect of poly(ethylene glycol)- and poly(phosphoester)-coated nanocarriers. *Nat. Nanotechnol.* 11, 372–377 (2016).
177. Salvati, A. et al. Transferrin-functionalized nanoparticles lose their targeting capabilities when a biomolecule corona adsorbs on the surface. *Nat. Nanotechnol.* 8, 137–143 (2013).
178. Akinc, A. et al. The Onpattro story and the clinical translation of nanomedicines containing nucleic acid-based drugs. *Nat. Nanotechnol.* 14, 1084–1087 (2019).
179. Akinc, A. et al. Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms. *Mol. Ther.* 18, 1357–1364 (2010).
180. Miao, L. et al. Synergistic lipid compositions for albumin receptor mediated delivery of mRNA to the liver. *Nat. Commun.* 11, 2424 (2020).
181. Sago, C. D. et al. Modifying a commonly expressed endocytic receptor retargets nanoparticles in vivo. *Nano Lett.* 18, 7590–7600 (2018).
182. Chen, S. et al. Influence of particle size on the in vivo potency of lipid nanoparticle formulations of siRNA. *J. Control. Rel.* 235, 236–244 (2016).
183. Nakamura, T. et al. The effect of size and charge of lipid nanoparticles prepared by microfluidic mixing on their lymph node transitivity and distribution. *Mol. Pharm.* 17, 944–953 (2020).
184. Reinhard, K. et al. An RNA vaccine drives expansion and efficacy of claudin-CAR-T cells against solid tumors. *Science* 367, 446–453 (2020).
185. Nair, J. K. et al. Multivalent *N*-acetylgalactosamine-conjugated siRNA localizes in hepatocytes and elicits robust RNAi-mediated gene silencing. *J. Am. Chem. Soc.* 136, 16958–16961 (2014).
186. Prakash, T. P. et al. Targeted delivery of antisense oligonucleotides to hepatocytes using triantennary *N*-acetyl galactosamine improves potency 10-fold in mice. *Nucleic Acids Res.* 42, 8796–8807 (2014).
187. Agarwal, S. et al. Impact of serum proteins on the uptake and RNA interference activity of N-acetylgalactosamine-conjugated small interfering RNAs. *Nucleic Acid Ther.* 31, 309–315 (2021).
188. Foster, D. J. et al. Advanced siRNA designs further improve in vivo performance of GalNAc-siRNA conjugates. *Mol. Ther.* 26, 708–717 (2018).
189. Nair, J. K. et al. Impact of enhanced metabolic stability on pharmacokinetics and pharmacodynamics of GalNAc-siRNA conjugates. *Nucleic Acids Res.* 45, 10969–10977 (2017).
190. Zanardi, T. A. et al. Safety, pharmacokinetic, and pharmacodynamic evaluation of a 2′-(2-methoxyethyl)-d-ribose antisense oligonucleotide-triantenarry *N*-acetyl-galactosamine conjugate that targets the human

transmembrane protease serine 6. *J. Pharmacol. Exp. Ther.* 377, 51–63 (2021).
191. Janas, M. M. et al. The nonclinical safety profile of GalNAc-conjugated RNAi therapeutics in subacute studies. *Toxicol. Pathol.* 46, 735–745 (2018).
192. Biscans, A. et al. Diverse lipid conjugates for functional extra-hepatic siRNA delivery in vivo. *Nucleic Acids Res.* 47, 1082–1096 (2019).
193. Osborn, M. F. et al. Hydrophobicity drives the systemic distribution of lipid-conjugated siRNAs via lipid transport pathways. *Nucleic Acids Res.* 47, 1070–1081 (2019).
194. Nagata, T. et al. Cholesterol-functionalized DNA/RNA heteroduplexes cross the blood–brain barrier and knock down genes in the rodent CNS. *Nat. Biotechnol.* https://doi.org/10.1038/s41587-021-00972-x (2021).
195. Zhou, J. & Rossi, J. Aptamers as targeted therapeutics: current potential and challenges. *Nat. Rev. Drug Discov.* 16, 181–202 (2017).
196. Yoon, S., Wu, X., Armstrong, B., Habib, N. & Rossi, J. J. An RNA aptamer targeting the receptor tyrosine kinase PDGFRα induces anti-tumor effects through STAT3 and p53 in glioblastoma. *Mol. Ther. Nucleic Acids* 14, 131–141 (2019).
197. Sugo, T. et al. Development of antibody-siRNA conjugate targeted to cardiac and skeletal muscles. *J. Controlled Rel.* 237, 1–13 (2016).
198. Avidity corporate presentation. *Avidity Biosciences* https://avidity biosciences.investorroom.com/events-and-presentations (2021).
199. Kedmi, R. et al. A modular platform for targeted RNAi therapeutics. *Nat. Nanotechnol.* 13, 214–219 (2018).
200. Veiga, N. et al. Cell specific delivery of modified mRNA expressing therapeutic proteins to leukocytes. *Nat. Commun.* 9, 4493 (2018).
