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Beyond the injection site: identifying the cellular targets of mRNA vaccines

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Abstract

Vaccines against COVID-19 based on the mRNA technology have broken many records, from the speed of development and production, to the number of doses administered and have overall proven safe, with only very rare reported adverse events. The accelerated rollout and the permissive regulatory framework had the major caveat that manufacturers did not provide biodistribution and pharmacokinetics data for their products in humans, despite this being essential for interpreting both the dynamics of the immune response and any potential toxic effects. Thankfully, in the past two years, the scientific community has attempted to fill the gaps, which will undoubtedly help in fine-tuning the next generation of mRNA vaccines.

Here we review existing data on the biodistribution and pharmacokinetics of the commercially available mRNA vaccine platforms, focusing on human studies, where available. We structure this review by tissue type and we discuss potential correlations between vaccine mRNA uptake and pathogenic effects, if applicable. We find that many studies have focused on the heart, due to the medical and social impact of myocarditis, especially in adolescents. We conclude by observing critical data is still missing for many organs and we suggest potential avenues for future research.

Keywords:

mRNA vaccines, LNP, lipofection, S-protein, biodistribution.

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Introduction

Two messenger RNA (mRNA) vaccines against COVID-19 were developed and approved at remarkable speed for combating the spread of the disease, Comirnaty/BNT162b2 by Pfizer-BioNTech and Spikevax by Moderna. Recently, the mRNA technology was awarded the Nobel prize (Callaway and Naddaf, 2023), having initially contributed significantly to preventing symptomatic COVID-19, and then, as SARS-CoV-2 accumulated mutations in the spike protein gene, to protecting against severe disease and hospital admission (Bobrovitz *et al.*, 2023; Feikin *et al.*, 2023). Moreover, this technology has immense potential for the prevention of other infectious diseases (Chaudhary *et al.*, 2021) and for personalized cancer therapies (Liu *et al.*, 2023), with numerous ongoing clinical trials (Wang *et al.*, 2023).

