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Review

Beyond current treatment of Fanconi Anemia: What do advances in cell and gene-based approaches offer?

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ABSTRACT

Fanconi anemia (FA) is a rare inherited disorder that mainly affects the bone marrow. This condition causes decreased production of all types of blood cells. FA is caused by a defective repair of DNA interstrand crosslinks and to date, mutations in over 20 genes have been linked to the disease. Advances in science and molecular biology have provided new insight between FA gene mutations and the severity of clinical manifestations. Here, we will highlight the current and promising therapeutic options for this rare disease. The current standard treatment for FA patients is hematopoietic stem cell transplantation, a treatment associated to exposure to radiation or chemotherapy, immunological complications, plus opportunistic infections from prolonged immune incompetence or increased risk of morbidity. New arising treatments include gene addition therapy, genome editing using CRISPR-Cas9 nuclease, and hematopoietic stem cell generation from induced pluripotent stem cells. Finally, we will also discuss the revolutionary developments in mRNA therapeutics as an opportunity for this disease.

1. Introduction

Fanconi anemia (FA) is a rare human genetic disorder, whose symptoms include hematopoietic failure, birth defects and high risk of cancer [1,2]. FA can be detected at birth or during childhood by the presence of one or more physical traits (including short stature, hand, arm and other skeletal anomalies, kidney problems and small head or eyes). FA leads to diverse complications that require specific clinical care approaches. FA patients suffer potential hearing loss or structural abnormalities of the eardrums and/or middle ear bones, therefore an otolaryngologist may consider possible surgical intervention to improve their hearing. On the other hand, all patients with FA should limit sun exposure and wear sunscreen protection to reduce the risk of skin cancer [3]. Endocrine problems, including growth hormone deficiency, hypothyroidism, pubertal delay or diabetes are also linked to this pathology [4,5]. A significant number of patients with FA have gastrointestinal symptoms, such as poor oral intake, nausea, abdominal pain, and/or diarrhea resulting in a failure to thrive. These problems may affect nutrition and quality of life in patients with FA. Finally, the most common complication in FA patients is probably the development of a bone marrow failure (BMF) linked to DNA damage accumulation [6].

Since some patients may have no obvious physical traits, FA is often diagnosed only when cytopenia is detected. The median age at diagnosis of FA is 7 years, although cases from birth to more than 50 years of age have been described [7]. The average lifespan for people with the disorder is between 20 and 30 years old. Eighty percent of 15 year-old FA patients develop BMF, and the risk of BMF exceeds 90% in older FA patients [8]. Half of the FA patients develop myelodysplastic syndrome (MDS) and/or acute myeloid leukemia (AML). The usual period of development of MDS/AML is the late teenage years or young adulthood. AML in the setting of FA is uneasy to treat and associated with a grave prognosis [9]. Additionally, the risk of head and neck, esophageal, gastrointestinal, vulvar, and anal cancers is approximately 50-fold higher in patients with FA [1,10].

The tumor suppressor function of FA proteins reflects their roles in genome maintenance, that include preventing replication stress and

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repairing endogenous DNA interstrand crosslinks (ICLs) induced by aldehyde metabolisms [11]. ICLs occur when two DNA nucleotides on opposite strands of DNA are covalently linked together, which blocks the DNA replication and transcription processes [12]. FA cell hallmarks are genomic instability, cell cycle alterations, chromosome anomalies, elevated cell death, slow growth, and defects in cell reprogramming and p53-p21 axis activation [13]. FA diagnostic is based on chromosome breakage analysis following an alkylating agent exposure (ICL-causing agents), such as diepoxybutane (DEB) or mitomycin C (MMC) [14].

