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The clinical progress of mRNA vaccines and immunotherapies

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The emergency use authorizations (EUAs) of two mRNA-based severe acute respiratory syndrome coronavirus (SARS-CoV)-2 vaccines approximately 11 months after publication of the viral sequence highlights the transformative potential of this nucleic acid technology. Most clinical applications of mRNA to date have focused on vaccines for infectious disease and cancer for which low doses, low protein expression and local delivery can be effective because of the inherent immunostimulatory properties of some mRNA species and formulations. In addition, work on mRNA-encoded protein or cellular immunotherapies has also begun, for which minimal immune stimulation, high protein expression in target cells and tissues, and the need for repeated administration have led to additional manufacturing and formulation challenges for clinical translation. Building on this momentum, the past year has seen clinical progress with second-generation coronavirus disease 2019 (COVID-19) vaccines, Omicron-specific boosters and vaccines against seasonal influenza, Epstein–Barr virus, human immunodeficiency virus (HIV) and cancer. Here we review the clinical progress of mRNA therapy as well as provide an overview and future outlook of the transformative technology behind these mRNA-based drugs.

The medical promise of mRNA has been finally realized with the full approval of two rapid-response mRNA vaccines against COVID-19: Comirnity (BNT162b2) and Spikevax (mRNA-1273). Moderna's mRNA-1273, one of several mRNA vaccines directed against the SARS-CoV-2 spike (S) protein, was first administered to human volunteers on 16 March 2020, within weeks of the virus sequence being published on 11 January 2020 (refs. [1,2]). This remarkable achievement was facilitated by almost a decade's worth of clinical experience with mRNA vaccines for infectious disease and cancer (summarized in Fig. 1).

The concept of using mRNA to encode proteins for either vaccination or protein replacement received its first in vivo validation in 1990, when Wolff et al. demonstrated the production of a target protein after intramuscular (i.m.) injection in mice[3]. It took several decades, however, before the promise of this technology was clinically validated, a delay due, in part, to technical difficulties with mRNA stability and delivery and an interim shift in research priorities, funding efforts and industry focus to DNA vaccines during the 2000s[4]. In the meantime, the potential advantages of mRNA as a vaccine moiety (ease and speed of design and testing, inherent immunogenicity, rapid scale up and manufacture[5], and negligible risk of insertional mutagenesis[6,7]) meant that a small number of dedicated academics continued to work on this single-stranded nucleic acid.

One particularly important advantage of mRNA technology arises from its biological role as a template for protein translation. Whereas conventional vaccine technology relies on bulk production of a vaccine using mammalian cells in a bioreactor or chicken eggs, mRNA vaccines turn into the final product only once inside a patient's cells. In effect, mRNA uses the human body as its own vaccine-production facility, with several accompanying advantages.

First, it allows human post-translational modification (PTM) of protein products with the potential for less immunogenicity and full functionality. Second, multimeric proteins that are not amenable to production in a bioreactor can be translated, folded and assembled correctly in the patient's cells[8] (for example, Moderna has designed a cocktail of five mRNA species for an investigational cytomegalovirus (CMV) vaccine (mRNA-1647) that produces a pentameric protein[9,10]). And third, mRNA therapy can produce transmembrane and intracellular proteins and traffic them to their appropriate site in the cellular environment.

Compared with viral vector-based modalities, mRNA also has a reduced potential for either pre-existing antibodies against the vector (which limit the patient pool eligible for treatment) or the generation of post-dosing antibodies, which decrease the efficacy of subsequent doses[11]. Although the generation of anti-vector antibodies for RNA nanoformulations has been observed in preclinical models, methods have been developed to eliminate these responses, enabling repeat dosing without reduced efficacy[12–14]. There are also examples of successful repeat human dosing[15] and repeated human dosing with related short interfering RNA nanoformulations[16].

Similar to other drugs, the dose of an mRNA can easily be titrated up or down, with a longer or shorter interval, depending on an individual patient's need, weight and disease state. In addition, the duration of action is intrinsically limited, reducing the likelihood of irreversible side effects and enabling treatment of acute indications[7,8]; as mRNA degradation is regulated by normal cellular processes, in vivo half-life can be regulated through modifications to the molecule and the delivery methods[17–20].

In the following Review, we provide a broad overview of the clinical landscape of mRNA medicines. We give particular emphasis to technological innovations in manufacturing and formulation that have turned this approach from a vision into approved vaccines, the lessons learned so far from clinical trials and the challenges that we envision for future research, including the prospects of other modalities, such as mRNA-encoded protein and cellular

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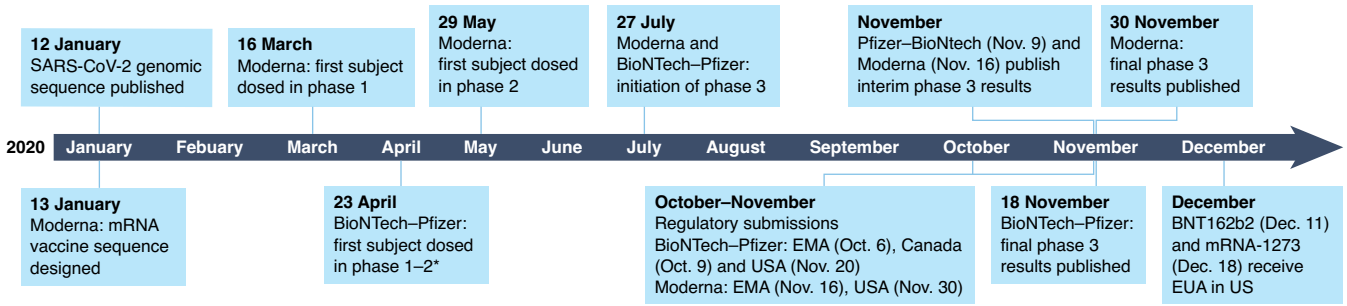

Fig. 1 | 2020 timeline showing rapid development of mRNA vaccines against SARS-CoV-2. *BNT162 phase 1–2 trial investigated several drug candidates, with BNT162b2 selected for phase 3 trials. EMA, European Medicines Agency.

immunotherapies. Because of space constraints, we refer readers to several recent reviews that cover mRNA-transfected dendritic cells (DCs)[10,21] or self-replicating mRNA derived from viruses[7,9,10], which are not covered here in detail.

mRNA as a medical product. mRNA medicines fall into three basic categories: preventative vaccines, therapeutic vaccines and protein-encoding therapies. Although each application has its own unique set of challenges, one challenge common to all is the requirement for intracellular delivery of the mRNA moiety to target cells while preserving mRNA stability. RNA is intrinsically an unstable molecule, and much of the early work on turning the concept of mRNA medicines into a reality focused on stabilization. Various techniques have been used for this, including optimizing the 5′ cap structure and the 3′ poly(A) tail length as well as regulatory elements within the 5′ and 3′ untranslated regions[22] (the reader is referred elsewhere for a detailed discussion of these techniques)[23–25].

In addition to these advances made in improving mRNA stability, effective in vivo mRNA medicines also required efficient intracellular delivery. A decade's worth of experimentation, which started with naked mRNA and then explored the condensation of mRNA into nanoformulations, has converged toward an increased focus on lipid formulations to achieve delivery. A typical lipid nanoparticle (LNP) formulation is composed of (1) an ionizable or cationic lipid to interact with the polyanionic RNA, (2) a helper phospholipid (for example, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC)) that resemble the lipids in the cell membrane and support the bilayer structure), (3) a cholesterol analog to adjust the fluidity of the lipid bilayer and (4) a polyethylene glycol (PEG)–lipid to improve colloidal stability and decrease opsonization (Fig. 2).

Although substantially less advanced clinically than LNPs, polymeric nanoparticles (PNPs) have also shown promise as delivery systems. These formulations are generally composed of a biodegradable, amine-containing polymer that can self-assemble with RNA. Depending on the application, PNPs may also be formulated with helper phospholipids, cholesterol and PEG–lipid (Fig. 2). Both LNPs and PNPs may be further modified with specific ligands to facilitate cell-specific targeting. The specific compositions of non-viral vector formulations in development greatly vary and can have substantial effects on the efficiency of intracellular delivery and the cell types targeted by the nanoparticle–mRNA complex as well as immunogenicity of the administered mRNA medicine.

Eliciting appropriate immunogenicity when desirable (vaccines) or to elude it for other indications (mRNA protein-replacement therapy) is an important aspect to consider when manufacturing and formulating mRNA medicines. RNA, as the genetic material of RNA viruses or a byproduct of the replication of DNA viruses, can be a powerful stimulus to the innate immune system. Microbial RNA has a number of structural and sequence characteristics that distinguish it from self RNA that can be recognized by pattern-recognition receptors in host cells. Two systems of pattern-recognition receptors have evolved to orchestrate an appropriate immune response by the production of type I interferons (IFNs) and inflammatory cytokines: the first, the Toll-like receptor (TLR) system, is located in the plasma membrane, endosomes and lysosomes of epithelial and immune cells, including DCs, monocytes and macrophages[26]; the second, the retinoic acid-inducible gene I (RIG-I)-like receptors, are located in the cytosol of most cells[27]. TLR3 is activated by double-stranded RNA (dsRNA), whereas TLR7 and TLR8 are activated by single-stranded RNA. RIG-I and melanoma differentiation-associated protein 5 (MDA5) are differentially activated in the cytosol by 5′-triphosphorolyated short (18–19 bp) dsRNA and long (>1,000 bp) dsRNA, respectively[27]. TLR3 activation leads to the production of type I IFN via the TIR domain-containing adaptor molecule 1 (TICAM-1) pathway, whereas the other TLRs use a MYD88-dependent cascade that leads to a nuclear factor (NF)-κB-dependent or an IFN regulatory transcription factor (IRF)3-dependent production of pro-inflammatory cytokines[28]. The extent to which these pathways are activated, if at all, by the mRNA product and its delivery vector greatly depends on the application and is a key aspect in the development of mRNA medicines.

