CKJ REVIEW

mRNA as a medicine in nephrology: the future is now

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ABSTRACT

The successful employment of messenger RNA (mRNA) as vaccine therapy for the prevention of COVID-19 infection has spotlighted the attention of scientific community onto the potential clinical application of these molecules as innovative and alternative therapeutic approaches in different fields of medicine. As therapy, mRNAs may be advantageous due to their unique biological properties of targeting almost any genetic component within the cell, many of which may be unreachable using other pharmacological/therapeutic approaches, and encoding any proteins and peptides without the need for their transport into the nuclei of the target cells. Additionally, these molecules may be rapidly designed/produced and clinically tested. Once the chemistry of the RNA and its delivery system are optimized, the cost of developing novel variants of these medications for new selected clinical disorders is significantly reduced. However, although potentially useful as new therapeutic weapons against several kidney diseases, the complex architecture of kidney and the inability of nanoparticles that accommodate oligonucleotides to cross the integral glomerular filtration barrier have largely decreased their potential employment in nephrology. However, in the next few years, the technical improvements in mRNA that increase translational efficiency, modulate innate and adaptive immunogenicity, and increase their delivery at the site of action will overcome these limitations. Therefore, this review has the scope of summarizing the key strengths of these RNA-based therapies and illustrating potential future directions and challenges of this promising technology for widespread therapeutic use in nephrology.

Keywords: *in vitro* transcription, kidney diseases, mRNA-based therapies, nephrology, translational medicine

INTRODUCTION

Over the past decade, messenger RNA (mRNA) has been recognized as a potential therapeutic tool against several intractable or genetic diseases (comprising genetic/hereditary kidney diseases) and, thanks to the rapid development of innovative technological skills for its large-scale production, it has been successfully employed as vaccine therapy against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

mRNAs as therapy could target almost any genetic component within the cell, many of which are unreachable using other technologies (including both small molecules and antibodies), and encode proteins/peptides in the cytoplasm of the target cells without being transported into the nuclei (thus allowing protein production in post-mitotic cells) [1]. Therapeutic applications of mRNAs include: synthesis of a single protein for replacing the function in the case of monogenic disease; mRNA coding for transcription or growth factor used to modulate cell behavior; and mRNA-encoded factors involved in immune response.

The advantages of mRNAs include the low risk of adverse effects and toxicities because of their transient nature, and absence of insertional mutagenesis because they do not integrate into the genome [1, 2].

mRNA is easily synthesized through the *in vitro* transcription (IVT) process and is more effective, rapid in design and production, flexible, and cost-effective than conventional therapeutics [3]. In fact, once the chemistry of the RNA and its delivery system are optimized, the cost of developing novel variants of these

medications for new selected clinical disorders is significantly reduced.

Nevertheless, several challenges have significantly hampered the employment of mRNA as a therapy in medicine [4]. First, mRNA is vulnerable to ubiquitous RNases which are highly abundant in the extracellular space and tissues; secondly, due to its negative charge, it cannot be easily transported across the cell membrane [5]; and finally, mRNA is able to stimulate the innate immune system [6–9] by activating Toll-like receptors and pattern recognition receptors.

Thanks to the recent advances in bioinformatics and nanotechnology these hurdles have been, at least partially, overcome leading to an increase in the potential applications of mRNA therapeutics.

MAIN STRATEGIES TO MANUFACTURE, AND REDUCE IMMUNOGENICITY, IMPROVE INTRACELLULAR STABILITY AND FACILITATE DELIVERY OF mRNA

Synthesis and optimization of mRNA

After IVT mRNA synthesis, using linearizing plasmid DNA or a PCR product as template and T3, T7 or SP6 RNA polymerase, a capping step is required to avoid degradation of mRNA by RNase and/or activation of the immune system by the 5′-ppp group [10, 11].

The capping of mRNA can be performed through a co-transcriptional or a post-transcriptional method [12, 13]. The latter uses capping enzymes from vaccinia virus that add a 7-methylguanosine cap at the 5′ end of the RNA using GTP and S-adenosyl methionine as donors (Cap 0 structure). Furthermore, 2′ ribose position of the first cap-proximal nucleotide is 2′O-methylated to form a Cap 1 structure (^{m7}GpppN$_{2'Om}$N), and, in ∼50% of transcripts, the second cap-proximal nucleotide is 2′O-methylated to form a Cap 2 structure (^{m7}GpppN$_{2'Om}$N$_{2'Om}$), which reduces mRNA immunogenicity [14, 15].