201. Dammes, N. et al. Conformation-sensitive targeting of lipid nanoparticles for RNA therapeutics. *Nat. Nanotechnol.* https://doi.org/10.1038/s41565-021-00928-x (2021).
202. Li, Q. et al. Engineering caveolae-targeted lipid nanoparticles to deliver mRNA to the lungs. *ACS Chem. Biol.* 15, 830–836 (2020).
203. Zhuang, X. et al. mRNA vaccines encoding the HA protein of influenza A H1N1 virus delivered by cationic lipid nanoparticles induce protective immune responses in mice. *Vaccines* 8, 123 (2020).
204. Paunovska, K. et al. A direct comparison of in vitro and in vivo nucleic acid delivery mediated by hundreds of nanoparticles reveals a weak correlation. *Nano Lett.* 18, 2148–2157 (2018).
205. Paunovska, K., Loughrey, D., Sago, C. D., Langer, R. & Dahlman, J. E. Using large datasets to understand nanotechnology. *Adv. Mater.* 31, e1902798 (2019).
206. Lokugamage, M. P., Sago, C. D. & Dahlman, J. E. Testing thousands of nanoparticles in vivo using DNA barcodes. *Curr. Opin. Biomed. Eng.* 7, 1–8 (2018).
207. Yaari, Z. et al. Theranostic barcoded nanoparticles for personalized cancer medicine. *Nat. Commun.* 7, 13325 (2016).
208. Dahlman, J. E. et al. Barcoded nanoparticles for high throughput in vivo discovery of targeted therapeutics. *Proc. Natl Acad. Sci. USA* 114, 2060–2065 (2017).

209. Lokugamage, M. P., Sago, C. D., Gan, Z., Krupczak, B. R. & Dahlman, J. E. Constrained nanoparticles deliver siRNA and sgRNA to T cells in vivo without targeting ligands. *Adv. Mater.* 31, e1902251 (2019).
210. Lokugamage, M. P. et al. Mild innate immune activation overrides efficient nanoparticle-mediated RNA delivery. *Adv. Mater.* 32, 1904905 (2019).
211. Riley, R. S. et al. Ionizable lipid nanoparticles for in utero mRNA delivery. *Sci. Adv.* 7, eaba1028 (2021).
212. Havel, P. J., Kievit, P., Comuzzie, A. G. & Bremer, A. A. Use and importance of nonhuman primates in metabolic disease research: current state of the field. *ILAR J.* 58, 251–268 (2017).
213. Paunovska, K. et al. Increased PIP3 activity blocks nanoparticle mRNA delivery. *Sci. Adv.* 6, eaba5672 (2020).
214. Li, R. et al. Therapeutically reprogrammed nutrient signalling enhances nanoparticulate albumin bound drug uptake and efficacy in KRAS-mutant cancer. *Nat. Nanotechnol.* 16, 830–839 (2021).
215. Patel, S. et al. Boosting intracellular delivery of lipid nanoparticle-encapsulated mRNA. *Nano Lett.* 17, 5711–5718 (2017).
216. Yin, W. et al. Plasma lipid profiling across species for the identification of optimal animal models of human dyslipidemia. *J. Lipid Res.* 53, 51–65 (2012).
217. Rampado, R., Crotti, S., Caliceti, P., Pucciarelli, S. & Agostini, M. Recent advances in understanding the protein corona of nanoparticles and in the formulation of “stealthy” nanomaterials. *Front. Bioeng. Biotechnol.* 8, 166 (2020).
218. Delprato, A. et al. Systems genetic analysis of hippocampal neuroanatomy and spatial learning in mice. *Genes Brain Behav.* 14, 591–606 (2015).
219. Harrill, A. H. et al. A mouse diversity panel approach reveals the potential for clinical kidney injury due to DB289 not predicted by classical rodent models. *Toxicol. Sci.* 130, 416–426 (2012).
220. Church, R. J. et al. A systems biology approach utilizing a mouse diversity panel identifies genetic differences influencing isoniazid-induced microvesicular steatosis. *Toxicol. Sci.* 140, 481–492 (2014).
221. Leist, S. R. et al. Influenza H3N2 infection of the collaborative cross founder strains reveals highly divergent host responses and identifies a unique phenotype in CAST/EiJ mice. *BMC Genomics* 17, 143 (2016).
222. Jaxpheno2 project protocol: morphometric (organ weight) survey of 11 strains of mice (2006). *Mouse Phenome Database at the Jackson Laboratory* https://phenome.jax.org/projects/Jaxpheno2/protocol?method=organ+weights (2006).
223. Sugimoto, K. et al. Background data on organ weights and histopathological lesions in Cej:CD(SD)IGS rats for 4-, 13- and 26-weeks repeated-dose toxicity studies. Biological reference data on CD(SD)IGS rats. In *IGS Databook 2000* 79–87 (Charles River Laboratory, 2000).