The landscape of biomedical uses of mRNA continues to rapidly evolve. Below, we divide our discussion into three areas: direct in vivo administration of mRNA for preventative vaccines against infectious disease (Table 1), therapeutic mRNA vaccines against cancer (Table 2) and mRNA-encoded immune therapies (Table 3). We refer the reader to Supplementary Table 1 for a complete list of all mRNA species in clinical testing at the time of writing. To provide up-to-date information on clinical advances, we have used as sources papers indexed in PubMed, company press releases, postings at https://clinicaltrials.gov/ and US Securities Exchange Commission filings through August 2021.

Vaccines for infectious disease. Following the US Food and Drug Administration (FDA) approvals for COVID-19 vaccines, mRNA is now recognized as a potentially transformative vaccine modality in infectious disease (Table 1). The field eagerly awaits further validation of mRNA vaccines directed against pathogens other than SARS-CoV-2.

The mRNA molecule's inherent immunostimulatory nature and ability to function as an immunoadjuvant were seen as a key strength for vaccine application[22]. Turning these characteristics into a safe and efficacious clinical product presents the challenge of balancing immune stimulation with expression of the encoded antigen. Thus far, the most clinically advanced products are non-replicating mRNA vaccines featuring chemically modified and unmodified nucleotide bases. The two approved mRNA products, Pfizer–BioNTech's BNT162b2 and Moderna's mRNA-1273, are vaccines with chemically modified uradine bases.

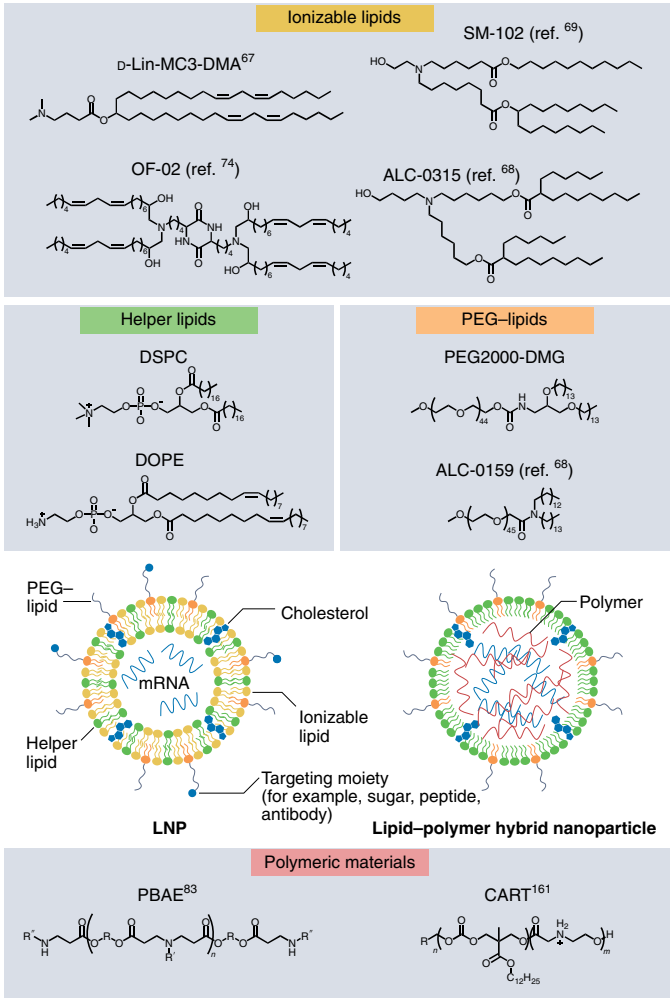

Fig. 2 | Composition of mRNA-delivery nanoparticles. A typical LNP is composed of RNA, an ionizable lipid, a helper lipid, cholesterol and PEG-lipid. Representative members from these categories are shown, including D-Lin-MC3-DMA[65], SM-102 (ref. [67]), OF-02 (ref. [72]), ALC-0315 (ref. [66]) and ALC01596 (ref. [66]). Although less advanced clinically, PNPs are composed of RNA and primarily a biodegradable, ionizable polymer. An example of a polymer from the hyperbranched poly(β-amino ester) (PBAE) class[81] and one from the charge-altering releasable transporter (CART) class[77] are shown. PNPs may include helper lipids, cholesterol and PEG lipids, depending on the application. Delivery nanoparticles may be modified with targeting ligands (small molecules, antibodies, etc.) to alter cellular tropism. DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine.

By contrast, the results of unmodified RNA vaccine trials for COVID-19 thus far have been disappointing. Although definitive data remain to be released, CureVac reported that its unmodified CureVac COVID-19 vaccine (CVnCOV) shows only 47% protection against coronavirus infection[29]. Various explanations have been put forward to account for the lower efficacy of the unmodified CureVac vaccine compared with that of modified vaccines. CureVac has pointed to the changing SARS-CoV-2 variant landscape during the conduct of its trial, which was not the case for BNT162b2 or mRNA-1273; others have noted CureVac's use of a lower dose (12 μg) versus those of BNT162b2 (30 μg) and of mRNA-1273 (100 μg) (chosen as unmodified RNA is more reactogenic than modified RNA) may have been insufficient to produce an effective neutralizing antibody response; others have hypothesized that translation efficiency of unmodified RNA may be lower, resulting in lower epitope levels[30].

Apart from the above non-replicating mRNA vaccines, several groups are also pursuing self-amplifying constructs encoding RNA-dependent RNA polymerases that amplify the delivered RNA and thus increase antigen protein expression[13]. As of yet, these have only completed early-stage clinical testing. One potential drawback of this last type of vaccine is that any mRNA-delivery technology must contend with the substantially larger mRNA construct sizes associated with self-replicating mRNA vaccines.

Similar to recombinant protein vaccines, all the above mRNA vaccines have the advantage of not producing infectious particles. Thus, concerns associated with live attenuated viral vaccines or replication-competent viral vectors and their potential to revert to a pathogenic form or cause some form of exacerbated disease (as has been observed with a live attenuated respiratory syncytial virus (RSV) vaccine) do not apply. The absence of risk of insertional mutagenesis caused by integration into the recipient's DNA is another major advantage of mRNA vaccines compared with DNA vaccines or certain viral vectors.

Accelerated discovery and development times. The rapid spread of the SARS-CoV-2 pandemic across the globe highlighted the importance of vaccine technologies capable of rapid deployment for human trials. The speed of mRNA vaccine development was such that the first products had already entered clinical trials before studies in non-human primates confirmed that protective immunity could be achieved either by infection with SARS-CoV-2 (ref. [31]) or by a DNA vaccine[32].

The unprecedented speed with which mRNA companies pivoted toward producing SARS-CoV-2 vaccine candidates is illustrated by the chronology shown in Fig. 1 (refs. [1,2]). The remarkably similar timelines of two independent efforts of Pfizer–BioNTech[33] and Moderna[34] indicate a trajectory from genetic identification of a pathogen to EUA by regulatory agencies ~11 months or years shorter than the typical vaccine-development timeline.

Other mRNA companies have also been able to leverage their prior expertise in vaccine development in comparably rapid ways. For instance, CureVac announced approval to start a phase 1 study of CVnCOV in June 2020 (NCT04449276)[35] and interim results of its phase 2–3 trial (HERALD, NCT04652102) a year later; Arcturus announced interim results of a phase 1 study of ARCT-021 (NCT04480957) on 9 November 2020 (ref. [36]); and Sanofi Pasteur–Translate Bio (NCT04798027)[37], the Imperial College of London (ISRCTN17072692, Eudract 2020-001646-20) and Yunnan Walvax Biotechnology (ChiCTR2000034112) all took mRNA vaccines to human testing in less than a year from publication of the SARS-CoV-2 sequence.

The COVID-19 pandemic has challenged traditional approaches to vaccine development and created a unique environment to galvanize mRNA vaccine research. One key differentiator was the large injection of funding that companies received from the Biomedical Advanced Research and Development Authority and the Coalition for Epidemic Preparedness Innovations[1,38–40]. The public health emergency served to catapult development efforts into high gear and prompted manufacturers to find ways to reduce the time to clinic (for example, by parallelizing different parts of the serial development process, minimizing pilot studies and conducting minimal product-quality release testing); conversely, extensive validation of the new mRNA technology against established vaccines (as had been done previously in the side-by-side evaluation of mRNA vaccine CV7202 versus an inactivated strain vaccine, Rabipur[41]) was de-prioritized. For in-depth discussions and comparisons of the various technologies currently established or in development for vaccines, we refer the reader to some excellent reviews[39,42–44].