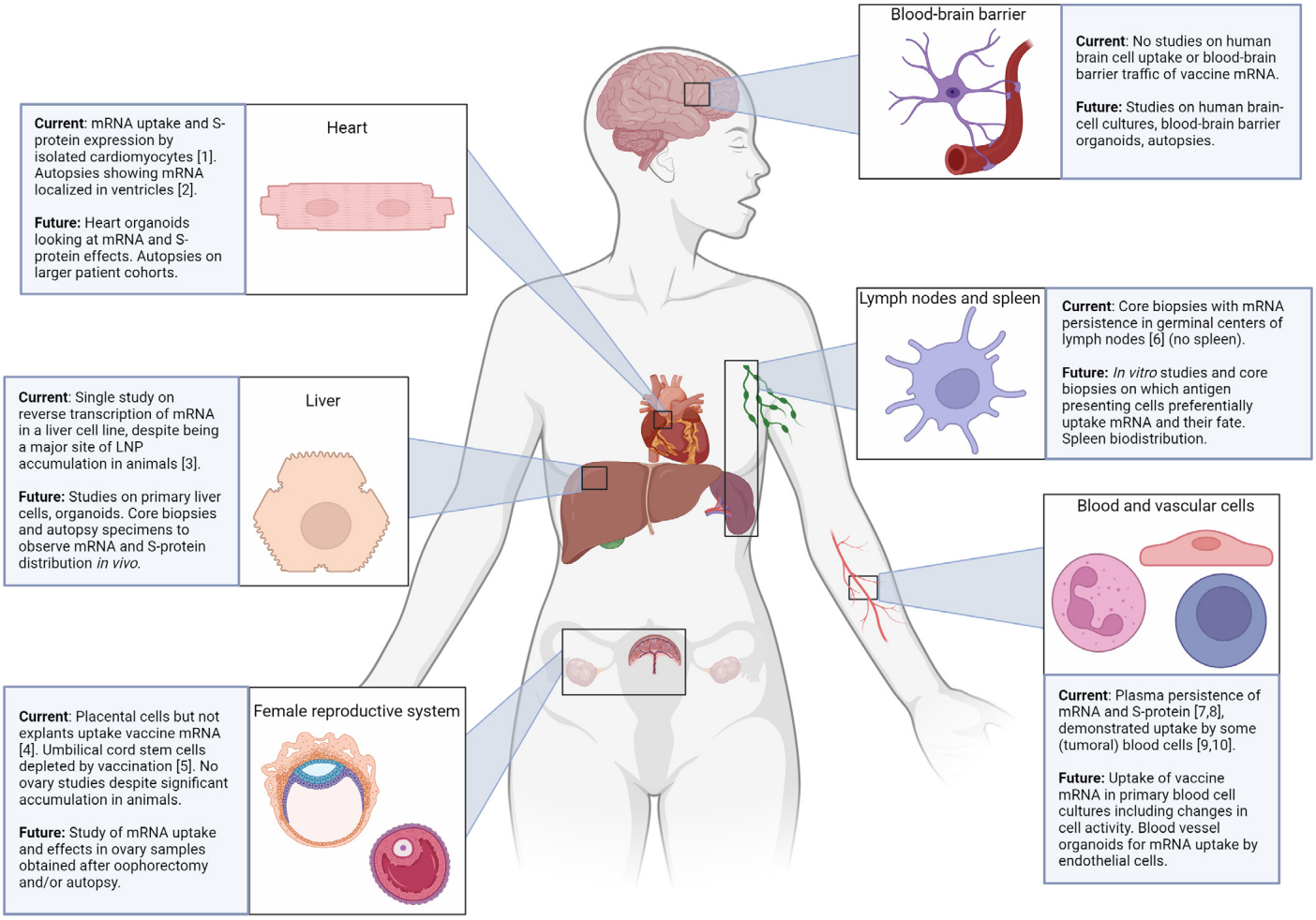
Overall, these products have been proven safe, with only rare side effects. Notable adverse reactions include anaphylaxis (Hatziantoniou et al., 2021), myocarditis, especially in adolescents (Alami et al., 2023), increased menstrual cycle length (Edelman et al., 2022), onset or exacerbation of autoimmune diseases (Chen et al., 2021; Hinterseher et al., 2023), thromboembolic events (Berild et al., 2022) and even a Long COVID-like syndrome similar to postural orthostatic tachycardia syndrome (POTS, Kwan et al., 2022). Especially in the case of myocarditis it is unclear to what extent these adverse reactions are due to off-target effects of vaccine particles or are the result of non-specific, systemic immune responses (Li et al., 2021; Trougakos et al., 2022; Bozkurt, 2023). Although manufacturers have done extensive testing in animal models for similar mRNA-based constructs, the pharmacokinetics, biodistribution and tissue tropism of the final vaccine formulations in humans were unknown at rollout. This lack of human biodistribution data was permitted under the existing regulatory framework in the US and EU, which arguably lags behind the rapid development of RNA therapeutics and vaccines (Vervaeke et al., 2022) and is additionally complicated by confidentiality agreements (Tinari, 2021). In the past two years, a growing number of independent research groups, including our own, have looked at the biodistribution and tissue tropism of commercially available mRNA products in humans and this review aims to summarize these studies (Figure 1). We believe this data is critical to further fine-tune this promising platform and improve cellular targeting for the next generation of mRNA therapies and vaccines.

Figure 1
Current knowledge and perspectives for the study of vaccine mRNA biodistribution in humans.
Figure created with *BioRender.com*.
Organs and cells not shown to scale.
[1] Schreckenberg *et al.*, 2023;
[2] Krauson *et al.*, 2023;
[3] Aldén *et al.*, 2022;
[4] Gonzalez *et al.*, 2023;
[5] Estep *et al.*, 2022;
[6] Röltgen *et al.*., 2022;
[7] Fertig *et al.*, 2022;
[8] Brogna *et al.*, 2023;
[9] Cari *et al.*, 2023;
[10] Zurlo *et al.*, 2023.