Unfortunately, because of the presence of a 3′-OH on both the 7-methylguanosine and guanosine moieties, up to half of the mRNAs contain caps incorporated in the reverse orientation, which cannot be recognized by the ribosome and hinder overall mRNA translation activity [15–19]. This problem was overcome by the introduction in the transcription reaction of anti-reverse cap analogs bearing modified m^{7}G at the 2′ or 3′ position (2′-O-methyl, 3′-O-methyl, 3′-H) ensuring correct orientation and higher translation efficiency [20, 21].

The mature mRNA also includes a 3′ poly(A) tail that can be added post-transcriptionally using the poly-A-polymerase enzyme or incorporated in the DNA template [1, 22, 23]. Optimization of the poly(A) tail length (100–300 nucleotides) has proven critical in balancing the translation efficacy of mRNAs [24–26].

Furthermore, other modifications that can enhance translational efficiency and reduce immunogenicity include changes in the open reading frame by replacing rare codons with more frequently occurring variants (codon optimization) [27], elimination of structural motifs able to activate innate immune response and the introduction of chemical alterations that render the mRNA more similar to an endogenous molecule [28–30].

Purification of mRNA

After synthesis, IVT mRNA is mixed with unwanted side products such as DNA templates, short mRNA, uncapped mRNA, double-stranded RNA (dsRNA) and mRNA fragments. All these contaminant impurities must be removed in order to avoid interference with mRNA translation, activation of innate immunity or overestimation of the total functional mRNA cargo [25, 31, 32].

Purification of IVT mRNA can be carried out by different procedures, including acidic phenol-chloroform extraction, precipitation with LiCl, elution based on silica matrices or chromatographic methods [33]. All these procedures eliminate proteins, nucleotides and other components of the IVT reaction but cannot remove dsRNA impurities.

The established way to eliminate dsRNA contaminants from long IVT mRNAs is by using ion pair reversed-phase high-performance liquid chromatography (HPLC) [31, 34]. However, this method has some disadvantages: it is not scalable, the toxic effects of acetonitrile and the high cost [31, 35].

Based on the selective binding of dsRNA to cellulose in ethanol-containing buffer, Baiersdörfer *et al.* [35] have developed a feasible cellulose-based chromatography method for the elimination of dsRNA contaminants with a quality comparable to that of the corresponding HPLC-purified mRNA.

Another possible approach is to use the dsRNA-specific nuclease RNase III [36]. A potential drawback is that this enzyme may cleave the double-stranded secondary structure formed by single-stranded RNA.

Finally, short RNAs can be removed by polyacrylamide gel electrophoresis (PAGE) followed by excision and elution of the band of interest from the gel, and long RNAs can be separated by denaturing agarose gel electrophoresis [37, 38].

Delivery methods for mRNA

Targeted delivery of IVT mRNA is a great challenge for the *in vivo* application of mRNA-based therapeutics. The large molecular weight (approximately 1–15 kb) [27] and high negative charge of this nucleic acid impair its permeation across cellular membranes [39]. Moreover, it is highly susceptible to degradation by nucleases and its median intracellular half-life is only approximately 7 h [40]. Therefore, different strategies have been developed to protect mRNA from degradation and optimize its delivery at the tissue target [27].

In general, IVT mRNA delivery can be obtained by three strategies: physical methods, viral-based approaches and nonviral vectors.

Physical methods such as electroporation (which uses high voltage electric pulse to increase cell permeability) transiently disrupt the barrier function of the cell membrane with frequent damage to the cells, and are therefore not suitable for *in vivo* applications [41, 42].

The recombinant viruses use the naturally occurring biological modes of uptake but are associated with several limitations such as potential reverse genome insertional risks, difficulties in controlling the gene expression and vector-size limitations, as well as strong immunologic side effects [43, 44].

The nonviral vectors that use rationally designed and easily developed chemical nanocarriers have huge potential for the delivery of nucleic acids [42].