Manufacturing and scale up. Many of the advantages of mRNA (and some types of DNA) vaccines relate to the speed and flexibility of

Table 1 | Summary of past and ongoing clinical studies with mRNA vaccines for infectious disease, phase 2 or 3 only

Name of product	Payload (for example, antigen or protein)	Disease	Population	Route of admin	*N*	CTrials.gov or EudraCT number	Study phase	Trial status	Sponsor/ collaborators	Comments/ references
Infectious disease										
mRNA-1893	Structural proteins of Zika virus	Zika virus	Adults in endemic and non-endemic flavivirus areas	i.m., two doses given 28 d apart	800	NCT04917861	2	Active, recruiting	Moderna	[164]
mRNA-1647	Six mRNAs coding for pentamer viral antigen and gB protein of CMV	CMV infection	Healthy adults seronegative and seropositive for CMV	i.m. vaccine	446	NCT04232280	2	Active, recruiting	Moderna	[67]
mRNA-1647	Six mRNAs coding for pentamer viral antigen and gB protein of CMV	CMV infection	Healthy participants 16–40 years	i.m.	6,900	NCT05085366	3	Active, recruiting	Moderna	[102]
mRNA-1345	Stabilized prefusion F protein	Respiratory syncitial virus	Adults >60 years	i.m.	34,000	NCT05127434	2–3	Active, recruiting	Moderna	[102]
iHIVARNA-01	An HIV immunogen to induce T cell responses against relatively conserved, vulnerable portions of the virus, HTI	HIV	Adults with HIV	i.n. vaccine	33	NCT02888756	2	Terminated	Hivarna consortium, Etherna	Study terminated; interim analysis did not show sufficient immunogenicity compared with placebo[98,112,113]
mRNA-1273	Stabilized S protein of SARS-CoV-2	SARS-CoV-2	Healthy adults	i.m., deltoid muscle	600	NCT04405076	2	Active, not recruiting	Moderna	
mRNA-1273	Stabilized S protein of SARS-CoV-2	SARS-CoV-2	Healthy adults	i.m., deltoid muscle	30,402	NCT04470427	3	Active, not recruiting	Moderna	EUA[70]; rolling biologic license application submission[164]
mRNA-1273	Stabilized S protein of SARS-CoV-2	SARS-CoV-2	Children 12–17 years	i.m., deltoid muscle	3,732	NCT04649151	3	Active, not recruiting	Moderna	'TeenCOVE' study[164]
mRNA-1273	See above	SARS-CoV-2	Children 6 months–11 years	i.m.	6,975	NCT04796896	2–3	Active, recruiting	Moderna	'KidCOVE' study[164]
mRNA-1273	See above	SARS-CoV-2	Adults 65 years and older who have received two doses	Third dose	300	NCT04969276	2	Active, recruiting	Moderna	Fluzone 2020–2021 quadrivalent flu vaccine and third dose of mRNA-1273 either alone or concomitantly
mRNA-1273	See above	SARS-CoV-2	Adults 18–29 years	i.m.	37,500	NCT04811664	3	Active, recruiting	NIAID	
mRNA-1273	See above	SARS-CoV-2	Healthy adults	i.m.	1,200	NCT04894435	2	Active, recruiting	Canadian Immunization Research Network	MOSAIC study, investigating 'mix and match' of distinct vaccines
mRNA-1273.211	Combines mRNA-1273 and mRNA-1273.351 in a single vaccine	SARS-CoV-2	Patients who received two vaccinations under EUA	Third shot, i.m.	800	NCT04889209	1–2	Active, recruiting	NIAID	Delayed heterologous SARS-CoV-2 vaccine dosing (boost) after receipt of EUA vaccines
mRNA-1273.351	Codes for the full-length prefusion stabilized S protein of the SARS-CoV-2 B.1.351 variant	SARS-CoV-2 B.1.351 variant	Healthy adults	i.m.	135	NCT04785144	2	Active, not recruiting	Moderna	
BNT162 (four types)	Four different SARS-CoV-2 vaccines: BNT-162a1, BNT162b1, BNT162b2, BNT162c2	SARS-CoV-2	Healthy adults, including older ones	i.m.	456	NCT04380701	1–2	Active, recruiting	BioNTech–Pfizer	
BNT162b2	SARS-CoV-2	SARS-CoV-2	Adults who received two vaccinations under EUA	Third shot, i.m.	800	NCT04889209	1–2	Active, recruiting	NIAID	Delayed heterologous SARS-CoV-2 vaccine dosing (boost) after receipt of EUA vaccines
BNT162b2	SARS-CoV-2	SARS-CoV-2	Adults who have recovered from COVID-19 in the ACTIV-3–TICO trial	i.m.	640	NCT04969250	3	Active, recruiting	NIAID	
BNT162b2	SARS-CoV-2	SARS-CoV-2	Healthy children <12 years	i.m.	4,500	NCT04816643	1–3	Active, recruiting	BioNTech	
CVnCOV	SARS-CoV-2	SARS-CoV-2	Adults	i.m.	674	NCT04515147	2a	Active, not recruiting	CureVac	[47,84]
CVnCOV	SARS-CoV-2	SARS-CoV-2	Adults	i.m.	36,500	NCT04652102	2–3	Active, not recruiting	CureVac	[29,30,84,106]
ARCT-021	SARS-CoV-2	SARS-CoV-2	Adults	i.m., deltoid muscle	600	NCT04668339	2	Active, not recruiting	Arcturus	

Admin, administration; CTrials.gov, https://clinicaltrials.gov/; EudraCT, European Union Drug Regulating Authorities Clinical Trials; NIAID, National Institute of Allergy and Infectious Diseases.

Table 2 | Summary of past and ongoing clinical studies with mRNA vaccines for cancer, phase 2 or 3 only

Name of product	Payload (for example, antigen or protein)	Disease	Population	Route of administration	*N*	CTrials.gov or EudraCT number	Phase	Trial status	Sponsor/ collaborator	Comments/ references
BNT122 (RO7198457)	Up to 20 patient-specific neoantigens	Melanoma	Previously untreated advanced melanoma	i.v.	132	NCT03815058	2	Active, recruiting	BioNTech–Genentech	Pembrolizumab alone versus BNT122 + pembrolizumab[128]
BNT122 (RO7198457)	As above	Colorectal cancer	ctDNA$^+$ resected stage II and III colorectal cancer	i.v.	201	NCT04486378	2	Active, recruiting	BioNTech	
mRNA-4157	Can encode up to 34 neoantigens	High-risk melanoma	Resectable cutaneous melanoma metastatic to a lymph node and at high risk of recurrence	i.m.	150	NCT03897881	2	Active, recruiting	Moderna–Merck	KEYNOTE-942 study; combination with pembrolizumab[38,123,124]
BNT111	Mix of four melanoma-associated antigens	Melanoma	Anti-PD1 refractory/relapsed stage III or IV melanoma	i.v.	180	NCT04526899	2	Active, recruiting	BioNTech	BNT111 and cemiplimab in combination or as single agents[128]
BNT113	HPV16-derived tumor antigens (E6 and E7 viral oncoproteins)	HPV16$^+$ head-and-neck squamous carcinoma	Head-and-neck cancer positive for HPV16 and expressing PD1	i.v.	285	NCT04534205	2	Active, recruiting	BioNTech	AHEAD-MERIT; BNT113 + pembrolizumab versus pembrolizumab alone
CV9202	NY-ESO-1, MAGE C1, MAGE C2, TPBG (5T4), survivin, MUC1	NSCLC	NSCLC	i.d.	56	NCT03164772	1–2	Active, not recruiting	CureVac, Ludwig Institute	[136]

ctDNA, circulating tumor DNA; NSCLC, non-small cell lung cancer.

manufacturing, which is largely based on in vitro processes with chemical constituents. Because mRNA codes for the immunogenic protein of interest and no live virus is required, there is no need for specialized facilities or biosafety laboratories[44]. In contrast to egg-based vaccines, mRNA vaccines are not limited by egg-production capacity and allow vaccination of individuals with egg allergies[39]. Production in cell-free systems minimizes the risk of bacterial contaminants and eliminates the need for bioreactor processes[39].

All nucleic acid-based vaccines (whether mRNA or DNA) encode the immunogen of interest, but their characteristics are independent of that immunogen. The manufacturing of different vaccines with the mRNA platform relies on the same chemical components, which means that, once an investment has been made in the platform, it can readily be adapted to new pathogens as they are identified[44]. This is a particularly attractive feature in the context of preparedness for emerging epidemics or seasonal vaccines.

Furthermore, the same manufacturing processes can be used for vaccines and other mRNA-based medicines, providing efficiency and flexibility. In view of the emergence of new strains of SARS-CoV-2 while the rollout of the first-generation vaccines is underway, this flexibility to switch out the coding mRNA in the same delivery vehicle is especially useful. Although there is no consensus about the cost of manufacturing, the technology is expected to be more cost-effective than older methods[43].

Rapid antigen-specific sequence optimization. Another advantage of mRNA technology is the ability to design and redesign the antigen based on introducing changes in nucleic acids, which is a relatively straightforward process compared to the bioengineering of distinct proteins or peptides. For SARS-CoV-2, this has mainly taken the form of introducing prolines to stabilize the immunogenic S protein into the prefusion configuration.

BioNTech has applied this technical flexibility by putting no fewer than five different COVID-19 mRNA vaccine candidates into the clinic (three using nucleoside-modified mRNA, one using uridine-containing mRNA and one using self-amplifying mRNA). The BNT162b1 vaccine candidate uses nucleoside-modified mRNA to encode the SARS-CoV-2 S protein receptor-binding domain modified by the addition of a T4 fibritin-derived foldon trimerization domain to increase its immunogenicity[45]. BNT162b2, the final selected candidate, encodes full-length S protein modified by two proline substitutions to lock it in the prefusion conformation[46]. The other COVID-19 mRNA vaccines from CureVac[29,47], Moderna[48] and Translate Bio[49] also use the S protein with various modifications.