Vaccine mRNAs are modified and lipid-encapsulated for increased persistence

The principle behind the mRNA vaccine platforms is that modified mRNAs coding the antigen of interest, are delivered to susceptible cells parenterally (generally by intra-muscular injection), and these cells then express the desired protein for immune recognition. In the case of the two commercially available mRNA vaccines against COVID-19, the coded antigen is the SARS-CoV-2 pre-fusion stabilized spike glycoprotein (S-protein). For both sequences, pre-fusion is achieved by two proline substitutions in the S2 region of the S-protein, which stabilize expression possibly by preventing misfolding and/or proteolytic cleavage, leading to superior antigenicity compared to unmodified S-proteins (Hsieh *et al.*, 2020).

To increase stability and efficiency of translation, synthetic mRNAs are codon optimized and altered by changes of the 5'-cap and poly(A) tail and insertion of modified nucleosides (*e.g.*, N1-methypseudouridine), with significant differences between the two approved COVID-19 vaccines (Xia, 2021). The biological impact of these modifications have been extensively reviewed elsewhere (Boo and Kim, 2020; Cui *et al.*, 2022), but it is worth noting that changes to sequence and structure allow these exogenous mRNAs to avoid nuclease degradation in solution and to remain functional inside transfected cells for extended periods of time compared to endogenous mRNAs (Yang *et al.*, 2003), for some constructs longer than 48 hours (Pardi *et*



al., 2015; Gampe *et al.*, 2018; Mauger *et al.*, 2019; Leppek *et al.*, 2022). Documentation provided by Pfizer-BioNTech to the European Medicines Agency (EMA) showed activity of a luciferase-coding modRNA continued up to 9 days in mouse tissues[1].

By contrast, data on rats provided by Moderna, albeit also using a different modified mRNA construct from the COVID-19 vaccine, showed mRNA half-lives of up to 63 h[2]. However, the interpretation of the publicly available EMA synopsis of the data is that these durations refer to physical half-life (*i.e.*, persistence of mRNA in tissue), rather than functional half-life (*i.e.*, duration of intra-cellular protein translation).

Indeed, whereas the extended functional half-life of exogenous mRNAs in the cytosol of cells is the result of sequence and structure modifications, the persistence of modRNA in the extracellular environment is the result of its containment within protective lipid nano-particles (LNPs). These lipid shells offer not just protection from ubiquitous endogenous RNAses, but, based on their lipid composition, also allow relatively precise tissue targeting and cell entry.

The chemical composition, structure and morphology of LNPs from COVID-19 vaccines have been described elsewhere (Hou *et al.*, 2021; Jung *et al.*, 2022; Wang *et al.*, 2023; Zhang *et al.*, 2023; Szebeni *et al.*, 2023). Briefly, LNPs comprise four main components: a ionizable lipid, a helper lipid (for example phosphatidylcholine), cholesterol and a polyethylene glycol (PEG)-functionalized lipid. The ionizable lipids (for example the proprietary ALC-315 component of the BNT162b2 vaccine) regulate surface charge, remaining neutral at physiological pH, but becoming protonated at the acidic pH of endosomes, which allows membrane destabilization and endosomal escape (Hou *et al.*, 2021). Variations of any of the four components directly impacts stability, persistence and can determine preferential accumulation in some major organs (for a comprehensive review see Zadory *et al.*, 2022),

[1] **https://www.ema.europa.eu/en/documents/assessment-report/comirnaty-epar-public-assessment-report_en.pdf**
[2] **https://www.ema.europa.eu/en/documents/assessment-report/spikevax-previously-covid-19-vaccine-moderna-epar-public-assessment-report_en.pdf**

but precise cellular targeting remains elusive and may be impossible to achieve in absence of targeting molecules (e.g. antibodies) on the LNP surface (Rurik *et al.*, 2022).

Animal biodistribution studies preceding vaccine rollout

Experiments on animal models by manufacturers of the two anti-COVID19 mRNA vaccines indicated slightly different biodistribution patterns. Both companies reported that following injection, vaccine particles remain mostly concentrated at the injection site and are gradually distributed to draining lymph nodes, but after reaching systemic circulation (plasma) LNP-mRNA reach most organs, including the brain. Aside from muscle and lymph nodes, organs with significant accumulation of vaccine differ between the two studies: Moderna reported the spleen and the eye as having comparatively strong signals[3], whereas Pfizer-BioNTech reported the liver (over 20%), spleen, adrenal glands and ovaries as sites of accumulation in the first 48 hours[4]. This highlights potential limitations of the different measurement methods used and/or the impact of LNP formulations on biodistribution. The maximum half-life for the modRNA was measured in the spleen for Spikevax (63 hours), whereas for BNT162b2 luciferase signal slowly subsided by the 9th day, after which no further measurements were done[5]. Notably, ALC-0315, the proprietary lipid used in BNT162b2, was estimated to be cleared by the liver after as long as 6 weeks[6], suggesting that when discussing vaccine persistence, LNP-mRNA physical half-life should be decoupled from mRNA functional half-life in tissue.