224. Durbin, P. W., Jeung, N., Williams, M. H., Kullgren, B. & Parrott, M. W. Weights of bones and tissues at maturity and growth of the skeleton of rhesus (*Macaca mullata* and cynomolgus (*Macaca fascicularis*) monkeys. *escholarship* https://escholarship.org/content/qt6kw7682s/qt6kw7682s.pdf (1996).

225. Molina, D. K. & DiMaio, V. J. Normal organ weights in men. Part II — the brain, lungs, liver, spleen, and kidneys. *Am. J. Forensic Med. Pathol.* 33, 368–372 (2012).
226. Molina, D. K. & DiMaio, V. J. Normal organ weights in women. Part II — the brain, lungs, liver, spleen, and kidneys. *Am. J. Forensic Med. Pathol.* 36, 182–187 (2015).
227. Molina, D. K. & DiMaio, V. J. Normal organ weights in women. Part I — the heart. *Am. J. Forensic Med. Pathol.* 36, 176–181 (2015).
228. Molina, D. K. & DiMaio, V. J. Normal organ weights in men. Part I — the heart. *Am. J. Forensic Med. Pathol.* 33, 362–367 (2012).
229. Hatit, M. Z. C. et al. Species-dependent in vivo mRNA delivery and cellular responses to nanoparticles. *Nat. Nanotechnol.* https://doi.org/10.1038/s41565-021-01030-y (2021).
230. Zhang, X., Goel, V. & Robbie, G. J. Pharmacokinetics of patisiran, the first approved RNA interference therapy in patients with hereditary transthyretin-mediated amyloidosis. *J. Clin. Pharmacol.* 60, 573–585 (2019).
231. Zhang, X. et al. Patisiran pharmacokinetics, pharmacodynamics, and exposure-response analyses in the phase 3 APOLLO trial in patients with hereditary transthyretin-mediated (hATTR) amyloidosis. *J. Clin. Pharmacol.* 60, 37–49 (2020).
232. Center for Drug Evaluation and Research application number: 210922Orig1s000. *FDA* https://www.accessdata.fda.gov/drugsatfda_docs/nda/2018/210922Orig1s000MultiR.pdf (2018).
233. Maier, M. A. et al. Biodegradable lipids enabling rapidly eliminated lipid nanoparticles for systemic delivery of RNAi therapeutics. *Mol. Ther.* 21, 1570–1578 (2013).
234. Cheng, Z., Al Zaki, A., Hui, J. Z., Muzykantov, V. R. & Tsourkas, A. Multifunctional nanoparticles: cost versus benefit of adding targeting and imaging
235. Gilleron, J. et al. Image-based analysis of lipid nanoparticle-mediated siRNA delivery, intracellular trafficking and endosomal escape. *Nat. Biotechnol.* 31, 638–646 (2013).
236. Wittrup, A. et al. Visualizing lipid-formulated siRNA release from endosomes and target gene knockdown. *Nat. Biotechnol.* 33, 870–876 (2015).
237. Alberer, M. et al. Safety and immunogenicity of a mRNA rabies vaccine in healthy adults: an open-label, non-randomised, prospective, first-in-human phase 1 clinical trial. *Lancet* 390, 1511–1520 (2017).
238. Zhao, P. et al. Long-term storage of lipid-like nanoparticles for mRNA delivery. *Bioact. Mater.* 5, 358–363 (2020).
239. Gerhardt, A. et al. A thermostable, flexible RNA vaccine delivery platform for pandemic response. Preprint at *bioRxiv* https://doi.org/10.1101/2021.02.01.429283 (2021).
240. Besin, G. et al. Accelerated blood clearance of lipid nanoparticles entails a biphasic humoral response of B-1 followed by B-2 lymphocytes to distinct antigenic moieties. *Immunohorizons* 3, 282–293 (2019).
241. Machin, N. & Ragni, M. V. An investigational RNAi therapeutic targeting antithrombin for the treatment of hemophilia A and B. *J. Blood Med.* 9, 135–140 (2018).
242. Habtemariam, B. A. et al. Single-dose pharmacokinetics and pharmacodynamics of transthyretin targeting *N*-acetylgalactosamine–small

interfering ribonucleic acid conjugate, vutrisiran, in healthy subjects. *Clin. Pharmacol. Ther.* 109, 372–382 (2021).
243. Wang, Y., Yu, R. Z., Henry, S. & Geary, R. S. Pharmacokinetics and clinical pharmacology considerations of GalNAc(3)-conjugated antisense oligonucleotides. *Expert Opin. Drug Metab. Toxicol.* 15, 475–485 (2019).
244. Paunovska, K., Loughrey, D., & Dahlman, J. E. (2022). Drug delivery systems for RNA therapeutics. *Nature Reviews Genetics*, *23*(5), 265-280.