FA is caused by mutations in any of the 23 genes that are involved in the FA/BRCA pathway, named *FANC* genes, with bi-allelic mutations in *FANCA* being the most common as it occurs in 60–70% of patients [15]. FANCA is involved in the recognition of damage together with other members of the FA core complex including FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FAAP20 and FAAP100 [16] along with the FANCM-FAAP24 complex. This complex can monoubiquitinate FANCD2 and FANCI [17]. Monoubiquitinated FANCD2/FANCI localizes to the ICL and promotes dual incisions on either side of the ICL by recruiting a complex containing the scaffold protein SLX4 and the flap endonuclease XPF-ERCC1 [18]. The unhooking of the lesions generates a double-strand break (DSB) in one of the sister chromatids, leaving a DNA adduct on the other sister. The adducted chromatid is restored by translesion synthesis (TLS) polymerases in a two-step reaction. Finally, the DSB is repaired by Rad51-mediated homologous recombination (HR) using the intact sister chromatid as a homology donor, and the remaining mono-adduct is likely to be removed by nucleotide excision repair. However, this is a simplistic view, as ICL repair is very complex and diverse and does not always involve the FA pathway. Different models of ICL repair have been extensively discussed elsewhere in recent reviews [19,20]. The FA pathway also contributes to various aspects of normal or challenged DNA replication independently of ICLs [21]. Moreover, many other functions of FANC proteins have been described ranging from mRNA metabolism, ribosome biogenesis to mitochondial homeostasis or inflammation [22–26], the deregulation of which could also contribute to BMF in addition to the DNA repair defects of FA cells.

2. Treatment option for FA

2.1. Bone marrow transplant (BMT)

The current standard treatment for FA patients is hematopoietic stem cell transplantation (HSCT). It can be curative mainly for lethal BMF, but also for hematological malignancy manifestations of the disease (*i.e.*, leukemia, myelodysplasia, and severe aplastic anemia). However, not all patients can benefit from it. Additionally, due to the intrinsic role of FA proteins, FA patients have a higher risk of morbidity during or after HSCT. This is mainly due to the toxicity of the radiation or chemotherapy used during pre-transplant conditioning.

HSCT can also increase the risk of developing cancer and others complications. A 10-year retrospective analysis of 22 patients with FA and other BMFs who underwent HSCT showed that 61% of the patients had persistent hemochromatosis, 22% developed hypothyroidism, 39% had insulin resistance, 27% developed hypertriglyceridemia, 68% developed gonadal dysfunction and 9% died of squamous cell carcinoma (SCC) [27]. Another study reported that FA patients who received an HSCT were 4.4 times more likely to develop SCC than FA patients who did not receive transplants [28]. In addition, the source of stem cells is also important, as the risk of secondary malignancies is higher when peripheral blood is used compared to bone marrow transplantation [29].

Graft-*versus*-host disease (GVHD) represents another complication observed after HSCT. It is the major life-threatening complication of allogeneic hematopoietic cell transplant. It occurs when immune competent T cells in the donated tissue (the graft) recognize the recipient (the host) as foreign. The resulting immune response activates donor T cells to gain the cytolytic capacity to eliminate antigen-bearing host cells. It affects the skin, digestive tract, or liver and can manifest as a rash, elevated liver enzymes, or gastrointestinal symptoms such as nausea, vomiting or diarrhea. The inflammatory process can be acute (aGVHD) or chronic (cGVHD). aGVHD grades are grade I (mild), II (moderate), III (severe), and IV (very severe). Incidences of aGVHD grade II-IV significantly decreased from 40% for 1990–1995, to 28% for 2010–2015 [30]. If the inflammatory process is continuous in time, the patient develops a cGVHD. Almost any organ system can be affected by cGVHD, with common symptoms such as dry mouth, dry eyes, skin tightness, joint tightness and even lung disease. In contrast to aGVHD, cGVHD incidence has not decreased over the years [31]. Moreover, the presence of cGVHD also strongly increases the risk of secondary cancers [29].

To prevent the occurrence of GVHD, T-cell depletion is usually performed [32]. The depletion can be done *in vivo* using immunosuppressors, as anti-thymocyte globulin (ATG), usually in combination with alkylants agents as busulfan or fludarabine [33] or *ex vivo* using immunomagnetic beads to make a positive selection of CD34+ cells [34]. Currently, there is no standardization, so more studies are needed to conclude which combination is more effective [35]. Also, *ex vivo* T-cell depletion is not available in every country [36]. The general GVHD treatment consists of steroids administration and there are standardized treatment schedules and regular follow-ups to ensure the best possible patient outcome, but it would be necessary for multi-armed trials to strengthen the present recommendations [36].