One of the most well-developed methods for mRNA delivery is co-formulation into lipid nanoparticles (LNPs) [45], typically composed of four components: (i) neutrally charged phospholipids (structural lipids); (ii) cholesterol as a stabilizing agent for the lipid bilayer; (iii) pH-sensitive ionizable cationic lipids needed for the loading of negatively charged nucleic acids into LNPs; and (iv) stealth lipids (mainly polyethylene glycol (PEG) polymer–conjugated lipids) to reduce immunogenicity [46–48].
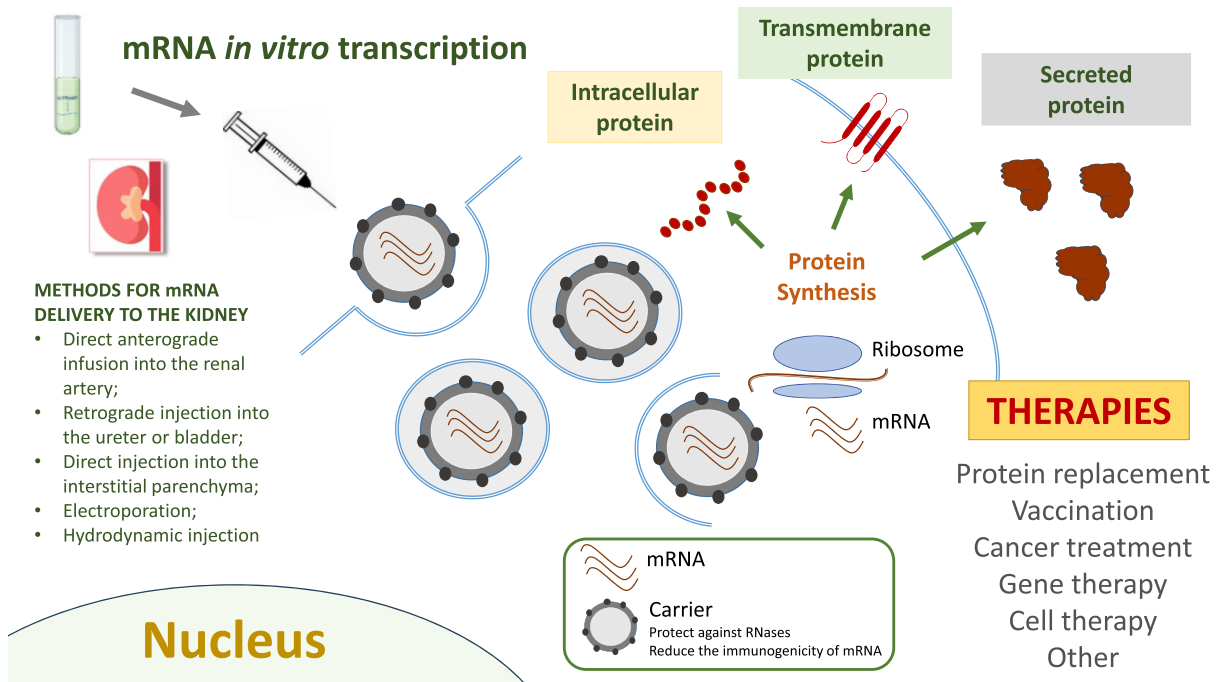
Figure 1: A schematic representation of the steps of mRNA therapy in kidney diseases. After IVT, mRNA is purified and administered via several possible routes (direct anterograde infusion into the renal artery; retrograde injection into the ureter or bladder; direct injection into the interstitial parenchyma; electroporation; hydrodynamic injection) to reach the kidney. Once in the organ, mRNA is translated by ribosomes in cytoplasmatic, transmembrane or secreted proteins. The potential applications of this therapy are: protein replacement, vaccination, cancer treatment, gene therapy and cell therapy.

More recently, the use of lipid polymer hybrid nanoparticles, which integrate the properties of lipids with polymeric nanomaterials, has shown higher efficiency of mRNA delivery [49].

A critical hinderance for the correct delivery of LNPs is their rapid uptake by antigen-presenting cells and macrophages. The usual approach to prolong the circulation of mRNA-loaded LNPs is the addition of PEG units on the surface of LNPs which cause formation of a hydration layer that prevents clearance by the mononuclear phagocyte system [50]. Moreover, the addition of a ligand to cell-membrane to imitate "self" materials can avoid the uptake by phagocytes [51, 52].