Accumulation of vaccine in lymph nodes

Mechanisms for gradual accumulation of LNP-mRNA in lymph nodes have been previously described not just in rodents, but also in primates (Lindsay *et al.*, 2019). Antigen-presenting cells (particularly monocytes) are quickly recruited to the injection site where they uptake vaccine particles, after which mRNA is found predominantly in dendritic cells and B-lymphocytes within the draining lymph nodes. The same study showed that these cells are not just passive vectors, but also likely candidates for protein expression (Lindsay *et al.*, 2019). In humans, both mRNA and spike protein were found in the germinal centers of axillary lymph nodes up to 60 days after vaccination (Röltgen *et al.*, 2022).

Persistence of vaccine and expressed S-protein in plasma

Accumulation of LNP-mRNA in various tissues aside from the injection site and lymph nodes is directly conditioned by plasma persistence. Animal studies indicated that vaccine particles would be terminally cleared from plasma by day 6 for BNT162b2 and as quickly as 1 day for Spikevax[7]. However, when our team used qPCR to measure synthetic mRNA levels, at different time points, in the blood of 16 individuals vaccinated with BNT162b2, we found that it persists up to 15 days after injection (Fertig *et al.*, 2022). Our results were confirmed by a later study, employing RNA sequencing of plasma from patients with chronic hepatitis C virus, which found both BNT162b2 and Spikevax sequences persisting in circulation at up to 28 days from injection (Castruita *et al.*, 2023). It is likely that the mRNA detected in both studies circulated protected by LNPs, otherwise it would have been rapidly degraded by host nucleases.

This is significant, as LNP-encapsulated mRNA retains its potential protein expression in almost any contacted cell and may therefore explain prolonged S-protein detection in some study cohorts. Continuously occurring, low-level lipofections over weeks is a more parsimonious explanation to S-protein persistence than the suggested genomic integration of vaccine mRNA, or alternatively, protein expression by blood microbiota (Brogna *et al.*, 2023).

Indeed, a number of studies have now identified the circulating S-protein in the blood of vac-

[3] https://www.ema.europa.eu/en/documents/assessment-report/spikevax-previously-covid-19-vaccine-moderna-epar-public-assessment-report_en.pdf
[4] https://www.ema.europa.eu/en/documents/assessment-report/comirnaty-epar-public-assessment-report_en.pdf
[5] https://www.ema.europa.eu/en/documents/assessment-report/spikevax-previously-covid-19-vaccine-moderna-epar-public-assessment-report_en.pdf; https://www.ema.europa.eu/en/documents/assessment-report/comirnaty-epar-public-assessment-report_en.pdf
[6] https://www.ema.europa.eu/en/documents/assessment-report/comirnaty-epar-public-assessment-report_en.pdf
[7] https://www.ema.europa.eu/en/documents/assessment-report/comirnaty-epar-public-assessment-report_en.pdf

cinated individuals, each using different techniques, with different sensitivities and pushing persistence intervals further. In one study, the S-protein was detected by single molecule array as early as 1 day and as late as 29 days after inoculation (Ogata et al., 2022), another found undetectable levels in 4 individuals after 10 days using photonic biosensors (Cognetti and Miller, 2021) and in a more recent study mass spectrometry identified S-protein in blood as late as 187 days from vaccination (Brogna et al., 2023). It appears the S-protein is not only shed by cells in its free form, but also attached to extracellular vesicles. Western blotting showed S-protein-decorated exosomes peaked at 14 days from injection and remained detectable even at 4 months (Bansal et al., 2021). It is expected that the circulating S-protein or its fragments, just like the LNP-mRNA, would reach most tissues including the brain (Rhea et al., 2021). While known that the virus-associated SARS-CoV-2 S-protein exerts toxic effects in tissues during infection, it is still under debate (Trougakos et al., 2022; Parry et al., 2023; Bozkurt, 2023) if the less abundant and proline-stabilized recombinant protein would have similar effects, for example through ACE2 deregulation (Lei et al., 2021), abnormal inflammatory responses (Nuovo et al., 2021) and premature cell death (Kucia et al., 2021).