Despite all these possible complications, HSCT remains the treatment of reference for FA patients. Best results after HSCT have historically been reported from HLA-identical sibling donors [29], although recent advances in conditioning regimen, pharmacologic prophylaxis and graft manipulation techniques have remarkably improved outcomes of alternative donor HSCT [37–39] . So far, the best results are limited to young patients (less than 10 years) and before AML/MDS development. If the patients have developed AML/MDS, the 5-year overall survival is halved, independent of primary diagnosis, conditioning regimen or donor type [40] [41]. Because of this, it is necessary to find an alternative treatment for patients who cannot receive an HSCT and to try to avoid side effects.

2.2. Gene therapy by gene addition

As an alternative treatment option to HSCT, more and more studies are advocating the use of gene therapy to restore the hematopoietic system and reduce the risk of AML/MDS in FA patients. The general strategy is to introduce *ex vivo* the wild-type version of the patient's mutated gene (gene addition) or to correct the mutated gene (gene editing) using viral vectors.

There are different types of viral vectors used in gene therapy, the most common being adenoviruses and retroviruses. Adenoviruses have been used in 50% of total worldwide gene therapy trials. They have been mainly applied to novel vaccines, like Ebola [42] or COVID-19 [43] and cancer therapies [44]. However, adenoviruses can trigger strong immune responses and cellular toxicity in humans, reinforcing safety concerns for their use, and their scope of application is restricted to therapies that are not impacted by an immunological response [45], but not for FA patients. On the other hand, retroviruses were also used in drug development, with lentiviral vectors being the most common due to their safety profile. Lentiviruses are medium size (80–100 nm) enveloped single-stranded RNA viruses that are converted into double-stranded DNA during the replication process with a packaging capacity of up to 9 kb. Lentiviral systems derived from the HIV-1 virus have evolved through the years for safety reasons and to improve transduction efficiency. Lentiviral vectors have become the most widely used tools for *ex vivo* transgene delivery for FA gene therapy because they have many attractive features, such as being able to transduce non-dividing cells and their low immunogenicity [45].

FA, as mentioned above, is caused by the loss of function of one of the 23 genes involved in the FA/BRCA pathway. Therefore, the "classical"

approach of gene therapy has been to insert the wild-type gene into the patient's deficient cells. Inserting the "normal" gene synthesizes a functional protein, thus restoring the affected pathway.

First studies demonstrating that it is possible to correct the phenotype of FA cells through the use of *ex vivo* gene therapy date back to the beginning of the 21st century. Before that, some preclinical trials were performed with little success due to the inability to efficiently grow *in vitro* hematopoietic progenitors and in their consequent resistance to retroviral transduction. In 2001, Grompe lab has shown that it is possible to correct hematopoietic progenitor cells from FANCC deficient mice using retroviral vectors to increase survival after MMC treatment [46]. However, due to the success of third generation lentiviruses, which were able to infect quiescent cells, studies focused on this method of transduction [47]. In this way, Walsh lab was the first to demonstrate the transduction efficacy of the lentiviral vectors using FANCC deficient Epstein-Barr virus-transformed lymphoblasts [48]. One year later, the Verma laboratory demonstrated that quiescent hematopoietic progenitors from FANCA- and FANCC-deficient mice can be corrected using a transduction protocol that does not include *ex vivo* expansion of progenitors, as resistance to DNA-damaging agents was fully restored, allowing *in vivo* selection of corrected cells [49].