More recently, other new procedures, developed for delivery of nanoparticle-based drugs, include the mononuclear phagocyte system blockade by low-toxicity "blocking" agents [53], macrophage depletion by means of the administration of clodronate/gadolinium chloride [54] and pre-induced depletion of erythrocytes by administration of a low dose of allogeneic anti-erythrocyte antibodies [55, 56]. It is plausible that these methods could be used in future also to prolong the circulation time of mRNA and to optimize delivery to target tissue.

To efficiently reach their target to deliver the cargo, LNPs can be also conjugated with specific ligands to the surface that help the identification and the uptake by the intended cells. These ligands can be peptides, antibodies, nucleic acid aptamers, carbohydrates or small molecules [22].

Other systems developed as alternatives to LNPs are biological delivery vehicles such as cells or extracellular vesicles. The main advantages of this methodology comprise biocompatibility, wide range of customization, extended longevity in circulation and reduced toxicity [57–60].

However, current challenges in their clinical use include the characterization, the isolation method and their purification [61, 62].

DELIVERY METHODS FOR mRNA THERAPY IN KIDNEY DISEASES

mRNA delivery in the kidney is difficult due to its architecture and the large number of different cell types within the organ [22]. Moreover, the glomeruli that eliminate proteins above 50 kDa and the slit diaphragm with a diameter of 10 nm prevent entry of most molecular therapies from the blood into the kidney [63].

Some methods previously developed for gene therapy in kidney diseases can be used for mRNA delivery into the kidney: direct anterograde infusion into the renal artery targeting the glomeruli and tubular epithelium; retrograde injection into the ureter or bladder, and directly into the interstitial parenchyma [64].

In particular, recently, renal artery injection of transforming growth factor (TGF)-β/Smad-small interfering RNA (siRNA) has been used for the treatment of glomerulonephritis and renal vein injection of FAS-siRNA for improving survival after ischemia/reperfusion injury in mouse models [65, 66].

Other ways to directly deliver drug to kidney include physical methods such as electroporation [67], pressure stimulation, hydrodynamic injection, magnetically guided oligonucleotide-loaded nanoparticles [68], light-triggered lipid-based nanoparticles and aptamers, which have been applied to cancer therapy [69, 70] (Fig. 1).

Electroporation has been used for delivery of siRNA targeting TGF-β1 to the kidney to reduce the progression of matrix expansion in an animal model of glomerulonephritis [67]. However, membrane destruction associated with this method may lead to the loss of cytoplasmic content with significant cytotoxicity [71].

Pressure stimulation such as pushing or suction after normal intravenous injection has been previously tested to introduce plasmid DNA (pDNA) or siRNA into the kidney with good efficiency and no renal dysfunction [72, 73].

The addition of hydrodynamic injection to pressure stimulation has recently been developed to administer mRNA-loaded polyplex nanomicelles via renal pelvis injection into the kidney [74, 75]. The administration of mRNA-loaded nanomicelles by this route in kidneys of ICR mice induced protein expression in a greater number of tubular epithelium cells for some days compared with naked pDNA and naked mRNA, although introduced in the same way [75]. The renal function after administration remained similar to those of the sham-operated controls, without marked changes in histological sections, demonstrating the safety of the methodology.

In order to efficiently reach their target tissue, the nanoparticles containing the IVT mRNA can be conjugated with specific ligands (peptides, antibodies, nucleic acid aptamers or small molecules) to the surface that help the identification and the uptake by the intended cells [22]. Nevertheless, none of them has yet been used for mRNA delivery, but we expect that they could be employed in the future.

For example, the cyclo-(Arg-Gly-Asp-D-Phe-Cys) peptide has been used as a specific ligand of αvβ3 integrin receptor on the podocyte surface [76]. Likewise, megalin in the proximal tubular cells has been used as a target for specific peptides [77].

Antibodies can be attached to the delivery vehicle by means of Fc-binding peptides in combination with a surface linker [78]. In addition, the LNPs can be noncovalently coated with targeting antibodies via a recombinant lipoprotein (named ASSET) that is incorporated into siRNA-loaded LNP and interacts with the antibody Fc domain [79]. Several studies have also developed the use of antibody fragments instead of whole immunoglobulins in order to reduce immunogenicity, increase loading capacities and, thereby, improve the efficacy [80]. This methodology has been used for anti-cancer and siRNA therapies [81].