Vaccine interactions with circulating blood cells

Various white blood cell phenotypes have been shown to uptake vaccine during the inflammatory response at the injection site, mainly monocytes, dendritic cells (Lindsay *et al.*, 2019) and neutrophils (Verbeke *et al.*, 2022). It is reasonable to assume vaccine particles would also randomly collide with white blood cells or platelets in circulation, potentially leading to lipofection events. Our group looked at *in vitro* interactions between LNP-mRNA and human blood cells, by exposing both myeloid and lymphoid phenotypes to varying concentrations of the BNT162b2 vaccine, for various durations (Fertig *et al.*, 2022). Specifically, we treated tumor cells of myeloid lineage (HL-60, K562) but also peripheral blood mononuclear cells (lymphocytes, monocytes) from vaccine-naïve individuals with up to 10 µg of product. Although LNPs accumulated in the endosomal compartments of these cells (especially monocytes/macrophages), we could only confirm S-protein expression in less than 5% of K562 cells when using electron microscopy (Fertig *et al.*, 2022). Since our publication, Cari *et al.* has confirmed expression of the S-protein in K562, as well as Jurkat cells, after treatment with BNT162b2 and Spikevax, using a combination of flow cytometry and ELISA (Cari *et al.*, 2023). There are two interesting take-aways from this study:

(1) there was significantly higher expression with the Moderna product in both cell lines, and

(2) Jurkat T-cells had much higher expression compared to K562, thereby confirming K562 as non-optimal candidates for vaccine mRNA translation (Cari *et al.*, 2023). Despite this, in a recent preprint, Zurlo and colleagues showed that exposure of K562 to BNT162b2 suppresses erythroid differentiation of these cells and inhibits globin gene expression in a dose-dependent manner (Zurlo *et al.*, 2023). These results are congruent with documentation submitted to EMA by both Pfizer-BioNTech and Moderna, showing decreases in red blood cell precursors and parameters (*e.g.* hemoglobin, hematocrit) in vaccine toxicology studies on rodents[8].

Other evidence of effects on hematopoiesis comes from a study showing that vaccination, more than SARS-CoV-2 infection, leads to a decrease in umbilical cord $CD34^+$ hematopoietic stem cells through apoptosis, likely via an interferon-γ dependent pathway (Estep *et al.*, 2022). The virus-associated S-protein is also directly toxic to stem cells, inducing Nlrp3 inflammasome activation and cell death via pyroptosis (Kucia *et al.*, 2021).

Vaccine mRNA reaches milk

Plasma persistence of vaccine mRNA also impacts lactational transfer. Although it was initially reported that mRNA was not detectable in the milk of 7 breastfeeding mothers (Golan *et al.*, 2021), later studies showed it is in fact present up to 45 hours after inoculation, likely within extracellular vesicles (Hanna *et al.*, 2022). Cu-

[8] **https://www.ema.europa.eu/ en/documents/assessment-report/comirnaty-epar-public-assessment-report_en.pdf; https://www.ema.europa.eu/en/documents/assessment-report/spikevax-previously-covid-19-vaccine-moderna-epar-public-assessment-report_en.pdf**

riously, this mRNA was mostly non-integral and was therefore unable to induce S-protein expression in HT-29 cells (Hanna *et al.*, 2023).

Vaccine uptake in the heart

An area of great interest remains the biodistribution of the vaccine to the heart, mainly due to the extensively reported and highly publicized organ-associated side effects, such as myocarditis and pericarditis (Alami *et al.*, 2023). Accurate evaluations of biodistribution in the human heart are obviously technically challenging, with heart biopsies being not only high risk, but also ethically and medically difficult to justify.