Many preclinical studies have demonstrated the efficacy of FA complementation. Most of them use a FA Subtype A model (FA-A) that is deficient in FANCA, because it is the most common mutation in FA patients (about 60–70% of the cases) [50] [8,51]. Moreover, there are some phase I/II clinical trials for FANCA complementation. The aim of these studies is, on the one hand, to improve the safety and efficacy of transduction and, on the other hand, to improve CD34+ mobilization since this is a major problem for FA patients. One of the most promising trials about the search for an optimized CD34+ mobilization protocol is FANCOSTEM study (NCT02931071). This is a phase II trial that began in 2013 designed to assess the safety and efficacy of CD34+ cells mobilization with Plerixafor (Mozobil) and Filgrastim (G-CSF, Granulocyte Colony Stimulating Factor). Results showed that 9 of the 11 patients in the trial achieved the mobilization peaks required for hematopoietic progenitor stem cell (HPSC) correction and engraftment. The oldest patients (15 and 16 years old) were the only ones who did not reach that threshold [52]. Amazingly, same research groups in Spain, led by Dr. Bueren, have conducted the first clinical trial that aim to evaluate the safety and efficacy, in non-conditioned FA-A patients, of gene-corrected-hematopoietic stem cell (HSC) engraftment (NCT03157804). In FANCOLEN study, they used lentiviral vector carrying *FANCA* gene to do an *ex vivo* transduction. During the 30-month follow-up period of the 4 patients, a significant engraftment of gene-corrected HSCs was observed with no serious adverse events or genetic abnormalities [53]. Another clinical trial is currently underway to evaluate the long-term consequences of FANCA complementation in these patients (NCT04437771). The trial is planned to end in 2035. Actually, there are three more lentiviral-mediated clinical trials in phase I and phase II to evaluate the efficacy of hematopoietic cell-based gene therapy for pediatric patients with FA (Table 1).

The disadvantage of this method is the uncontrolled integration into the patient's genome, so it is necessary to locate the integration and study the long-term health risks due to the fact that mutations can be generated that can lead to the development of cancer. This is the reason for its high cost and long development times. The future for gene therapy is to develop non-integrative systems to reduce insertional mutagenesis, improve their safety application and reduce potential serious adverse effects. One of the most promising approaches is to correct the mutation in the patient's cells using genome editing tools. *In situ* genetic correction also presents the advantage to preserve regulatory DNA sequences for endogenous gene expression, in contrast to viral gene delivery.

2.3. Gene editing by CRISPR-Cas9 technology

Genome editing with designer nucleases has recently made tremendous progress with the advent of the CRISPR-Cas9 technology which shows strong promise for the correction of human gene mutations *in situ*. Other nucleases have also been used for genome engineering such as transcription activator-like effector nucleases (TALENs) or zinc-finger nucleases (ZFNs). In the context of FA, ZFNs have been used to target a wild-type FANCA transgene encoded by an integrase-deficient lentiviral vector at the safe AAVS1 locus in FA-A cells [54]. Nevertheless, the versatility and simplicity of the CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 system makes it currently the tool of choice for genome editing. It contains two components: the Cas9 nuclease and a guide RNA (gRNA). The gRNA serves to recruit the Cas9 protein and is designed to target a unique genomic locus where Cas9 induces a DNA double-strand break (DSB) [55]. To repair Cas9-induced DSBs, there are two main repair pathways: non-homologous end joining (NHEJ) and homologous recombination (HR). While NHEJ directly fuses two broken ends to seal DSBs, which is frequently accompanied by small insertions or deletions, HR requires external homologous donor DNA to produce precise insertions, deletions or base substitutions [56]. The use of a Cas9 nickase mutant allows to favor HR events over NHEJ.

The HR pathway is the most desirable DNA repair pathway for repairing CRISPR-Cas9 DSBs. However, this process is relatively inefficient in FA cells, as they are inherently deficient in HR DNA repair [57]. Moreover, the FA pathway was reported to be critical for genome editing when single-stranded DNA is used as a donor [58]. Nevertheless, several groups showed the feasibility of HR-based gene editing strategies to restore different wild-type FANC gene sequences in FA cells or FA mouse models [59,60] [61,62]). However, the gene editing efficiency remains often moderate and can sometimes require a selection strategy such as the use of poly ADP ribose polymerase (PARP) inhibitors to select gene-corrected FANCD1 primary patient fibroblasts [61]. Alternatively, different research groups have been working on NHEJ-mediated repair of a CRISPR-Cas9-induced DSB to introduce compensatory mutations restoring a functional FANC protein [62] [63]. In 2019, Paula Rio's lab proved the validity of NHEJ editing approaches to correct the mutation of FANCA, FANCB, FANCC, FANCD1/BRCA2 or FANCD2 in lymphoblasts or HSCs from FA patients [63]. Although this is not applicable to all types of mutations, this strategy without the need for a donor DNA is simpler and can be particularly interesting in the setting of a HR-

Table 1
Active clinical trials of gene therapy in FA disease.