Several of the vaccines currently in development have gone through at least one iterative optimization step, which is a feature of mRNA product development. For instance, mRNA-1777, which targets RSV, was tested in a phase 1 trial, and interim data showed humoral immune responses as measured by neutralizing antibody levels after a single dose[50]. However, development of this drug has been paused in favor of mRNA-1172, which was shown to be more potent than mRNA-1777 in animal models[38]. Similarly, development of the Zika virus vaccine candidate mRNA-1325 (NCT03014089) was halted in favor of mRNA-1893 (ref. [38]), which uses a different sequence and was reported to be 20-fold more potent than mRNA-1325 in non-human primate studies. As of February 2020, 90 participants, both flavivirus seropositive and seronegative, have been administered mRNA-1893 or placebo in a dosing regimen of two doses 1 month apart, at doses of 10, 30 and 100 µg (NCT04064905)[38,51].

Encoding multiple proteins and/or protein subunits. For SARS-CoV-2, the S protein immunogen is a homotrimer[52], and thus only a single mRNA sequence needs to be introduced. For pathogens for which the main immunogen is composed of multiple subunits, the challenges for producing recombinant protein subunits and successfully reconstituting them with the correct stoichiometry into a full protein can readily be imagined. By contrast, mRNA lends itself easily

to this application. The separate subunits can either be coded in a single long mRNA or as separate mRNA strands. For instance, mRNA-1647, a vaccine targeting CMV, contains six mRNA species, five of which encode five different proteins that combine to form a pentameric protein, with the sixth encoding the CMV glycoprotein B (gB) protein[38]. In a related approach, a single vaccine can target two different pathogens, as is the case for mRNA-1653, which combines two mRNA species, targeting the F protein of human metapneumovirus (hMPV) and parainfluenza virus type 3 (PIV3)[38].

Modulating mRNA immunogenicity. Apart from iterative optimization of sequence to optimize the immunogenicity of antigens, innate immunogenicity of the full mRNA transcript itself and other RNA products produced during its manufacture can also be exploited to boost immune responses to mRNA vaccines. The innate potential immunogenicity of RNA may be advantageous in vaccinations because it can activate immune response pathways, such as the TLR system, that lead to DC maturation and subsequently robust B and T cell immune responses[53–55]. As mentioned previously, CureVac's CVnCoV features unmodified mRNA[47]. This RNA-driven immunostimulation, however, can also be detrimental, leading to clinical side effects as well as reduced expression of the protein of interest. Activation of RNA-dependent protein kinase R (PKR), for instance, has been implicated in translational inhibition[56].

In recent years, progress has been made in our understanding of how to modulate in vitro transcribed mRNA immunogenicity. One of the main methods for modulating mRNA immunogenicity has been substitution of unmodified nucleotides with chemically modified versions. Work by Kariko, Weissman and colleagues reported that certain nucleoside modifications, such as pseudouridine and 5-methylcytidine, significantly reduce TLR signaling and PKR activation, leading to increased levels of protein expression in mice[56,57]. Notably, both approved COVID-19 mRNA vaccines from BioNTech and Moderna (BNT162b2 and mRNA-1273) feature complete substitution of uridine with N_1-methyl pseudouridine[2,58]. When compared with mRNA with modified nucleosides, however, others have subsequently demonstrated that incorporation of unmodified nucleosides actually leads to higher levels of protein expression in HeLa cells and similar levels of expression in the liver of mice[59]. We hypothesize that improvements in mRNA purification and the removal of RNA contaminants may in part explain these differences from earlier work. Removal of dsRNA by column purification (high pressure liquid chromatography (HPLC) or fast protein liquid chromatography) and more recently by less-expensive filter-binding technology leads to substantially improved translation efficiencies[60,61]. Researchers at CureVac reported that HPLC-purified, sequence-optimized, unmodified nucleoside mRNA is not immunogenic and produced higher levels of protein expression in mice than chemically modified nucleoside mRNA[18]. Recently, scientists at Genentech have reported that interleukin (IL)-1β and IL-1 receptor agonist (IL-1RA) are key regulators that control systemic responses to mRNA, suggesting that differences between these regulatory elements in mice, primates and humans may explain the observed differences in reactogenicity of uridine-modified and unmodified mRNA in vivo in these species[62].

Nanoformulations for mRNA delivery. Although early efforts for mRNA vaccine delivery focused on naked mRNA or the use of protamine, recent trends in mRNA vaccine development have converged on LNPs for delivery of mRNA. An early rabies vaccine (CV7201) was formulated with protamine, but development was discontinued because the level of immunogenicity seemed critically dependent on the method of vaccination, with only a needle-free system providing the desired immune response after intradermal (i.d.) administration (NCT02241135)[63]; the development of this product was halted in favor of an LNP-formulated vaccine candidate, CV7202 (NCT03713086)[64]. Elsewhere, a naked mRNA agent (iHIVARNA-01), which combines TriMix (a mix of three mRNA species encoding constitutively activated TLR4, CD40 ligand and CD70, all of which are immunostimulatory molecules) and an HIV immunogen, has been evaluated for safety and efficacy in patients with HIV after three intranodal (i.n.) injections (NCT02413645); however, this study has since been discontinued due to lack of immunogenicity (NCT02888756).

Most mRNA medicines in the clinic now use LNPs for delivery. The first RNA-based oligonucleotide drug approved by the FDA (patisiran, a short interfering RNA drug for the treatment of the polyneuropathy of hereditary transthyretin (TTR)-mediated amyloidosis) is an LNP formulation comprising an ionizable lipid, D-Lin-MC3-DMA (MC3), together with DSPC, cholesterol and 1,2-dimyristoyl-*rac*-glycero-3-methoxypolyethylene glycol-2000 (PEG2000-DMG)[65]. The BioNTech COVID-19 vaccine BNT162b2 is formulated using ((4-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate) (ALC-0315), 2-((PEG)-2000)-*N*,*N*-ditetradecylacetamide (ALC-0159), DSPC and cholesterol[66]. It appears that the identification, testing and production of the appropriate lipid formulation may have been important in determining the speed of entry into clinical trials. For instance, mRNA-1273 uses the same LNP as mRNA-1647 (CMV virus vaccine) and mRNA-1653 (hMPV–PIV3 virus vaccine), for which some clinical and regulatory precedent had been established (Table 1)[67].

The composition of an LNP formulation can substantially affect intracellular delivery efficiency, determine cell specificity of delivery and modulate immunogenicity. Although all lipid components are important for LNP function, the ionizable lipid component of LNPs has received much attention given its key role in multiple aspects of RNA delivery, including particle formation, cellular uptake and endosomal escape[10,24]. The structural diversity of ionizable lipids found within LNP formulations is vast, and, to facilitate rapid synthesis and evaluation of ionizable lipids, combinatorial, high-throughput methods for synthesizing large libraries of new lipids and evaluating them in vivo have been developed[68–71]. The list of potent ionizable lipids capable of delivering mRNA in vivo continues to expand, with advances in both the potency of delivery vehicles[72] and tolerability through the introduction of biodegradable linkages[73,74]. Although the recent trend in lipid development has focused on the incorporation of hydrolysable bonds to facilitate clearance, these degradable bonds may affect formulation stability, which continues to be a challenge for LNP formulations. Advances in lyophilization of mRNA LNPs seem likely to improve formulation stability, but, for low-dose applications (for example, vaccines), hydrolytically resistant lipids may prove advantageous.

In addition to systemically delivered RNA nanoparticles, other modes of RNA application offer the potential to provide therapy to the nervous system[75], eye[76,77], heart[78,79] and lung[80–83]. Of particular note, nanoformulations based on both biodegradable polymers[81] and oligo polymers[82] as well as lipids[83] have been developed to facilitate delivery to the lung epithelium by nebulization. For instance, patients with cystic fibrosis have been dosed repeatedly with MRT5005, a nebulized formulation of an LNP-formulated mRNA coding for the cystic fibrosis transmembrane regulator protein (NCT03375047).

Storage and shipping. An important aspect related to formulation is storage and shipping conditions. The challenge of maintaining cold-chain shipping and storage for vaccines was highlighted during the Ebola epidemic of 2014–2016, when an investigational vaccine based on an attenuated recombinant vesicular stomatitis virus (approved in 2019 as Ervebo) had to be stored at −80 °C to −60 °C, which was not always practicable in the regions of Africa where the vaccine was most needed. COVID-19 affects all continents; from a global emergency preparedness perspective, it is imperative that

Table 3 | Summary of past and ongoing clinical studies with mRNA for protein-replacement applications, phase 2 or 3 only

Name of product	Payload (for example, antigen or protein)	Disease	Population	Route of administration	*N*	CTrials.gov or EudraCT number	Phase	Trial status	Sponsor/ collaborator
BNT141	Secreted IgG antibody	Cancer	CLDN18.2-positive tumors	i.v.	48	NCT04683939	1–2	Not yet recruiting	BioNTech
BNT311/ GEN1046	Bispecific antibody PD-L1×4-1BB	Cancer	Patients with solid cancers	i.v.	512	NCT03917381	1–2	Active, recruiting	Genmab–BioNTech
BNT312/ GEN1042	Bispecific antibody CD40×4-1BB	Solid tumors	Patients with solid metastatic or unresectable cancers	i.v.	447	NCT04083599	1–2	Active, recruiting	Genmab–BioNTech
BNT211	CAR T for CLDN6$^+$ tumor + mRNA for CLDN6	CLDN6$^+$ tumors	CLDN6$^+$ advanced or relapsed tumors	i.v.	36	NCT04503278	1–2	Active, recruiting	BioNTech

CLDN18.2, claudin 18 isoform 2; CLDN6, claudin 6; PD-L1×4-1BB, bispecific antibody targeting both the human PD-ligand 1 (PD-L1) receptor and 4-1BB (CD137; tumor necrosis factor receptor superfamily member 9); CD40×4-1BB bispecific antibody targeting both cluster of differentiation 40 receptor (CD40) and 4-1BB (CD137; tumor necrosis factor receptor superfamily member 9).

millions of doses could be shipped worldwide and across a range of extreme temperatures to countries with widely differing standards of health care infrastructure[39].