A recent study has broken this barrier and tested the presence of synthetic mRNA in the heart of deceased individuals that were recently vaccinated, showing that 3 of 20 individuals had mRNA in the right, left or both ventricles (Krauson *et al.*, 2023). The vaccine mRNA was found in areas of healing injury which preceded vaccination, rich in macrophages, suggesting either that healing tissue is more susceptible to vaccine uptake, or that inflammatory cells carried vaccine particles to these regions of tissue. Surprisingly, although the study also showed lymph node localization of vaccine mRNA, it failed to detect it in the liver or spleen, organs highlighted as accumulation sites by the manufacturers. The authors interpret this discrepancy as possibly related to dosing (higher in animals than humans) which would make detection more difficult (Krauson *et al.*, 2023), however other possibilities are that

(1) tissue tropism and/or traffic of vaccine particles differs between species

or

(2) accidental intra-venous injection of the vaccine would alter biodistribution.

Indeed, injecting mice with BNT162b2 via an intra-venous but not intra-muscular route, led to acute multifocal myopericarditis, which was exacerbated by a second dose. In this study, mouse cardiomyocytes expressed S-protein *in situ*, suggesting successful lipofection by LNP-mRNA, which the authors suggest may reach these cells through the sponge-like action of the T tubule system (Li *et al.*, 2021).

The fact that vaccine mRNA can be uptaken by cardiomyocytes as well as non-myocytic cells (endothelial cells and fibroblasts) has been recently confirmed by Schreckenberg *et al.*. on an *ex vivo* rat heart-perfusion model (Schreckenberg *et al.*, 2023). They further showed that both isolated rat and human AC16 cardiomyocytes can also readily express the S-protein and have altered function when exposed for 48 hours to either Spikevax or BNT162b2. However, whereas exposure to the former induced arrhythmia and subsequent loss of function, the latter only led to changes resembling adrenergic stimulation with isoprenaline (Schreckenberg *et al.*, 2023).

Based on this data, there are at least three possible mechanisms to explain vaccine-induced myocarditis:

(1) uptake of mRNA and subsequent S-protein expression in cardiac tissue, which drives localized inflammatory responses and leads to extensive cell lysis in the affected territory,

(2) pro-inflammatory and cytotoxic effects in the heart by circulating S-protein expressed in any other tissue

or,

(3) exacerbated systemic immune responses, with upregulation of cytokines and cardiac tissue damage by activated lymphocytes (the latter described by Barmada *et al.*, 2023).

For all three hypotheses the S-protein remains a common denominator. At least one study has linked its plasma levels to myocarditis, showing that in these patients the S-protein circulated unbound by anti-S antibodies for weeks and correlated with troponin T levels and cytokine release (Yonker *et al.*, 2023).

Interactions of the vaccine with other cells and tissues

Only a very limited number of human cell types were tested with either mRNA vaccine, *in vitro*. Pfizer-BioNTech have provided to EMA data showing successful transfection of HEK293T cells, including surface expression of S-protein[9], whereas Moderna has previously published experiments on HeLa cells, albeit using an mRNA vaccine directed against influenza (Bahl *et al.*, 2017). Undoubtedly, this data was essential to show the ability of LNP-mRNA constructs to undergo endocytosis and lead to protein expression in eukaryotic cells, however HEK293T and HeLa cells are phenotypically dissimilar to cell types that the LNPs may encounter in muscle,

[9] **https://www.ema.europa.eu/en/documents/assessment-report/comirnaty-epar-public-assessment-report_en.pdf**

lymph nodes, blood, heart or any of the tissues in which they preferentially accumulate in vaccinated individuals.