Clinical trial	Status	Study title	Locations	Phase	Description
NCT01331018	Active, not recruiting	Gene Therapy for FA	Fred hutch/ University of Washington cancer consortium (United States)	I	Access the toxicity and efficacy of infusion of gene modified cells using a lentiviral vector carrying the FANCA gene.
NCT04248439	Recruiting	Gene Therapy for FA-A	Stanford University and University of Minnesota (United States)	II	Enriched CD34+ cells will be transduced *ex vivo* with the therapeutic lentiviral vector and infused *via* intravenous infusion following transduction without any prior conditioning.
NCT04069533	Estimated completion date in January 2023	Lentiviral-mediated Gene Therapy for pediatric patients with FA-A	Hospital Infantil Universitario Niño Jesús (Spain)	II	HSC mobilized peripheral blood of FA-A patients will be transduced *ex vivo* with a lentiviral vector carrying the *FANCA* gene.

defective background.

CRISPR technology also has risks, such as off-target effects. To minimize the off-target effect several improvements are being developed, such as limiting the expression of Cas9 in the cells. For example, Ding lab has developed a new strategy, using an additional cassette with a gRNA targeting Cas9 itself, that was co-expressed with the gRNA that recognizes the target gene. In this way, the viral vector would be simultaneously targeted along with the target gene, resulting in a much-reduced expression duration of Cas9 [64]. Another interesting approach to minimize risk involves mRNA editing. This has been notably developed by the Zhang lab by fusing a catalytically inactive type VI CRISPR-associated RNA-guided RNAse Cas13b to the deamination domain of ADAR2. This system allows the correction of pathogenic G > A mutations as deamination of adenosine leads to inosine, which is functionally equivalent to guanosine in translation, and has been applied to a particular mutation of FANCC with up to 23% of the transcripts being corrected [65]. In addition, different bioinformatics methods are being developed to identify the outcomes induced by CRISPR-Cas9 DNA repair, which are able to capture and quantify off-targets [56].

CRISPR technology also allowed the development of elegant DNA base editor systems without the need of introducing a DSB. The system is based on engineered base deaminases fused to a catalytically impaired CRISPR-Cas9. There are two different classes: cytosine base-editors (CBEs) and adenine base-editors (ABEs), allowing the four base transition mutations (C → T, T → C, A → G, and G → A). Both perform precise nucleotide substitutions in a programmable manner, without requiring a donor template [66,67]. In the context of FA, Moriarity lab used both CBEs and ABEs systems to correct FANCA mutations in primary patient fibroblasts and lymphoblasts [68]. Very recently, the Corn lab also employed ABEs to restore a functional FANCA expression in FA-HSPCs. Moreover, base editing of HSPCs from healthy donors did not affect their long-term repopulating capacity [69].

Recently, a new gene editing technique, called prime editing, has further expanded the CRISPR-base-editing tool. Lui lab has succeeded in improving its own DNA base editor technology, increasing versatility and accuracy and decreasing off-target effects. Prime editing is composed of an engineered reverse transcriptase fused to catalytically impaired Cas9 nickase that introduces a nick in the R-loop at the target DNA site and a specific prime editing guide RNA (pegRNA). The pegRNA anneals to the nicked target DNA strand and contains the desired edit which serves as a template for the reverse transcriptase. This generates a 3′ DNA flap containing the edited sequence that will be incorporated *via* the cellular DNA repair machinery. Using this technology, it is possible to introduce any base-to-base change or targeted insertions and deletions [70].