CureVac's CVnCoV has been reported to be stable and within defined specifications for at least 3 months when stored at a standard refrigerator temperature of +5 °C (+41 °F) and up to 24 h as ready-to-use vaccine when stored at room temperature[84]. Moderna's mRNA-1273 remains stable at −20 °C for up to 6 months, at refrigerated conditions for up to 30 d and at room temperature for up to 12 h[85]. By contrast, BioNTech's vaccine originally needed to be stored at −70 °C, and BioNTech's collaborator Pfizer had developed specific shipping boxes containing dry ice to facilitate the logistics of distribution. Subsequently, the sponsor submitted additional information supporting up to 2 weeks of storage at standard freezer temperatures[86,87].

The first-in-human trial, which usually involves only a limited number of participants and clinical trial sites, is sometimes performed with a less-than-optimal formulation, with the development of improved storage conditions proceeding in parallel with the clinical program. For instance, Moderna's mRNA-1647, a CMV vaccine candidate, was provided as a frozen liquid formulation for the phase 1 study but as a lyophilized formulation, stable at refrigerated temperatures for 18 months, for the phase 2 study[40].

The importance of these considerations of shipping, storage and stability is highlighted by the case of mRNA-1443, which targets the phosphoprotein 65 T antigen of CMV. This was evaluated in the same trial as mRNA-1647, but, in August 2018, the clinical material for mRNA-1443 failed to meet internal specifications after 1 year of storage and was subsequently the subject of a clinical hold[88,89]. Development of this vaccine appears to have paused[89].

Turning the formulation into a dry powder form is among the most commonly used methods for shipping and long-term storage of many pharmaceutical products. However, the stresses generated by crystallization and vacuum dehydration during the lyophilization process may decrease the stability of macromolecules or LNPs, inducing the loss of activity[90]. Adding appropriate cryoprotectants, such as trehalose, sucrose and mannitol, is reported to preserve the stability of LNPs in a formulation-specific manner[90,91]. Pfizer has initiated a phase 3 study to compare the safety and tolerability of the lyophilized BNT162b2 formulation to those of its frozen liquid BNT162b2 formulation (NCT04816669).

Route of administration. There is no consensus yet as to the best route of administration, although the approved SARS-CoV-2 mRNA vaccines employ i.m. administration. Global rollout of pandemic vaccines is ideally supported by a low-tech route of administration requiring little training of the numerous health care providers called upon to administer the vaccine. BTN162b2, CVnCoV, ARCT-021 and mRNA-1273 are administered via i.m. injection. A group at the Imperial College of London is considering studying their self-amplifying mRNA COVID-19 vaccine after inhalation, similar to what has been done for seasonal influenza[92]. An inhaled or intranasal vaccine may elicit both cellular and humoral responses that are particularly effective at neutralizing infectious respiratory viruses such as SARS-CoV-2 (refs. [93–95]); however, studies of intranasal mRNA vaccines have been limited to preclinical animal models[96], with further development of LNP carriers likely required to effectively target appropriate cell types in the upper respiratory tract.

In the broader mRNA field, both i.d. and i.m. injections have been used for the evaluation of candidate vaccines, sometimes for the same vaccine within the same study. The two routes of administration can yield divergent results, both in terms of immunogenicity and in terms of tolerability. mRNA-1440 (VAL-506440), which is directed against the hemagglutinin (HA) protein of the H10N8 strain of influenza, was tested in a phase 1 study in healthy volunteers (NCT03076385), using both i.m. (25–400 µg) and i.d. administration (25–50 µg) routes[97].

The i.d. route caused more injection-site reactions than the i.m. route and was not pursued, even though, at 25 µg, it appeared to be more immunogenic than the i.m. route. Two of the three participants vaccinated with 400 µg i.m. experienced severe adverse events of headache and erythema, and the safety committee stopped further dosing at this level[38,97]. As we have seen, the rabies vaccine CV7201 elicited an immune response only when administered via a needle-free system, and this was both for the i.m. and i.d. route (NCT02241135)[63]. An early HIV vaccine (iHIVARNA-01) was administered i.n., but this seems to have been the only such study[98]. At the present time, it seems that i.m. injection is the most widely used route of administration of infectious disease mRNA vaccines, identical to the case with protein and DNA vaccines.

Dosing regimens. The ideal dosing regimen, especially for global prevention, is a single dose with 100% seroconversion soon after the dose. But because of the phenomenon of booster immunity, most dosing regimens include at least two shots, typically a few weeks apart. This is also true for the SARS-CoV-2 mRNA vaccines: BNT162b2 is given as two i.m. injections 21 d apart; for CvnCOv and mRNA-1273, a booster shot is given 4 weeks after the prime. BNT162b2 and mRNA-1273 obtained EUA for their respective dosing regimens. However, due to early vaccine shortages and the partial protection observed after a single dose, some advocate immunizing

larger populations with a single dose, rather than reserving part of the supply for the second shot[99], or to space out the injections longer than studied in the pivotal clinical trials. Data from a small trial in adults older than 80 years have indicated that spacing the two BNT162b doses approximately 3 months rather than 3 weeks apart enhances the peak antibody generation, while the results regarding cellular immunity are less clear[100]. In parallel, there is a growing public realization that a third (booster) shot is indicated for optimal control. Recent data showing a slight waning of the effectiveness of the vaccine after 6 months[101] and the emergence of new strain variants have infused new urgency into this question.

Self-amplifying mRNA vaccines, such as ARC-021 and BNT162c2, are intended to be given as a single dose. More elaborate regimens have also been described. For instance, mRNA-1647, directed against CMV, was administered in a phase 1 study (NCT03382405) to healthy volunteers, who received three doses of 30, 90, 180 or 300 µg mRNA-1647 or placebo at months 0, 2 and 6 (ref. [38]). The RSV vaccine mRNA-1345 is being investigated in a phase 1 study (NCT04258719) as a three-dose injection regimen with doses 2 months apart, and this program has now entered phase 3 as a single-dose regimen[102].

Despite the similarities in technology and choice of antigen, the SARS-CoV-2 mRNA vaccines in development cover a wide dose range. As is to be expected based on the technology, self-amplifying mRNA vaccines use smaller amounts per dose: the Imperial College self-amplifying mRNA COVID-19 vaccine is being tested in doses between 0.1 and 1 µg. ARCT-021 was tested at doses between 1 and 10 µg as a single dose and a prime–boost regimen; the 7.5-µg dose will be taken forward for further development[36]. BNT162b2 and mRNA-1273 were successful in preventing approximately 95% of COVID-19 cases at doses of 30 µg and 100 µg[103–105], respectively. CVnCOV is being tested in a phase 3 trial at a dose of 12 µg (NCT04652102)[106]. Outside the SARS-CoV-2 field, the dose range is equally broad. Across different vaccines, the dose levels studied have ranged 300-fold, from 1 µg (CV7202)[107] to 300 µg (mRNA-1653 and mRNA-1657)[38]. Dosing amounts and regimens, along with the storage logistics of the mRNA vaccine, have enormous implications for global immunization plans: the most impactful COVID-19 vaccine or vaccines for future pandemic viruses may not be the first to receive EUA but the first to produce millions of doses and deliver them effectively to the point of service.

Role as adjuvant. As discussed above, RNA can have inherent immune-activating properties. As a supplement or alternative to immune stimulation via innate RNA sensing, some groups have added stimulatory molecules to their vaccines to potentiate the immune response to the encoded antigen with varying degrees of success. For instance, CureVac has used CV8102, a noncoding uncapped single-stranded RNA complexed with a cationic peptide carrier to boost the immunogenicity of a rabies vaccine[108,109]. CureVac's RNActive vaccine technology platform, the basis for the discontinued CV7201 vaccine mentioned previously, relies on a two-component mRNA vaccine in which naked mRNA is used for antigen expression while the same mRNA complexed with protamine is used as an adjuvant that activates TLR7 and TLR8 signaling[53,110,111]. Stimulation of TLR signaling pathways then leads to activation of DCs as part of the innate immune response to the protamine complex[53]. Another product, iHAVARNA-01, which combines DC-activating mRNA species encoding TriMix and an mRNA encoding HIV immunogen (derived from the consensus Gag protein of HIV-1 clade A and a string of $CD8^+$ T cell epitopes)[10,98,112,113].

However, the use of adjuvants for mRNA vaccines seems to be an exception; both BNT162b2 and mRNA-1273 rely solely on mRNA–LNP formulations without the use of adjuvants, and most companies developing mRNA vaccines in the clinic follow the adjuvant-free approach. This may be because LNP components themselves stimulate specific elements of the immune system, such as the stimulator of IFN-γ (STING) pathway and the TLR–RIG-I-like receptor (RLR)-independent mediator of innate immune responses[114]. The ability of nanoformulations to both deliver mRNA to appropriate cellular targets and selectively stimulate the immune system by design is an additional strength of mRNA as a vaccine platform.