Since the rollout of this generation of mRNA vaccine there was concern, especially with some members of the public, that mRNA can be integrated into the genome of lipofected cells. This concern was heightened by a recent study from Rudolf Jaenisch's group showing integration of SARS-CoV-2 mRNA sequences in the DNA of cultured human cells (Zhang *et al.*, 2021). There are mechanisms described for reverse transcription of random cytosolic mRNAs, mainly involving long interspersed nuclear element-1 (LINE-1), an autonomous retrotransposon implicated in pathogenesis (Kazazian and Moran, 2017). In a somewhat controversial series of experiments, Aldén *et al.* incubated the human liver cell line Huh7 with BNT162b2 and observed reverse transcription of exogenous vaccine mRNA to DNA within 6 hours (Aldén *et al.*, 2022). The choice of cell line was explained by data in animal models, from both manufacturers, showing the liver is a likely site of accumulation for vaccine particles, however one objection to the study is that these cells are an immortalized line with active DNA replication, which is different from *in vivo* conditions (Merchant, 2022). Other objections were high vaccine dosing and the lack of an immune environment to remove lipofected cells, like expected in tissue (Merchant, 2022). However, in the context of many billions of doses administered worldwide and knowing LINE1 expression is increased in some inflammatory diseases, like cancer (Xiao-Jie *et al.*, 2015), it may be clinically relevant to conduct further studies on potential integration events, for example on specimens obtained from patients undergoing biopsies for various, unrelated pathologies.

Another area of concern since vaccine rollout was if immunization of pregnant women can lead to complications or adverse neonatal outcomes, as caused by either circulating LNP-mRNA or expressed S-proteins. One study has looked at the ability of human placental explants to uptake vaccine at 0.5 and 4 h exposure and found that chorionic villi were not readily penetrated by vaccine (Gonzalez *et al.*, 2023). The same could not be said by the trophoblast cell lines BeWo and JEG-3, which were successfully lipofected. The discrepancy is explained by the authors as caused by the histological complexity of the explant with multiple layers of cells effectively acting as a barrier to LNP-mRNA passage (Gonzalez *et al.*, 2023). It has to be noted, however, that the relatively short incubation times with the vaccine in this study may not have been sufficient to allow LNP infiltration within the architecture of tissue.

Conclusions

Despite an ever-growing number of studies, the biodistribution and interactions of LNP-mRNA vaccines in humans are still poorly characterized. Notable examples of "missing data" at time of writing this review are the biodistribution and effects of the mRNA vaccines on liver, spleen and ovarian tissues, known sites of accumulation for these particles from pre-clinical testing. Due to the ability of both LNPs and S-proteins to cross the blood-brain barrier, pharmacokinetics and toxicity of vaccines in brain tissue also warrants further study. Although a good proportion of post-rollout biodistribution studies have focused on the heart due to myocarditis being a notorious adverse event, the exact molecular cascade leading to heart tissue inflammation remains unknown.

Unfortunately, studies done on cultured cell lines are limited by the absence of the tissue microenvironment and in particular, of the immune cells responsible for recognizing expressed antigens and clearing lipofected cells. Moreover, experiments on human tissues *in situ* and *ex vivo* are technically challenging and restricted to researchers working in clinical settings. A strong alternative for future studies may be tissue organoids, which offer a more complex microenvironment to single cell lines and are not limited by the ethical concerns of working with human tissues. Regardless of technique, it is unarguable that more biodistribution data in humans can only help fine-tune tissue targeting for the next generation of mRNA vaccines and therapies.

Abbreviations

ACE2 –	angiotensin-converting enzyme 2
CD34+ –	positive for cluster of differentiation 34
COVID19 –	coronavirus disease of 2019
DNA –	deoxyribonucleic acid
ELISA –	enzyme-linked immunosorbent assay
EMA –	European Medicines Agency
EU –	European Union
HEK293T –	Human Embryonic Kidney 293T
HL-60 –	Human Leukemia-60
HeLa –	Henrietta Lacks cells
LINE1 –	long interspersed nuclear element-1
LNP –	lipid nanoparticle
mRNA –	messenger ribonucleic acid
modRNA –	modified messenger ribonucleic acid
Nlrp3 –	NLR family pyrin domain containing 3
PEG –	Polyethylene glycol
POTS –	postural orthostatic tachycardia syndrome
qPCR –	quantitative polymerase chain reaction
RNAse –	ribonuclease
S-protein –	spike protein
S2 –	subunit 2 (of the spike protein)
SARS-CoV-2 –	severe acute respiratory syndrome coronavirus 2
US –	United States of America

Author contributions

All authors contributed equally to this study.

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Conflict of Interest

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no data was generated and analyzed for the current study.

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