2.4. HSC generation from iPSCs

Despite the progress in gene therapy and genome editing, the scarcity of hematopoietic progenitor and stem cells available for genetic correction/complementation remains a limitation for FA treatment [6]. One way to overcome this is to generate HSC or HPSCs from differentiated patient cells. This requires a first step to induce pluripotency in primary human cells to generate induced pluripotent stem cells (iPSC) through the expression of the OSKM gene cocktail (Oct4, Sox2, Klf4, and c-Myc) and then a reprogramming step to hematopoietic lineage to generate HSC/HPSC, which are then infused into the patient to increase blood counts.

The production and differentiation of iPSCs is a field in continuous development. iPSCs have been one of the most challenging cell types to grow in culture, but advances in reagent development now allow most laboratories to expand them using commercially available products. However, there are many studies on how to increase the reproducibility and efficiency of iPSC reprogramming and decrease timelines and costs, and protocols need to be improved to exploit their full potential in clinics.

In order to take advantage of this tool for FA therapy, it first appeared that complementation or genetic correction should be performed before cell reprogramming. Indeed, a defect in the FA DNA repair pathway strongly compromises iPSC derivation while no defect in iPSC generation and their differentiation capacity were observed after complementation with viral vectors [71,72]. However, using distinct strategies and protocol adaptations, several studies could also achieve FA cells reprogramming into iPCS although with moderate efficiency [72] [73] [60,74]. Interestingly, one way to obtain FA iPSCs is to down-regulate p53 during the reprogramming procedure [73], in line with the pathological overactivation of p53 in FA [6]. Tolar lab also showed how to reprogram fibroblasts from a FANCI deficient FA-patient to induce pluripotency before gene correction using CRISPR/Cas9 technology [60]. However, an inducible system to complement FANCA mutation in patient-derived iPSCs revealed that the FA pathway is required to prevent progressive exhaustion of iPSC cultures and accelerated cell differentiation [75,76].

Thanks to the development of these new gene editing strategies, the therapeutic application of gene editing in hematological disorders including FA is very close [77,78] but due to current cost and ethical questions, it may have rich its glass ceiling and may be difficult to apply to a large number of patients.

3. Is mRNA treatment an opportunity?

Technological advances are leading to the development of innovative, potentially curative DNA-directed gene therapies for the restoration of hematopoietic function and MDS/AML development prevention in FA patients. However, the hurdles to clinical implementation are high and ultimate success is uncertain. These include the long development times and high cost, associated with the necessity for clinical grade viral vectors under good manufacturing practice standards (GMPs), and long-term health risks due to erroneously triggered mutations (cancer risk) and the viral nature of the system. The solution can be to develop a non-viral gene-based therapy system that can be repeatedly administered and precisely targeted, in a dosed and controlled manner, to achieve a personalized outcome without those long-term health risks. The future of this therapy could lie in the use of messenger RNA (mRNA) to complement FA cells.

Synthetic mRNA was long considered insufficiently stable for pharmaceutical applications due to the high sensitivity to degradation by the omnipresence of RNA-degrading enzymes, RNAses, the difficulty to produce mRNA in sufficient amount, quality and the activation of an innate immune response after their administration because of their interaction with cellular RNA sensors, including Toll-like receptors (TLRs), PKR and RIG-I [79,80]. Over the last decades, extensive efforts on *in vitro* transcription (IVT) methods have allowed for synthetic mRNA to become an interesting class of gene therapy. Synthetic mRNA is a single stranded RNA molecule with four key elements; the 5′ cap that projects against exonucleases and promoting translation initiation, the untranslated regions (UTRs) that control the stability, translation efficiency, and subcellular location of mRNA, the open reading frame (ORF) that provides the sequence for translation, and the poly(A) tail that is involved in translation initiation. Tail length of the poly(A) can also affect stability and translation efficiency of the mRNA [81]. Optimization of these elements have led to increased mRNA uptake, intracellular stability and translational efficiency, and studies are still ongoing for further tuning of mRNA as therapeutic. Activation of the intracellular innate immune system is avoided by either of the two main strategies for de-immunization: i) chemical modification of nucleotides, and ii) sequence modifications to eliminate recognition motifs for innate immune receptors. The latter is achieved through synonymous codon optimization, but the majority of studies and drug development trajectories focus on various types of chemically modified nucleosides, being pseudouridine the most commonly used [82].