Adverse events. By their very nature as preventative, non-therapeutic agents, vaccines against infectious agents are held to a high standard of safety and tolerability. To date, the safety profile of the RNA vaccines discussed in this review is in line with that of protein-based vaccines. Local injection pain and local or systemic inflammatory reactions (fever, malaise) are the most frequently noted adverse events[38,63,97] The two COVID-19 mRNA vaccines that have been administered to more than 30,0000 healthy volunteers, including older people, represent the best dataset for evaluation of the side effect profile, but the comparison must bear in mind that there is a threefold difference in the dose level (and thus of both mRNA and lipid administered) between BNT162b2 (30 µg) and mRNA-1273 (100 µg). In addition, the trials have exclusion criteria that eliminate some of the highest-risk participants (for example, prior history of anaphylaxis) and are thus not necessarily representative of the complete population requiring protection. Both in the BNT162b2 and mRNA-1273 phase 3 studies, more than 80% of vaccine recipients reported local adverse events, mainly pain. The systemic events were mainly headache, fatigue, temperature elevation, myalgia and arthralgia[103]. For mRNA-1273, the frequency and severity of the adverse reactions tended to be more pronounced after the second dose[103]. It is not clear what the relative contribution of mRNA and LNP was to these adverse events, as the placebo in these phase 3 trials was 0.9% saline, not naked mRNA or empty LNPs.

An increase in the severity of the adverse events after the second dose may reflect increased reactogenicity and was also observed with the much smaller dataset of the phase 2 study of mRNA-1647, a CMV vaccine administered both to CMV-positive and CMV-negative participants. No difference was observed in safety profile between the two patient groups, but there was a trend toward more frequent and slightly more severe adverse events after the second vaccination[67].

Even very large trials, such as for the two mRNA COVID-19 vaccines, are limited in their ability to detect very rare but potentially worrisome adverse events. Reports of myocarditis occurring in young males in the days to weeks after vaccination have prompted the FDA Advisory Committee on Immunization Practices to review the benefit and risk of COVID-19 vaccines. According to their calculations, the risk of myocarditis is highest in the young male population (anticipated 39–47 occurrences per million vaccine doses administered in the group aged 12–29 years), but the benefits (prevention of 11,000 cases of COVID, 139 intensive care unit admissions and six deaths) outweigh the risks[115].

Therapeutic vaccines for cancer. The recent explosion and success of cancer immunotherapies has fueled interest in the use of mRNA therapies for this application[116] (Table 2). For mRNA cancer immunotherapies, one approach is modification of the immune-suppressive tumor microenvironment through the expression of deficient or altered tumor suppressor protein. However, current mRNA-delivery modalities are unlikely to reach every cancer cell in a patient. Instead, there is increasing focus on the use of mRNA as a therapeutic vaccine to train the immune system to seek out and kill cancer cells. Key characteristics of mRNA vaccines that enabled their success as SARS-CoV-2 vaccines and as vaccines for infectious diseases in general, including the ability to rapidly develop and manufacture the mRNA medicine as well as the ability for mRNA to encode whole antigens, make their use as cancer vaccines particularly promising. Furthermore, many patients have

tumors that are resistant to current immune-targeting drugs[117], creating a new opportunity for mRNA-based approaches.

The development of therapeutic cancer vaccines, regardless of modality, faces a number of challenges that must be addressed for successful clinical translation. Unlike prophylactic vaccines for infectious diseases for which protection against infection is largely, if not entirely, conferred by a robust humoral response, therapeutic cancer vaccines must also ensure that a strong cytotoxic $CD8^+$ T cell response is induced to eradicate cancerous cells. Although prophylactic vaccines for cancers are possible, there are currently only two FDA-approved cancer-related vaccines and both are against viruses known to cause cancer (human papillomavirus (HPV) and hepatitis B virus). Another challenge is the selection of proper antigens that are able to induce highly tumor-specific immune responses, due to the high variability of antigens across different individuals[118]. The increasing trend toward patient-specific neoantigens aims to address this challenge[119,120]. Finally, even if an antigen is able to induce a cellular immune response, the suppressive tumor microenvironment could prevent T cell infiltration into tumors and could lead to T cell exhaustion. Therefore, therapeutic vaccines may require administration in combination with another therapy designed to overcome the suppressive microenvironment such as immune checkpoint inhibitors, as has been posited for BNT111, as described below[121].

Tumor-associated antigens. Tumor-associated antigens (TAAs) are preferentially expressed on the surface of tumor cells and represent targets for immune killing of tumor cells. Cancer vaccines targeting TAAs involve the production of fixed, off-the-shelf TAAs for a variety of tumors. The most advanced of these, BNT111, is a mix of four melanoma-related antigens (New York esophageal squamous cell carcinoma 1 (NY-ESO-1), tyrosinase, melanoma antigen family A3 (MAGE A3) and transmembrane phosphatase with tensin homology (TPTE)) that is being evaluated in a phase 1–2 trial (Lipo-MERIT, NCT02410733) either as monotherapy or in combination with a checkpoint inhibitor. This vaccine is given as repeated intravenous (i.v.) administrations, starting with a series of eight injections and with the potential for additional monthly injections and has now progressed to phase 2 in combination with cemiplimab for advanced melanoma (NCT04526899).

The immunological effects of BNT111 in the above study have been reported in some detail[15]. The mRNA sequence for each of the four TAAs was optimized for translation in immature DCs. Each sequence also contains a signal peptide and the tetanus toxoid $CD4^+$ epitopes P2 and P16 as well as the major histocompatibility complex (MHC) class I trafficking domain for enhanced human leukocyte antigen (HLA) presentation and immunogenicity. Activation of lymphoid tissue was shown by an increase in metabolic activity in the spleen, as measured by ^{18}F-fluoro-2-deoxy-2-D-glucose positron emission tomography of the spleen. About 75% of the 50 evaluated patients showed an IFN-γ response against at least one of the four TAAs by enzyme-linked immune absorbent spot assay, indicating induction of an immune response. The antigen-specific T cells were of the $OD1^+CCR7^-DD27^{+/-}D45RA^-$ effector memory phenotype and secreted IFN-γ and tumor necrosis factor upon stimulation. In patients continuing to receive vaccinations, the TAA-specific cells remained stable or even increased in number, whereas in patients who stopped receiving the maintenance vaccinations, the T cells remained present for several months, with a decline thereafter. The ability of these cells to kill melanoma cells was demonstrated ex vivo by transfecting healthy donor $CD8^+$ cells with the cloned TAA-specific T cell receptor from a vaccinated patient and evaluating their ability to lyse melanoma cell lines.

After each dose, increased plasma levels of IFN-α, IFN-γ, IL-6 and other cytokines were found in patients, typically peaking a few hours after injection and normalizing within 24 h. This was in line with the observed adverse event profile, which was characterized by mild-to-moderate flu-like symptoms, equally transient and self-limiting. The first evaluation of 42 patients with radiographically evaluable disease was considered encouraging. In the group of 25 patients with vaccine monotherapy, three had partial responses and seven had stable responses, while six of 17 patients treated with the vaccine–anti-PD1 combination experienced a partial response. An interesting observation was that two patients who had progressed while on anti-PD1 therapy and had received vaccine monotherapy later responded again to anti-PD1 therapy, which is in line with the observation that the induced T cells were of the $PD1^+$ effector memory phenotype. BNT111 is now in a phase 2 study of melanoma (NCT04526899)[122]. mRNA-5671 (V941) is a concatemer designed to present KRAS antigens to the immune system and codes for the four most common KRAS substitutions (G12D, G12V, G13D, G12C)[38]. It is currently in a phase 1 study (NCT03948763) as monotherapy and in combination with pembrolizumab[10,38,123–125].

Additional examples include BNT112 (which encodes five prostate cancer-specific antigens[126]) and BNT113 (which encodes HPV16-derived tumor antigens E6 and E7 (viral oncoproteins)), BNT114 (ref. [127]) (which encodes a mix of selected breast cancer antigens) and BNT115 (which encodes a mixture of three ovarian cancer TAA-encoding RNA species)[128].

CureVac conducted early studies with unmodified mRNA species encoding TAAs, including naked mRNA species for autologous amplified tumor mRNA as an immunotherapeutic regimen[129], naked mRNA encoding six renal cancer-associated antigens[130] and protamine-stabilized mRNA for six different melanoma-associated antigens (NCT00204516, NCT00204607)[131]. These studies mainly provided safety and tolerability information about the formulations used. Other studies investigating tumor-associated antigens include CV9103 (mixture of four antigens for prostate cancer)[132], CV9104 (mixture of six different antigens for prostate cancer encoded by six different mRNA species[133] and CV9201 (mixture of five non-small lung cell cancer antigens)[134]. All these projects and/or drug candidates appear to have ceased development[64]. CV9202 contains six mRNA species that encode six different antigens (NY-ESO-1, MAGE C1, MAGE C2, trophoblast glycoprotein (TPBG (5T4)), survivin and mucin-1 (MUC1))[135] and is still in an active study (NCT03164772)[136,137].

Personalized neoantigens. During carcinogenesis, malignant cells acquire somatic mutations that lead to the production of protein sequences not expressed by normal cells[138]. These proteins are processed via the proteasome into peptides that are presented on the cell surface bound to MHC class I receptors, where they are recognized by T cell receptors. These neoantigens are typically unique to each patient and thus represent both the opportunity for and technical challenges associated with tumor-specific and patient-tailored immunotherapy[119].

To generate mRNA vaccines against patient-specific neoantigens, an individual patient's tumor is excised and patient-specific neoantigens are identified by next-generation sequencing. The mRNA encoding these neoantigens is then injected into the same patient, with the expectation that it will induce an immune response that will attack the patient's tumor[139]. It is of course imperative that this entire process should take a minimal amount of time so that the patient can be treated before the cancer evolves and progresses, and turnaround times as short as 30–40 d have been reported[128]. This poses additional challenges for manufacturing, which must satisfy the criteria for human use of investigational products.