Aptamers are short single-stranded oligonucleotides that can bind specific proteins to modulate their functions. For example, Emapticap pegol (NOX-E36) is an RNA aptamer that binds and inhibits the C-C motif ligand 2, currently in phase II trials for type 2 diabetes mellitus and albuminuria [82]. Aptamers are characterized by high affinity for their target molecules, being nonimmunogenic and, due to their small size, being able to bind to sites inaccessible to larger antibodies, and rapid synthesis and lower manufacturing costs [83].

THE APPLICATION OF mRNA-BASED THERAPIES IN KIDNEY DISEASES: SOME INITIAL EXAMPLES

No mRNA therapies for the kidney have yet been introduced in the clinic, and preclinical studies are very limited. However, the employment of these RNA-based therapies on systemic diseases with secondary kidney involvement (including primary oxaluria and Fabry disease) appears more promising.

Recently, Zhu *et al.* [84] carried out a preclinical study involving Fabry disease, a lysosomal storage disorder caused by the deficiency of alpha-galactosidase, which leads to cellular accumulation of glycosphingolipid [particularly globotriaosylceramide (Gb3) and the deacylated Gb3 analog globotriaosylsphingosine (lyso-Gb3)], and progressive damage in tissues such as kidney, heart and skin [85]. The mRNA encoding human alpha galactosidase A (h-α-Gal A), synthesized *in vitro* and packaged into LNP, was administered intravenously in α-Gal A–deficient Fabry mouse model at three different dosages, 0.5, 0.1 and 0.05 mg/kg. A single dose resulted in an increment in protein activity and glycosphingolipid reduction in tissues and plasma for up to 6 weeks. Likewise, repeated administration of 0.2 mg/kg or 0.5 mg/kg h-α-Gal A mRNA every week or 0.5 mg/kg h-α-Gal A mRNA every month for 3 months restored α-Gal A activity in tissues and reduced lyso-Gb3 and Gb3 in a dose-dependent manner.

The same procedure in non-human primates (0.5 mg/kg intravenously every week for four doses) confirmed the results obtained in mice, with the absence of an immune response to the protein encoded by the mRNA [84].

Another lysosomal storage disease with mRNA therapy preclinical study is cystinosis, caused by mutations in the *cystinosin* (*CTNS*) gene and consequent intra-lysosomal cystine accumulation. It initially affects the kidneys with defective proximal tubular reabsorption (renal Fanconi syndrome) and glomerular damage leading to kidney failure [86, 87].

Direct injection of CTNS mRNA (500 ng/mL) in a zebrafish model for cystinosis (ctns -/-) improved proximal tubular uptake of low molecular weight dextran (a marker for proximal reabsorption) and reduced overall proteinuria [88].

Kidney injury is also one of the symptoms of methylmalonic acidemia/aciduria (MMA), an autosomal recessive disease due to partial or complete deficiency of methylmalonyl-CoA mutase (MUT), a vitamin B12-dependent mitochondrial enzyme that catalyzes the isomerization of methylmalonyl-CoA to succinyl-CoA, the final step in the oxidation of odd-chain fatty acids, the amino acids valine, isoleucine, methionine and threonine, and cholesterol, providing metabolites for the tricarboxylic acid cycle. Other symptoms include growth retardation, acute metabolic decompensation with acidosis, vomiting, dehydration, hepatomegaly and psychomotor retardation with cognitive dysfunction. The intravenous administration of a single dose of *human MUT* (*hMUT*) mRNA (0.5 mg/kg) packaged into LNP in MMA mouse models resulted in 75%–85% reduction in plasma methylmalonic acid and was associated with increased hMUT protein expression and activity in the liver and substantially improved the biochemical abnormalities characteristic of the disorder [89].

The RNA technology can also be used for CKD-related comorbidities such as hypertension. In a recent phase I study, zilebesiran, an RNA interference therapeutic agent designed to achieve specific reduction in hepatic angiotensinogen mRNA levels, when administered to patients with hypertension induced dose-related decrease in both serum angiotensinogen levels and blood pressure after single subcutaneous doses. This effect was sustained for up to 24 weeks [90]. The great advantage of this agent is the specific hepatic target which limits the consequences of off-target renal renin–angiotensin–aldosterone system inhibition [90].