Regarding the difficulty to produce mRNA of sufficient quality,

purification methods have made incredible progress from the traditional lab methods such as lithium chloride precipitation. Purification is key to obtain mRNA that has minimal contaminants such as short transcripts or dsRNA, as these can activate the intracellular innate immune system and reduce translation efficiency [83]. For large scale purification, high performance liquid chromatography (HPLC) is often used given its selectiveness, versatility, scalability and cost-effectiveness. The most used methods are ion pair reverse-phase chromatography (IPC), ion exchange chromatography (IEC) and affinity-based separation. On one hand, using IPC, dsRNA impurities are effectively removed, but it is difficult to scale. On the other hand, IEC removes impurities and it is scalable and cost-effective, but must be performed under denaturing conditions, making this process more complex. Finally, affinity-based separation (such as poly-T tails that specifically bind to poly-A mRNA tails) can obtain high purity products, but the cost-effectiveness is lower [84]. While the recent success of the COVID-19 vaccines provided proof of the large-scale production, next steps are focused on optimizing the purification method together with in-process quality control.

Given the large, anionic nature of the mRNA, a delivery vehicle is required to mediate cellular entry. Lipid nanoparticles (LNPs) are among the most active non-viral delivery systems and have been approved by the FDA and EMA for an siRNA drug (Onpattro) and most recently for the COVID-19 vaccines [85]. The general structure of LNPs consists of four main components: a neutral helper lipid, cholesterol, a polyethylene-glycol (PEG)-lipid, and ionizable or cationic lipids. Neutral helper lipids create a lipid core to protect the mRNA in a hydrophobic medium and cholesterol maintains a balance between fluidization and condensation of the lipid bilayer by either creating or filling up bilayer defects. The PEG-lipids further increase particle stability by preventing particle aggregation, and they can act as shielding by inhibiting particle attachment to serum proteins to prolong the circulation time. Also, the amount of PEG-lipid can be used to control the particle size. Finally, PEG-lipids can be used to conjugate specific moieties that allow for targeted delivery. In contrast to ionizable lipids, cationic lipids have a permanent positive charge. This can be used for the interaction with the anionic mRNA and facilitates its internalization. Ionizable lipids are neutral a physiological pH but become protonated at low pH. As such, they improve biocompatibility of the LNP given a limited interaction with the anionic membranes of blood cells. Once taken up by cells, the ionizable lipids become positively charged due to the low pH in the endosomes and facilitate endosomal escape necessary for efficient protein expression [85]. While LNPs are most used, there are other types of delivery with potential for mRNA-based therapeutics such as polymer-based particles and polymer-lipid hybrid particles [86,87]. Additionally, extracellular vesicle-based systems have been utilized in both *in vivo* and *in vitro* systems successfully to facilitate the *in vivo* efficacy of mRNA-based therapeutics, increased stability and specificity, and prolonged circulation during systemic delivery [88–90]. Some preclinical work on hematopoietic cells demonstrated the potential of the nanoparticule strategy to target hematopoietic cells [91,92].

With these latest developments on mRNA technology and delivery vehicles, the field of mRNA therapeutics is growing significantly. This is also evidenced by the variety of clinical trials in phase I/II using mRNAs to treat different diseases such as solid tumors, heart disease, cystic fibrosis or melanoma [93]. Though, several aspects must be improved for mRNA therapeutics to reach full potential. This includes optimizations in dosing, delivery and stability [87].

Given the transient nature of mRNA, repeated administrations are required to sustain therapeutic levels of protein. The dosing frequency may depend on the mRNA stability, the half-life of the protein, its activity, as well as the turnover rate of the target cell. With regards to delivery, current LNPs are mostly taken up in the liver. This tropism is largely determined by the protein corona that covers the surface of the nanoparticle once it enters the bloodstream. While several groups demonstrated delivery to other organs (*i.e.*, lung, spleen) based on LNP charge, further studies are needed on 'active targeting'; the delivery of mRNA in on-target tissues or cells by conjugation of a targeting moiety (*e.g.*, antibody, ligand) [87]. Such targeting may also be relevant in terms of possibilities for lower dosing. Finally, efforts are focused on methods to optimize shipping and storage. On one hand this is needed for consistent activity of the mRNA drug product across multiple production runs and stability over longer time, and on the other hand it would reduce the need for cold-chain logistics.