Thus far, the majority of work in personalized neoantigen vaccines has involved the deployment of peptide-based neoantigen vaccines rather than mRNA vaccines; in general, these approaches have had limited success. Tumors with the highest mutational

burden, which are in theory the best candidates for this type of neoantigen approach, are also most likely to develop resistance to the treatments[140]. Compared with peptide vaccines, we hypothesize that mRNA-encoded neoantigen vaccines, with proper immune stimulation, may provide a stronger immunogenic response and clinical benefit. Unlike peptide-based vaccines, mRNA can encode whole antigen, thereby ensuring presentation of multiple epitopes without being restricted to a defined HLA type[141]. In addition, mRNA can be synthesized to express multiple neoantigens either as separate molecules or as a concatenation of multiple coding sequences. Certain tumor types can produce up to several dozens of neoantigens, and, from the perspective of inducing a broad immunological response, it is desirable to express multiple epitopes likely to evoke a T cell response.

BioNTech has developed several clinical neoantigen vaccine candidates for the treatment of cancer. BNT121 was studied via repeat administration in inguinal lymph nodes of 13 patients with metastatic melanoma (NCT02035956)[126,142]. The results from that study were considered encouraging, with robust immunological responses and some evidence of clinical activity. BNT122 (RO7198457), which can contain up to 20 individualized patient neoepitopes, is administered i.v. and is currently being evaluated in four studies (Table 2). Preliminary results indicated that BNT122, both with and without the anti-PD-L1 antibody atezolizumab, has an acceptable safety profile with mainly transient adverse events such as infusion-related reaction and/or cytokine-release syndrome manifesting as fever and chills[128]. BNT122 is also under evaluation in a phase 1 study of pancreatic cancer (NCT04161755), and a study in non-small lung cell cancer is expected to start soon (NCT04267237) as well as a study for an undisclosed adjuvant indication[126].

mRNA-4157 is another personalized cancer vaccine that can contain up to 34 neoantigens encoded on a single mRNA strand ('neoantigen concatemer') and is formulated in an LNP and administered i.m. This drug is currently in a phase 1 study of patients with resected primary solid tumors (monotherapy) and patients with metastatic unresected tumors (NCT03313778). As of February 2020, a total of 71 patients were reported to have received at least one dose of mRNA-4157 (ref. [38]). The most frequently noted adverse events were fatigue, injection-site soreness, colitis and myalgia. In parallel, a randomized phase 2 study as adjuvant in combination with pembrolizumab for patients with high-risk melanoma is also ongoing (NCT03897881). The compound NC-I4650 is closely related to mRNA-4157, the main difference being the neoantigen-selection protocols used[38].

Neoantigen vaccines, with their dependence on fast turnaround of patient-specific mRNA sequences, definitely benefit from the flexibility and speed inherent in the mRNA–LNP platform. Lastly, the variety of routes of administration in oncology is worth noting: intratumoral, i.n. and i.v. or i.m., with some of the same LNPs being used for more than one route of administration. This indicates the potential for wide applications of a single drug candidate: a tumor that cannot be reached by direct intratumoral injection or where there are no accessible lymph nodes may still respond to i.v. or i.m. administration of the relevant mRNA vaccine. The challenges are to identify the most effective protein or combination of proteins to encode to direct the immune system to attack cancers, to enable the immune system to penetrate deep into tumors and to personalize the therapies for each patient.

Protein and cellular immunotherapies. An area of renewed interest is the use of mRNA administration with the intent of generating therapeutic levels of immune or immunomodulatory proteins (Table 3), such as antibodies or cytokines. Compared with infectious disease and cancer vaccines, more protein has to be produced for such therapies to be effective, where, in certain cases, life-long treatment with repeated dosing may be required.

Another challenge for protein immunotherapies is delivery of mRNA to the desired organs and cell types to achieve optimal therapeutic outcomes. For instance, certain expressed proteins require further PTMs, such as glycosylation and proteolytic processing, to become fully functional. However, the manner in which PTMs are made to the protein can be tissue dependent and may not be dictated simply by the mRNA sequence, thus emphasizing the need for tissue-specific delivery of the mRNA.

When mRNA species are administered systemically in complex with LNPs, many tend to home to the liver due to binding of apolipoprotein E to the LNP surface, which leads to receptor-mediated uptake by hepatocytes[143]. Non-liver organ selectivity can be achieved through modification of lipid compositions, including adjusting lipid ratios and identities, leading to LNPs that target the lung endothelium or the spleen[144,145]. More recently, changes to LNP surface chemistry through modulation of the PEG–lipid structure have led to LNP targeting of bone marrow endothelial cells in the hematopoietic stem cell niche[146].

Thus, mRNA protein immunotherapy poses several unique challenges in terms of delivery, efficacy of protein production and tolerability compared with vaccines. This may explain why this application of mRNA has progressed more slowly than mRNA immunization.

mRNA-encoded monoclonal antibody therapy. Delivering an mRNA to a specific tissue or organ by direct injection is a barrier to development. Instead, systemic exposure can simplify clinical application as long as it is safe and a sufficient level of protein is expressed to gain a therapeutic effect. Encoding monoclonal antibodies (mAbs) in an mRNA medicine is an example of this approach and is exemplified by mRNA-1944, an mRNA–LNP encoding a neutralizing mAb against Chikunguya virus, identified in a patient with immunity (NCT03829384)[124]. Results from the first healthy volunteers treated indicated that, at all doses tested (0.1, 0.3 and 0.6 mg per kg, i.v.), neutralizing mAb levels could be detected. At the highest dose, however, three of four participants experienced infusion-related reactions, including grade 3 tachycardia and elevated white blood cell count in one participant, who also had grade 2 nausea, emesis, fever and transient inverted T waves on electrocardiogram[147]. A separate cohort at that same dose level but pretreated with steroids had no grade 3 adverse events, but the levels of Chikungunya-specific antibodies produced (E_{max}) were 1.7-fold lower[67]. Data from a cohort to which a dose of 0.3 mg per kg was administered twice, 2 weeks apart, indicated no exacerbation of adverse events after the first versus second dose and no lipid accumulation[67].

The application of mRNA to produce antibodies continues to be of interest, with several industry collaborations underway, such as partnerships between CureVac and Genmab (mRNA-based antibody anti-cancer therapeutics[148]) and between Neurimmune and Ethris (inhaled mRNA encoding mAbs against SARS-CoV-2 (ref. [149])). An important consideration here are the benefits of expressing an mAb from an mRNA, rather than administering the same antibody made through traditional recombinant manufacturing. Ultimately, the most promising approach will be a function of the doses required, the duration of effect, the types of PTMs needed and the relative therapeutic index ratio of the delivery system and the antibody of interest.

mRNA-encoded immunostimulatory proteins for cancer treatment. Another anti-cancer approach consists of the injection of mRNA encoding proteins expected to have a direct therapeutic effect, typically via stimulating the immune system, such as OX40 ligand (OX40L) or ILs. One such product, mRNA-2416, is an mRNA encoding the immune checkpoint modulator OX40L, administered intratumorally. Despite the first reported results as monotherapy in 41 patients with a variety of malignancies not meeting the Response

Evaluation Criteria in Solid Tumors for a partial response, the sponsor is currently taking it forward into a phase 2 expansion cohort in combination with durvalumab for ovarian cancer (NCT03323398)[38].

Other mRNA products encode several different immunomodulatory proteins. One example is ECI-006, a combination of TriMix (mRNA species encoding DC-activating molecules (CD40L, CD70 and caTLR4)) and mRNA species encoding melanoma-specific TAAs (tyrosinase, gp100, MAGE A3, MAGE C2 and PRAME)[150], which is administered i.n. and is being tested in a phase 1 study of resected melanoma (NCT03394937)[123] (TriMix alone is in a phase 1 study of breast cancer (NCT03788083) and given intratumorally[151]). An additional example is mRNA-2752 (three mRNA species encoding OX40L, IL-23 and IL-36γ), which is being evaluated in a dose-escalation study of solid tumors and lymphoma (NCT03739931). Similarly, BNT131 (SAR441000) encodes IL-12sc, IL-15sushi, IFN-α and granulocyte–macrophage colony-stimulating factor (GM-CSF) and is under investigation as an intratumoral injection intended to alter the tumor microenvironment[128].

Another type of product is immunomodulatory fusion proteins. MEDI1191 encodes a single chain fusion protein containing the IL-12α and IL-12β subunits, with a linker between the subunits. This agent was developed for intratumoral injection, with the aim of improved tolerability compared with systemic administration of recombinant IL-12 (ref. [38]).

mRNA in adoptive immune cell therapy. Adoptive cell transfer is a relatively new therapeutic approach that involves collecting and using a patient's own immune cells to treat their cancer[152]. This has been explored in humans with breast cancer for which Tchou et al.[153] observed that intratumoral injections of T cells transfected with mRNA encoding a chimeric antigen receptor (CAR) targeting c-Met were well tolerated and induced an inflammatory response within breast cancer tumor tissue; similarly, Maus et al.[154] reported on four individuals treated with autologous T cells electroporated with mRNA encoding a CAR derived from a murine antibody specific to human mesothelioma. One of the treated individuals experienced anaphylaxis and cardiac arrest within minutes after the third infusion, which the authors attributed to the production of immunoglobulin (Ig)E-type human anti-mouse antibodies (NCT01355965). More recently, Beatty et al.[155] evaluated T cells transfected with an mRNA encoding a mesothelin-directed CAR as a treatment for pancreatic cancer that avoided T cell priming; in phase 1 studies, these cells did not induce cytokine-release syndrome and did not elicit neurologic symptoms.