Another interesting application of mRNA as therapy is mRNA-based vaccines against infectious diseases and several types of cancer [91]. Two mRNA vaccines have been developed to treat renal cell carcinoma (RCC). The first is an *in vitro* transcribed naked mRNA, generated using plasmids coding

for human epidermal growth factor receptor 2 (Her-2/neu), carcinoembryonic (CEA), tumor-associated antigens mucin 1 (MUC1), telomerase, survivin and melanoma-associated antigen 1 (MAGE-A1). The trial involved 30 metastatic RCC (mRCC) patients divided into two cohorts. The vaccine was administered on Days 0, 14, 28 and 42 (20 μg/antigen) in the first 14 patients (Cohort A) and at Days 0–3, 7–10, 28 and 42 (50 μg/antigen) in the consecutive 16 patients (Cohort B) [92, 93]. In both cohorts, after this induction period, vaccinations were repeated monthly until tumor progression. The treatment was safe and well tolerated with no relevant side effects, and the median survival was longer than predicted according to the Memorial Sloan Kettering Cancer Center (MSKCC) risk score. Interestingly, the long-term survival update (after 10 years) showed a clear correlation with $CD4^+$ and $CD8^+$ T cell responses to tumor-associated antigens encoded by the naked mRNA vaccine [93].

More recently, in kidney renal clear cell carcinoma (KIRC), the identification of four genes [*neutrophil cytosol factor 4* (*NCF4*), *formin-like protein 1* (*FMNL1*), *DNA topoisomerase II alpha* (*TOP2A*) and *docking protein 3* (*DOK3*)] significantly up-regulated, positively associated with antigen-presenting cell infiltration and associated with decreased survival probability, suggested their use as potential effective neoantigens for KIRC mRNA vaccine development [94].

AGS-003 is an autologous dendritic cell vaccine prepared *ex vivo* from mature monocyte-derived dendritic cells (DCs) co-electroporated with the patient's amplified tumor RNA and synthetic CD40L RNA [95, 96]. When administered by intradermal injection, these RNA-loaded mature DCs are capable of presenting relevant patient-specific tumor antigens via major histocompatibility complex Class I presentation to T cells in the draining lymph node. Intradermal injections of AGS-003 in combination with sunitinib (the first-line treatment of mRCC) in an unselected, intermediate and poor-risk mRCC patient population was associated with a doubling of expected survival, encouraging long-term and 5-year overall survival, and an excellent safety profile [97]. These results have encouraged the current phase III (NCT01582672) trial.

However, more studies should be performed to assess the safety of this therapeutic approach. In fact, in the last few months, *de novo* vasculitis, cases of minimal change disease, acute interstitial nephritis and occasional recurrence of primary disease have been described after mRNA-based vaccines [98, 99]. Moreover, it is important to note that there are some limitations to its use. For example, the approved COVID-19 vaccines can be stored for several months depending on the formulation, but only at extremely low temperatures below freezing, which can lead to logistical barriers to distribution in certain areas. Furthermore, the need for multiple doses of the mRNA vaccines may pose a challenge for people to complete the series of their immunizations. There is also ongoing research looking into the duration of mRNA vaccines, as their development is still in early stages compared with other vaccines and more research is needed before they can widely be used for additional viral diseases.

Finally, at the moment, there is no specific guidance from the Food and Drug Administration (FDA) or European Medicines Agency (EMA) for mRNA products. However, there are numerous clinical trials, in particular for mRNA-based vaccines, under EMA and FDA oversight demonstrating that products are safe and acceptable for testing in humans [91]. Additionally, since mRNA can be considered a gene therapy product, the recommendations defined for DNA vaccines and gene therapy vectors can be applied, at least partially, to mRNA. However, it is likely that specific guidelines will be developed in the future to regulate the manufacture, quality control testing and administration of mRNA as therapy.

CONCLUSIONS

Although the use of mRNA therapy is a new, valid and potential therapeutic weapon against a series of pathologies and can represent a valid alternative to classical therapy, many doubts still exist about its use for the treatment of renal pathologies. Instead, its application for treating systemic diseases appears much more promising.

It is necessary to intensify research in this field and to start studies and clinical trials in order to assess the real potential of mRNA therapy in nephrology.

However, we expect that in the future, this technology could represent a therapy for many rare and neglected genetic kidney diseases. However, some hurdles should be overcome to permit the dissemination of its employment in several clinical settings.

DATA AVAILABILITY STATEMENT

There were no data generated or analysed during the current review.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

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