4. Conclusions and future directions

FA is one of the most frequent inherited causes of BM failure, whose symptoms usually appear at a median age of 7 years of life [94]. Regular blood transfusions can significantly improve blood counts for many FA patients, but this does not prevent progression of the underlying bone marrow disease and the development of cancer [95]. Currently, the only approved treatment for FA is HSCT. This is an important therapeutic option but donor sources are a limitation as well as the high morbidity, immunological complications and exposure to radiation or chemotherapy, which increase the incidence of several carcinomas in long term [96,97]. To overcome these limitations, autologous transplantation of patients' own cells after gene therapy seems to provide promising results. However, the development of a safe and effective gene therapy approach still faces some challenges.

Since the first FA gene therapy trial in 1993 (NCT00001399), in which retroviral vectors were designed to transfer a normal FANCC gene into deficient FA-C CD34+ cells, significant achievements have been made. Improved HSC mobilization and lentiviral transduction protocols have been developed to increase correction efficiency and facilitate successful engraftment, even in the absence of conditioning [53]. Moreover, the development of iPSCs holds great promise to overcome the low number of HSCs in FA patients. Great advances in the field of genome editing have also accelerated the future implementation of gene therapy for FA disease. In this area, the simplicity and versatility of the CRISPR/Cas-based RNA-guided DNA endonucleases have left ZFNs and TALENs behind. Recent development of DNA base editor and prime editing systems has increased the effectiveness, targeted scope and purity of the edited product and minimized off-target effects to increase safety [70]. These new technologies of gene therapy have promising outcomes in order to restore hematopoiesis in FA patients.

Unfortunately, the barriers to clinical application of FA gene therapy are still high and its success is not assured. Because of this, we would like to highlight mRNA-based therapeutics as a potential FA treatment. The recent success of mRNA vaccines in the fight against COVID-19 has significantly advanced the idea of mRNA therapeutics as a promising new class of medicine. Compared to DNA-based drugs, RNA is less biologically stable and does not come with the risk of foreign integration in the genome, making it inherently safer. Other advantages include the relatively simple, rapid and cost-effective development, and the ability of mRNA to be programmable (by sequence engineering), which allows for controlling translational efficacy and immunogenicity [93]. On the other hand, LNPs for mRNA delivery have also undergone significant progress, both in their production, stability, formulation and storage [85].

As a result of these developments, mRNA is increasingly considered for replacement therapy, where it is used as a drug to compensate for a defective gene/protein, or for cell therapy, where cells are modified with mRNA *ex vivo* and re-infused into the patient [98]. While this type of application still presents challenges in terms of repeated administration, tissue targeting and stability, mRNA therapeutics is a rapidly emerging field with unexplored capacity to treat FA disease.

Practice points

- Hematopoietic stem cell transplantation (HSCT) is the current standard treatment for FA patients.

- The use of lentivirus for semi-random gene insertion, of *ex-vivo* gene is a promising strategy at the clinical trial stage.
- Technical barriers to use CRISPR/Cas9-based gene correction are now behind and targeted gene therapy to correct FA mutation can be considered.

Research agenda

- Follow-up is needed to evaluate long-term safety and side effects of genome editing in FA patients.
- CRISPR/Cas9-based gene correction needs to be evaluated in a clinical trial.
- The use of mRNA-LNP to treat FA is still at the level of the idea and preclinical work is needed.

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Author contributions

EMB has written the original draft of the review with contributions from JHG, JAW has written the section on LNP-mRNA therapy, while ABPO and CL have designed, supervised, and edited the manuscript.

Declaration of Competing Interest

JAW is CEO of Mercurna BV, a company that develops mRNA therapeutics.

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