CAR T cells have been historically generated using retroviral gene transfer with substantial success and, more recently, using CRISPR–Cas9-mediated gene-integration systems. Success has been reported using not only mRNA but also ribonucleoprotein-mediated delivery systems. However, as discussed above, we see the potential of CAR T cell generation in vivo[156]. Although functional delivery of ribonucleoproteins in vivo has yet to be described, ultimately, mRNA may have substantial advantages over viral delivery in terms of both loading capacity and redosing, assuming safe and effective delivery to T cells can be demonstrated.

Nanoformulations capable of facilitating in vivo delivery to multiple classes of immune cells have also been described, including macrophages[157], B cells[158] and T cells[156,159–161], offering the promise of a range of immunotherapies. For example, with T cell-targeted mRNA delivery, in vivo CAR T cell generation may be possible, creating new types of therapy for cancer[156]. Retargeting LNPs to T cells has been achieved through the identification of specific lipid structures that facilitate delivery to these cells[160,161]. Furthermore, antibody-targeted mRNA nanoparticles, for which specificity is imparted by surface conjugation, enable affinity to immune cell-specific receptors, such as CD4 (ref. [162]).

For therapies for which the aim is to edit immune cells in vivo, mRNA may allow transient expression of the genome-editing nucleases or base editors, which last transiently in cells due to RNA degradation. Although, thus far, published reports of mRNA delivery to T cells have only shown disruption of green fluorescent protein marker in vitro[159], the success of gene editing via systemically delivered mRNA to hepatocytes has already been demonstrated in humans for TTR amyloidosis, for which an mRNA encoding Cas9 protein has been delivered in an LNP together with a guide RNA targeting TTR. In patients, there was a dose-dependent mean reduction from baseline in serum TTR protein concentrations with only mild adverse events[163]. This impressive outcome hints at the future potential use of mRNA in systemic gene editing.

Conclusions and future directions. mRNA occupies a distinctive niche between gene therapy and protein therapy, combining many of the advantages of both while addressing unique challenges faced by either one. For instance, multimeric proteins that would pose insurmountable technical challenges for production in a bioreactor can be produced in the patient's own body by an mRNA or combination of mRNA species encoding the different subunits, an opportunity for flexibility that has been put to use in a candidate CMV vaccine and for cancer applications and that takes on a new importance as the world is grappling with the emergence of distinct SARS-CoV-2 strains with anticipated different sensitivities to the authorized SARS-CoV-2 vaccines. As the field optimizes and refines the technology, it is likely that mRNA medicines will also be developed for indications beyond infectious disease and cancer.

The inherently transient duration of the expression of the target protein positions mRNA therapy as an ideal modality for situations in which a single or no more than a small number of episodes of protein expression are required, such as infectious disease vaccines. The ability to dose repeatedly, titrate the dose or vary the dosing interval offers the clinician the flexibility of classic drug therapy, making it an attractive choice for indications for which individual patient needs may vary or for which hesitations may exist about gene therapy. From the safety point of view, for two vaccine projects (mRNA-1273 for SARS-CoV-2 and mRNA-1647), it appeared that there was a more pronounced adverse event profile after the second dose than after the first dose. This does not seem to be a universal observation, however. The cancer vaccine BNT111 has been administered to some patients with more than eight doses, with apparently maintained efficacy[15]. The inhaled mRNA therapeutic MRT5005 has been given in up to five weekly doses, with no signs of worsening safety profile between the first and the fifth dose.

The potential of mRNA therapies will expand further through the evaluation of engineered, non-human and artificial protein constructs. Protein therapeutics have been engineered for extended half-life, for example, through fusion of the Fc domain to the therapeutic domain. The same can be encoded in mRNA therapies. Perhaps more exciting is the ability to express new intracellular therapeutics. The transient expression of gene-editing machinery from mRNA is appealing to reduce side effects from persistent expression. Additionally, the intracellular expression of antibodies, antibody fragments or other protein-binding motifs provides a distinct therapeutic class that can be combined with subcellular localization domains, for example, to the nucleus, to focus the action of the encoded protein.

The first applications of mRNA involved stimulation of the immune system, either for infectious disease vaccines or for cancer vaccines. The infectious disease application has been a proving ground for the platform; cancer vaccines have hitherto not been particularly successful as a class, but the encouraging results of BNT111 hint at the possibility that the combination of high protein expression and the immune-activation pathway may overcome some of the hurdles encountered by earlier protein vaccines.

Exploiting the immunostimulatory properties of RNA in products makes perfect sense thanks to the intrinsic ability of RNA to activate immune pathways via the TLR and RIG-I pathways. This is independent of the coded protein, as shown by CV8102, a noncoding RNA that is used as an immune adjuvant. The drawback of this immunostimulatory property is manifest upon a review of the safety and tolerability profiles of the mRNA drug candidates that have entered clinical trials. The emerging picture is that the most frequently noted adverse event associated with mRNA medicines as a class is some form of inflammatory reaction. This underlying pathway can manifest in a large number of ways: a local reaction in the case of i.m. or subcutaneous injection (local pain, redness, soreness) or as a more generalized febrile syndrome or flu-like reaction with i.v., i.m. or inhaled medicines. These seem to be typically treatable with classic anti-inflammatory drugs, although, in the case of i.v. administration of mRNA encoding a Chikungunya-specific mAb, prophylactic steroid use was used to mitigate the adverse events observed in the cohort receiving the highest dose[67]. This intervention did appear to be successful in tamping down the adverse events but was also associated with a decrease in protein expression.

These data offer interesting insights into some potential future directions for research into repeat dosing of mRNA therapeutics. Will steroids be effective and required for the mitigation of inflammatory side effect profiles of mRNA-encoded protein therapies? And if so, does that come at a price of less protein expression? Does the concomitant observation of fewer side effects with reduced expression hint that a certain level of inflammation might actually be a prerequisite for good protein expression? If this is the case, one clinical challenge will be to thread that needle and allow just enough subclinical inflammatory processes to be initiated to promote good translation without allowing them to rise to levels of severity that would jeopardize the clinical feasibility of repeat dosing.

These questions are somewhat complicated by the fact that the majority of mRNA applications do not involve simple naked mRNA but mRNA that is encapsulated in an LNP or PNP, all of which can contribute to the tolerability profile. The use of 'empty LNP' containing no mRNA in a control arm of clinical trials has been proposed to help elucidate the distinct contributions of mRNA versus LNP to the tolerability profile. A theoretical limitation to this approach is that the empty LNPs, when not complexed with negatively charged mRNA, have different physicochemical properties and thus do not represent a true comparator. Although a transient inflammatory reaction can be acceptable in the context of a single dose (vaccination) or life-threatening diseases (oncology), for indications for which chronic treatment is necessary, especially when administered i.v., the selection of appropriate, well-tolerated and safe lipid and formulation will be critical. It appears that animal experimentation will be of limited value here because it was noted that the concentrations of the cancer vaccine BNT111 that triggered cytokine release in humans were >1,000-fold lower in humans than those in mice[15], and the febrile reactions observed after inhalation of MRT5005 were not predicted based on animal toxicity experiments.

Beyond the immediate tolerability, longer-term questions about the potential for lipid accumulation will also have to be addressed. If the produced protein has a short half-life, then the dosing interval required to maintain expression and clinical efficacy may be shorter than ideal for elimination of the lipid. Repeat dosing could thus lead to lipid accumulation in target or off-target tissues, with difficult-to-predict long-term safety risks. Formulation science will be as much a part of the future of mRNA therapeutics as further understanding of the biology of mRNA itself.

The extraordinary potential of mRNA therapy is also illustrated by the different routes of administration introduced into the clinic: i.m., i.d., subcutaneous, i.n., intratumoral, i.v., epicardial and inhaled. Additional applications can readily be imagined: intranasal vaccines, eye or nose drops, skin ointments, suppositories, solutions for intravesical instillation, intrathecal drug-delivery devices or Ommaya reservoirs. We believe that the future breadth of mRNA therapy will be defined by advances in delivery nanoparticles. There is growing evidence that LNPs and PNPs can be engineered to deliver to a range of tissues in the body including the liver[65,72], the endothelium[144], the lung[81–83], the bone marrow[146] and multiple elements of the immune system[156–162]. Additional advances in potency and tissue targeting by improved delivery materials and inclusion of additional targeting elements will continue to open doors to new therapeutic applications for mRNA therapy.

The success of the two SARS-CoV-2 vaccines receiving EUAs has highlighted one of the pharmaceutical advantages of mRNA: the speed of production of clinical trial materials. Manufacturing technologies originally developed for the fast turnaround of individualized neoantigen vaccines from excised patient tumors toward an injection-ready clinical product have demonstrated their potential by enabling the start of clinical trials of candidate mRNA vaccines within weeks after the publication of the SARS-CoV-2 sequence.

At the same time, our experience with COVID-19 vaccines also highlights one of the current limitations of the technology: the dependency on cold-chain storage and transport. Freezers capable of handling temperatures of −80 °C are specialized equipment and not readily available in every pharmacy or clinical trial site. For treatments that are intended for self-administration in the patient's home, even storage at −20 °C can be challenging. The next frontier to bring mRNA medicines from the bench to the bedside may very well be formulation science.

The emergence of mRNA as a safe and effective platform in the race to produce a COVID-19 vaccine has provided the entire world with an accelerated education in the benefits and risks of mRNA–LNP technology. Although much attention has been lavished on the speed of manufacturing and storage conditions of BNT121 and mRNA-1273 as well as the side effects of a two-dose regimen, the scientific and medical communities are looking beyond these two vaccines and eagerly awaiting further validation of mRNA medicines in other indications[164,165].

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Competing interests

A.J.B. and R.W. are former employees of Translate Bio. D.G.A. is a consultant for Translate Bio. D.G.A is also a founder of oRNA Tx.

Additional information

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